BLOOD TRANFUSION IN CLINICAL MEDICINE

BY

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WITH A FOREWORD BY

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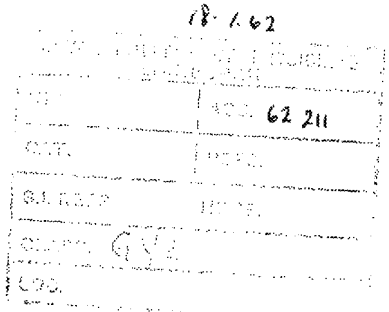

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CONTENTS

CHAPTER ONE

The Taking and Storing of Blood: Survival of Blood Components after Transfusion

	Page
THE WITHDRAWAL OF BLOOD	3
ANTICOAGULANTS	8
THE SURVIVAL OF TRANSFUSED RED CELLS AFTER STORAGE	11
THE SURVIVAL OF TRANSFUSED LEUCOCYTES	31
THE SURVIVAL OF TRANSFUSED PLATELETS	33
THE SURVIVAL OF TRANSFUSED PLASMA PROTEINS	36

CHAPTER TWO

Blood Volume

DISCREPANCY BETWEEN 'VENOUS HAEMATOCRIT' AND 'WHOLE BODY HAEMATOCRIT'	46
ESTIMATION OF PLASMA VOLUME	49
ESTIMATION OF PACKED CELL VOLUME	57
ESTIMATION OF RED CELL VOLUME	62
NORMAL PLASMA VOLUME	72
NORMAL RED CELL VOLUME	73
NORMAL TOTAL BLOOD VOLUME	79
VARIATIONS IN BLOOD VOLUME IN DISEASE	83

CHAPTER THREE

Haemorrhage and Transfusion

CIRCULATORY EFFECTS OF HAEMORRHAGE	89
SPONTANEOUS RESTORATION OF BLOOD VOLUME	93
THE CIRCULATION IN SEVERE ANAEMIA	97
RESTORATION OF BLOOD VOLUME BY TRANSFUSION	98
PLASMA SUBSTITUTES	106

RCPE0002068_0002

CHAPTER FOUR

Transfusion and Anaemia

	Page
INDICATIONS FOR TRANSFUSION	112
TRANSFUSIONS NOT INTO BLOOD VESSELS (INTRAPERITONEAL, ETC.)	119
'GRAFTING' OF BONE MARROW	123
EFFECTS OF TRANSFUSION ON THE CIRCULATION	127
TRANSFUSION IN SEVERE ANAEMIA	130

CHAPTER FIVE

Methods of Estimating the Survival Time of Red Cells *in vivo*; Results in Normals

THE METHOD OF DIFFERENTIAL AGGLUTINATION	135
SURVIVAL OF TRANSFUSED RED CELLS IN NORMAL SUBJECTS	144
LABELLING OF RED CELLS WITH ^{51}Cr	152
RESULTS OBSERVED WITH ^{51}Cr-LABELLED RED CELLS	156
OTHER METHODS OF LABELLING RED CELLS	169

CHAPTER SIX

Diminished Survival of Red Cells in Disease

ANAEMIAS ASSOCIATED WITH THE PRODUCTION OF FAULTY RED CELLS	177
HAEMOLYTIC ANAEMIAS IN WHICH RED CELL LIFE-SPAN IS DIMINISHED BY EXTRINSIC FACTORS	189
RANDOM LOSS OF RED CELLS DUE TO CHRONIC HAEMORRHAGE	207
SURFACE COUNTING IN HAEMOLYTIC ANAEMIAS	208

CHAPTER SEVEN

Blood Groups: General Considerations

DISCOVERY OF BLOOD GROUPS	211
INHERITANCE OF BLOOD GROUP ANTIGENS	213
NATURALLY-OCCURRING BLOOD GROUP ANTIBODIES	220
IMMUNE BLOOD GROUP ANTIBODIES: CHARACTERISTICS OF IMMUNE RESPONSES	223
COMPLEMENT	232
THE ANTIGLOBULIN SENSITIZATION TEST	237
THE CONGLUTINATION REACTION	244
SERUM PROTEIN GROUPS	248

CHAPTER EIGHT

ABO and Lewis Blood Group Systems

	Page
ABO GROUPS	250
ANTIBODIES OF THE ABO SYSTEM	260
IMMUNE CHARACTERISTICS OF ANTIBODIES OF THE ABO SYSTEM	267
THE LEWIS BLOOD GROUP SYSTEM	283
ANTIBODIES OF THE LEWIS SYSTEM	289

CHAPTER NINE

Rh Blood Group System

Rh ANTIGENS	294
Rh ANTIBODIES	304
FORMATION OF IMMUNE Rh ANTIBODIES FOLLOWING TRANSFUSION OR INJECTION	310
IMMUNIZATION BY PREGNANCY	317
PRODUCTION IN ANIMALS OF ANTIBODIES RESEMBLING ANTI-Rh	326

CHAPTER TEN

Other Blood Group Systems (other than ABO, Le and Rh)

THE MNSs SYSTEM	328
THE P SYSTEM	333
THE LUTHERAN (Lu) SYSTEM	337
THE KELL (K) SYSTEM	339
THE DUFFY (Fy) SYSTEM	341
THE KIDD (Jk) SYSTEM	343
THE DIEGO (Di) SYSTEM	344
THE SUTTER (Js) SYSTEM	345
ANTIGENS WITH A LOW INCIDENCE	345
HIGH INCIDENCE ANTIGENS	347

CHAPTER ELEVEN

Auto-antibodies and other causes of interaction between Red Cells and their own Serum or Plasma

NATURALLY-OCCURRING COLD AUTO-ANTIBODIES	350
PATHOLOGICAL AUTO-ANTIBODIES	354
ROULEAUX FORMATION	359
OTHER FACTORS CAUSING NON-SPECIFIC AGGLUTINATION	361
PHENOMENA DUE TO INFECTION OF RED CELLS OR SERUM	364

RCPE0002068_0003

CHAPTER TWELVE

Blood Grouping Techniques

	Page
STORAGE OF RED CELLS BEFORE TESTING	371
STORAGE OF BLOOD GROUPING SERA	376
AGGLUTINATION TESTS	378
AGGLUTINATION OF RED CELLS SUSPENDED IN A COLLOID MEDIUM	386
THE USE OF ENZYME-TREATED CELLS TO DETECT INCOMPLETE ANTIBODIES	393
TESTS FOR HAEMOLYSIS	398
USE OF SERUM CONTAINING IMMUNO-CONGLUTININ (I-K)	401
THE ANTI-HUMAN GLOBULIN TEST (COOMBS TEST)	402
COMPATIBILITY TESTS	418
ISOLATION OF ANTIBODIES	427
IDENTIFICATION OF ANTIBODIES	432
DETECTION OF WHITE CELL ANTIBODIES	435

CHAPTER THIRTEEN

Incompatibility: or Shortening of the Life-Span of Red Cells by Iso-Antibodies

METHODS OF STUDYING THE EFFECTS OF INCOMPATIBILITY ON RED CELL SURVIVAL	437
TYPES OF RED CELL DESTRUCTION OBSERVED	441
FACTORS AFFECTING THE DESTRUCTION OF INCOMPATIBLE RED CELLS	444
INCOMPATIBILITY WITHOUT DEMONSTRABLE ANTIBODIES	476
THE DESTRUCTION OF RED CELLS BY TRANSFUSED INCOMPATIBLE PLASMA	485
SYMPTOMS AND SIGNS PRODUCED BY INCOMPATIBLE TRANSFUSIONS	496

CHAPTER FOURTEEN

Haemolytic Transfusion Reactions

CLEARANCE OF HAEMOGLOBIN FROM THE PLASMA	505
PIGMENT KATABOLISM AFTER EXTRAVASCULAR DESTRUCTION OF RED CELLS	507
HAEMOLYTIC REACTIONS CHARACTERIZED BY HAEMOGLOBINAEMIA	508
HAEMOLYTIC REACTIONS CHARACTERIZED BY HYPERBILIRUBINAEMIA	515
A ROUTINE PROCEDURE FOR THE INVESTIGATION OF HAEMOLYTIC TRANSFUSION REACTIONS	529

CHAPTER FIFTEEN

Other Unfavourable Effects of Transfusion

	Page
FEBRILE REACTIONS	534
ALLERGIC REACTIONS	546
INFECTED BLOOD	549
TRANSMISSION OF DISEASE	556
TOXIC FACTORS IN PLASMA, INCLUDING ANTICOAGULANTS	564
TRANSFUSION HAEMOSIDEROSIS	570
THROMBOPHLEBITIS	572
AIR EMBOLISM	574

CHAPTER SIXTEEN

Some Normal Blood Findings in Newborn Infants: Indications for Simple Transfusion

SOURCES OF VARIABILITY IN HAEMOGLOBIN CONCENTRATION IN NEWBORN INFANTS	578
PLASMA BILIRUBIN VALUES IN NEWBORN INFANTS	585
INDICATIONS FOR TRANSFUSION IN INFANTS	587

CHAPTER SEVENTEEN

Haemolytic Disease of the Newborn

PLACENTAL TRANSFER OF ANTIBODIES	596
HAEMOLYTIC DISEASE OF THE NEWBORN DUE TO ANTI-Rh	603
ANTENATAL ASSESSMENT OF SEVERITY	604
ASSESSMENT OF SEVERITY FROM CORD BLOOD FINDINGS	611
TREATMENT OF HAEMOLYTIC DISEASE OF THE NEWBORN	623
PREMATURE INDUCTION OF LABOUR	624
TRANSFUSION	628
HAEMOLYTIC DISEASE OF THE NEWBORN DUE TO ABO INCOMPATIBILITY	655
SYNDROMES WHICH MAY HAVE TO BE DISTINGUISHED FROM HAEMOLYTIC DISEASE OF THE NEWBORN	670

RCPE0002068_0004

LIST OF FIGURES

NO.	TITLE	PAGE
1	Post-transfusion survival of red cells stored in ACD	12
2	Removal of non-viable red cells from the circulation	14
3	24-hour survival of red cells stored in trisodium citrate and ACD	16
4	24-hour survival of red cells stored in ACD, with or without adenosine	21
5	24-hour survival of red cells stored in glycerol at −20° C.	28
6	Reversal of electrolyte changes in stored red cells after transfusion	31
7	Concept of the 'body haematocrit'; effect of splenomegaly	47
8	Disappearance of T-1824 from the plasma following injection	52
9	Plasma trapped in the red cell column of the haematocrit	58
10	Effect on PCV of posture and of venous stasis	61
11	Mixing of injected red cells as observed in samples from a peripheral vein	64
12	Effect of splenomegaly on rate of mixing	65
13	Disappearance of ^{32}P, as labelled red cells, following injection	69
14	Relation between plasma volume and venous haematocrit	73
15	Relation between red cell volume and venous haematocrit	75
16	Relation between body haematocrit and venous haematocrit	77
17	Relation between total blood volume and venous haematocrit	81
18	Spontaneous restoration of blood volume after haemorrhage	94
19	Haemoglobin regeneration after blood donation	96
20	Effect of blood transfusion on red cell production	116
21	Changes in PCV to estimate red cell survival in aplastic anaemia	118
22	Entry of red cells into the circulation following intraperitoneal injection	120
23	Entry of red cells into the circulation following intraperitoneal injection (therapeutic quantity)	121
24	Automatic pipette	137
25	Direct differential agglutination	142
26	Normal survival of transfused red cells	145
27	Normal survival curve showing 'tailing'	146
28	Curvilinear survival curve in a female recipient	149
29	Survival of red cells obtained from the placenta	151
30	Disappearance of ^{51}Cr from plasma	155
31	Normal survival surve of ^{51}Cr-labelled red cells	157
32	True red cell survival curve and single-component Cr elution on a semi-log plot	158
33	Slow component of Cr elution deduced from an actual Cr survival curve	159
34	Over-estimate of '$T_{\frac{1}{2}}Cr$' due to ignoring changes in PCV	160
35	Normal Cr survival corrected only for single component elution	163
36	Cr survival curve after labelling red cells with too much $Na_2\,^{51}CrO_4$	165
37	Survival of 'placental' red cells, labelled with ^{51}Cr	167
38	Survival curve (in a rabbit) of reticulocytes labelled *in vitro* with ^{59}Fe	170
39	Hypothetical example of the survival of a population of short-lived and long-lived red cells	179

NO.	TITLE	PAGE
40	Effect of ignoring the existence of very short-lived red cells	180
41	Curve with four components	181
42	Three rapid components plotted as a single component	182
43	Effect of presence of transfused cells on survival curve	183
44	Survival of red cells in a patient with a mild 'intrinsic' red cell defect	184
45	Survival of red cells in paroxysmal nocturnal haemoglobinuria	187
46	Survival of red cells in acquired haemolytic anaemia, as a semi-logarithmic plot	190
47	Cr survival in a patient with a mild haemolytic anaemia compared with two normal Cr survival curves	191
48	Three different corrections for Cr elution applied to data of Fig. 47	192
49	Method of deducing mean life-span of patient's own red cells from survival of transfused red cells	195
50	Survival of transfused red cells in a very severe case of 'auto-immune' haemolytic anaemia	196
51	Survival of transfused red cells and of patient's own red cells in auto-immune haemolytic anaemia	197
52	Survival of patient's own red cells (e+) and of transfused red cells (e−) in auto-immune haemolytic anaemia	199
53	Survival in normal subjects of red cells from patients with auto-immune haemolytic anaemia and with hereditary spherocytosis	201
54	Separate inheritance of antigens of ABO and Rh blood group systems	214
55	A scheme to explain 'Bombay' phenotype	258
56	Iso-agglutinin titres at different ages	266
57	Iso-immune response to group B blood and group A serum	275
58	Iso-immune response to group A blood	276
59	Intravascular haemolysis	442
60	Prolonged survival of a proportion of incompatible red cells	444
61	Delayed red cell destruction; incompatibility due to anti-B	446
62	Delayed red cell destruction; incompatibility due to anti-Rh	447
63	Destruction of transfused Rh-positive red cells by anti-Rh	450
64	Slow destruction of incompatible red cells by low titre anti-c	452
65	Partial destruction of O Le (a−b+) red cells by anti-Le^a	454
66	Destruction of Jk^aJk^a and Jk^aJk^b red cells by anti-Jk^a	455
67	Destruction of *cc* and *cC* red cells by potent agglutinating anti-c	456
68	Destruction of A_1 red cells by anti-A_1 in patients of subgroups A_2 and A_2B	460
69	Destruction of *MM* red cells by anti-M	461
70	Destruction of *MN* red cells by anti-M	462
71	Destruction by anti-M of *MM* red cells injected as a warm suspension and as a cold suspension	463
72	Destruction of group O red cells by anti-O (H)	464
73	Destruction of Le(a+b−) red cells by anti-Le^a	465
74	Destruction by anti-P_1 of P_1+ red cells injected as a warm suspension and as a cold suspension	466
75, 76	Survival *in vivo* of Fy(a+) red cells sensitized *in vitro* with anti-Fy^a	467, 468
77, 78	Survival *in vivo* of Rh (+) red cells sensitized *in vitro* with anti-Rh	470, 471
79	Survival of Rh-sensitized red cells in a splenectomized subject	473

RCPE0002068_0005

NO.	TITLE	PAGE
80	Low-grade haemoglobinaemia following extravascular destruction	474
81	Destruction of all transfused red cells within 24 hours, without demonstrable antibody	477
82	Moderate shortening of survival of transfused red cells without demonstrable antibodies	480
83		481
84		482
85		483
86	Effects of transfusing plasma containing immune anti-A to a group A subject	490
87	Destruction of heated red cells	512
88	Extravascular destruction of 'non-viable' stored red cells	514
89	Severe febrile reactions	536
90	Effect of transfusing 'Buffy' coat to patient with leucocyte agglutinins	539
91	Rate of disappearance of anti-Rh acquired by placental transfer	601
92	Haemoglobin concentration of cord blood related to chance of survival in haemolytic disease of the newborn	612
93	Haemoglobin concentration and plasma bilirubin concentration in cord blood of infants with haemolytic disease of the newborn	615
94	Masking of anaemia on the first day of life	619
95	Rate of fall of haemoglobin concentration in a mild case of haemolytic disease of the newborn	620
96	Rate of fall of haemoglobin concentration in very mild cases of haemolytic disease of the newborn	621
97	Effect of treatment on chance of survival in haemolytic disease of the newborn	630
98	Changes in plasma bilirubin concentration in identical twins with haemolytic disease of the newborn	632
99	Rapid destruction of Rh-positive red cells in two infants with haemolytic disease of the newborn	634
100	Effect of growth on the red cell survival curve	635
101	Survival of Rh-negative cells in haemolytic disease of the newborn	636
102	Haemoglobin changes during first 3 months of life in infants treated by exchange transfusion.	644
103	Diagram of apparatus for exchange transfusion	647
104	Saline manometer for measuring venous pressure	654
105	Osmotic fragility of red cells in infants with haemolytic disease of the newborn due to anti-A	664
106	Osmotic fragility of red cells in normal infants	665
107	Osmotic fragility of red cells in infants with haemolytic disease of the newborn due to anti-Rh	666
108	Serial changes in osmotic fragility in haemolytic disease of the newborn due to anti-A	667
109	Survival of O and A cells in an infant with haemolytic disease of the newborn due to anti-A	669

LIST OF TABLES

NO.	TITLE	PAGE
I	Effect on red cell viability of preliminary incubation before storage	25
II	Plasma volume in normal subjects	72
III	Plasma volume in severe anaemia	74
IV	Red cell volume in normal subjects	75
V	Blood volume in newborn infants and in adults	82
VI	Needle sizes	104
VII	Testing a group O serum for use in differential agglutination	138
VIII	Normal Cr survival	162
IX	Survival of a mixed population of short-lived and long-lived red cells	178
X	Lower limits of normal Cr survival	193
XI	Discovery of different blood group systems	212
XII	Phenotypes and genotypes, illustrated by the ABO system	213
XIII	Blood group systems (excluding ABO) in which 'naturally-occurring' antibodies are most often found.	221
XIV	Relative frequency of some blood group antibodies, with estimates of 'potency' of antigens	228
XV	Reactions with different antiglobulin sera of red cells sensitized with various blood group antibodies	242
XVI	Agreement between different tests for complement-binding.	245
XVII	Antigens and antibodies in the ABO system	251
XVIII	Chances of giving incompatible blood if ABO groups are ignored	252
XIX	Reactions of group A cells from a newborn infant	255
XX	Varieties of anti-H(O)	264
XXI	Changes in anti-A following an injection of anti-tetanus serum	269
XXII	Discrepancies between iso-agglutinin titre and 'immune characteristics' of selected anti-A sera	274
XXIII	Interaction of Lewis genes and 'Secretor' genes to produce various phenotypes	285
XXIV	Frequency of common Rh genes	296
XXV	Errors of guessing Rh genotypes	299
XXVI	Antigens produced by Rh gene-complexes	303
XXVII	Terminology of the main antibodies of the Rh system	305
XXVIII	Transfusion history in Rh-immunized pregnant women	320
XXIX	Probability of responding to Rh	322
XXX	Frequencies of MN groups	328
XXXI	The antigen S	329
XXXII	The P blood group system	334
XXXIII	The Duffy blood group system	342
XXXIV	Routine ABO grouping	381
XXXV	Estimation of H substance in saliva	384
XXXVI	Estimation of Lewis substances in saliva	385
XXXVII	Effect of technique on value ascribed to agglutinin titre	391
XXXVIII	Preliminary testing of antiglobulin sera	408

RCPE0002068_0006

NO.	TITLE	PAGE
XXXIX	One- and two-stage antiglobulin tests in detecting anti-Le^a	413
XL	Request form for blood transfusion	420
XLI	A 'panel' of red cells for the identification of antibodies	433
XLII	Use of hydatid cyst substance to neutralize anti-P_1	434
XLIII	Use of gamma globulin to neutralize anti-gamma but not anti-beta globulin	435
XLIV	Absorption of agglutinating and incomplete antibodies	445
XLV	Effect of incompatible antibodies scarcely detectable at time of transfusion	478
XLVI	Incidence of thrombophlebitis after long infusions through rubber and plastic tubing	573
XLVII	Lower limits of normal haemoglobin concentration in first 3 months of life	584
XLVIII	Relation between indirect antiglobulin titre in mother's serum and chance of stillbirth (selected series)	608
XLIX	Relation between indirect antiglobulin titre in mother's serum and chance of stillbirth ('first-affected' cases)	609
L	Relation between colour of amniotic fluid and severity of haemolytic disease	611
LI	Incidence of kernicterus in relation to cord blood haemoglobin concentration	613
LII	Distribution of cord blood haemoglobin values in haemolytic disease of the newborn	614
LIII	Relation between cord plasma bilirubin concentration and mortality	614
LIV	Relation between plasma bilirubin concentration and kernicterus	623
LV	Stillbirths related to history and antibody titre	625
LVI	Risk of stillbirth in relation to previous history	627
LVII	Calculation of proportion of 'heterospecific' pregnancies	656
LVIII	Effect of ABO incompatibility on cord blood findings	659
LIX	Osmotic fragility of red cells in newborn infants	666
LX	Osmotic fragility: range of haemolysis in three saline solutions	667

FOREWORD TO FIRST EDITION

Within the past twelve years the sum total of new knowledge which has been derived from the practice of blood transfusion has been remarkable. In the United States the discoveries of Landsteiner, Levine and Wiener laid the foundations for a swift and astonishing expansion of the subject of blood groups. In Britain the impetus to extend knowledge of blood transfusion came from the outbreak of war. The interchange of knowledge between Britain and the U.S.A., which began soon afterwards and in which many other countries later participated, was an important factor in the speed of advance.

During the early part of the war Dr Mollison had an opportunity of sharing in many of these developments. He was particularly associated with the testing of new preservative solutions and with the growing realization that the ability of stored red cells to survive in the patient's circulation after transfusion was the real criterion of successful preservation. This work led naturally to the measurement of the survival of transfused erythrocytes as a method of investigating blood disorders, a field of investigation which till then had been largely neglected. After the war, Dr Mollison became Director of the Blood Transfusion Research Unit set up by the Medical Research Council and thus had an opportunity of taking up this work again. In a sense, the present book is a record of his experiences, and it shows that he has made good use of his opportunities.

The author has, it seems, three main objects in view: to lay down clearly the techniques necessary for successful transfusion in the varying circumstances in which it is required; to set out as much fundamental knowledge as is vital for intelligent transfusion, and to show how, by modifications in transfusion techniques, new knowledge in human physiology and pathology can be obtained. It is not an easy task, but Dr Mollison has succeeded admirably for he has written a book which reads easily and contains all the essential information.

Many readers will, I expect, on opening the book turn to the chapters on 'Blood Groups' to see 'what he has made of this'. I do not think they will be disappointed, for he has set down clearly and concisely just what is necessary for the clinician.

This wise selection of knowledge is a characteristic of the whole book, and those who read it should find that their understanding of blood

RCPE0002068_0007

transfusion has been greatly strengthened. It is written too for those who wish to share in the delight of acquiring knowledge through the successful application of new techniques. It is a stimulating book and I hope it will be widely read by those who practise transfusion.

ALAN N. DRURY

Lister Institute,
London.
5th June, 1951.

PREFACE TO THIRD EDITION

During the five years since the second edition of this book was published, there have been innumerable developments related directly or indirectly to blood transfusion and in order to put these into perspective it has again been necessary to re-write most of the chapters.

One new chapter has been added so that before considering the various blood group systems in detail, blood groups can be considered as a whole; the subjects discussed include the relative potency of the different blood group antigens; differences between naturally-occurring and immune blood group antibodies, including the distinction between 19*S* and 7*S* gamma globulins; and the role of complement. In the same chapter various other immunological topics are briefly discussed: for example, the production of antibodies against white cells; the existence of serum groups and the phenomena of tolerance and immunological paralysis. The inclusion of such topics is an acknowledgment that the parochial phase of blood group serology is over—a time when, secure in their sole possession of the antiglobulin test, many blood groupers felt no need to acquaint themselves with the classical concepts and techniques of immunology—and that now the benefits of studying immunology as a whole have become obvious to everyone.

As in preparing the two previous editions, I have been greatly helped by the many friends who have read the typescript and offered me advice. Professor A. C. Dornhorst read several chapters, particularly those dealing with the estimation of red cell survival in health and disease and the interpretation of the resulting curves, Dr W. d'A Maycock read the chapters dealing with the storage of blood and the unfavourable effects of transfusion, Dr E. B. Reeve those dealing with blood volume and haemorrhage, Dr D. T. M. Gairdner those dealing with normal blood values in the newborn and Dr W. Walker the chapter on haemolytic disease of the newborn.

In the many chapters dealing with blood groups, I had much useful advice from Miss Margaret Polley who also did a great deal of the work of checking references. Dr N. C. Hughes Jones was also constantly available for discussion and made many helpful suggestions relevant to the passages dealing with haemolytic syndromes.

Mrs Marie Crookston and Dr Eloise Giblett gave me an immense amount of help; they read the blood grouping chapters in typescript and

RCPE0002068_0008

later read through the entire book in the galley proof stage, and sent me long and helpful lists of additions and improvements.

Sir Alan Drury, from whom I have received so much encouragement, read the galley proofs of the earlier chapters and helped me in many other ways.

Thanks are due to the Athlone Press and to the publishers of *Blood*, the *British Journal of Haematology*, the *British Medical Journal*, *Clinical Science*, the *Journal of Physiology*, the *Lancet*, and the *Proceedings of the Royal Society B*, for permission to reproduce figures originally published by them.

I should again like to acknowledge the support I have had from Mr Per Saugman of Blackwell Scientific Publications. I found it hard work to get this edition ready for the press and would have found it very difficult to complete the task without the help given by his staff. Finally I am most grateful to Miss Diana Groundwater and Mrs Margaret Crisp for their unfailing patience during the preparation of the typescript.

PREFACE TO FIRST EDITION

Blood was once regarded as a fluid of infinite complexity, the very essence of life. The blood of each person seemed to carry in it the secrets of individuality. As recently as 1666 it was natural for Mr Boyle, in writing to Dr Lower, to speculate in the following terms about the possible effects of cross-transfusion: ' ... as whether the blood of a mastiff, being frequently transfused into a bloodhound, or a spaniel, will not prejudice them in point of scent'.

If each person's blood were as individual as this, transfusion would indeed be complex and would deserve to rank as the most refined branch of medicine. However, this early view of the subtlety of transfusion was eclipsed at the beginning of this century by the discovery that the blood of all human beings could be divided into four groups. It seemed that, provided blood of the same group was transfused, one person's blood was indistinguishable from another's. Indeed, it came to be believed that people who belonged to the common group O could give their blood to anyone whatsoever. This point of view reached its widest acceptance in the early 1940s, when hundreds of thousands of bottles of group O blood were given as a general panacea for the injuries of war, with remarkably satisfactory effects. As a result of this experience, a generation of medical men has grown up believing that blood transfusion is one of the simplest forms of therapy.

And yet, this view of the interchangeability of blood has to be reconciled with the growing knowledge of its immense complexity. There are so many possible combinations of blood group antigens that the commonest of them all occurs in only 2 per cent of the English population. Indeed, such is the individuality of the blood that, in Race's striking phrase, certain combinations 'may never have formed the blood of an Englishman'.

The explanation of this apparent paradox — the potential complexity of transfusion and its actual simplicity — lies in the fact that many blood group factors are so weakly antigenic in man that they are not recognized as foreign by the recipient. However, it can no longer be maintained that a knowledge of the ABO system constitutes an adequate equipment for the transfusionist, for the role of some of the other systems is by no means negligible. Thus, a book on blood transfusion requires a special account of blood groups, in which the emphasis laid on any one of

RCPE0002068_0009

the antigens depends mainly upon the part that it plays in incompatibility.

A good understanding of the effects of transfusion requires two further accounts: one of the regulation of blood volume and of the effects of transfusion on the circulation, and one of the survival of the various elements of blood after transfusion. The survival of transfused red cells has become a matter of special interest. Red cells survive for a longer period than any of the other components of blood, and their survival can be estimated with comparative precision by the method of differential agglutination. A study of the survival of transfused red cells has proved of great value in investigating haemolytic transfusion reactions. In addition, it has contributed strikingly to fundamental knowledge in haematology by demonstrating the diminished survival of pathological red cells and the existence of extrinsic haemolytic mechanisms in disease. Transfusions are now not uncommonly given for the purpose of investigation as well as of therapy.

This book is thus composed mainly of an account of blood groups from a clinical point of view and of descriptions of the effects of transfusion on the circulation and of the survival of transfused red cells; it also contains chapters designed to fill in the remaining background of knowledge about the results of transfusion in man. Finally it contains a rather detailed account of haemolytic disease of the newborn. It is addressed to all those who possess an elementary knowledge of blood transfusion and are interested in acquiring a fuller understanding of its effects.

In preparing this book I have had the help and advice of many friends. Dr J. V. Dacie read through almost all the typescript and made innumerable suggestions for improvements. Dr A. C. Dornhorst gave me the most extensive help in writing about the interpretation of red cell survival curves, and he is responsible for the simple rules for estimating mean cell life, which I hope that many besides myself will find useful; he has also read through the book during its preparation and given me the benefit of his very wide general knowledge. Dr J. F. Loutit, Dr I. D. P. Wootton and Dr L. E. Young are amongst those who have read certain sections and helped me with their expert advice.

I am even more indebted to Miss Marie Cutbush, who has given an immense amount of time to helping to prepare this book for the press and has, on every page, suggested changes to clarify the meaning of some sentence. In addition she has most generously encouraged me to quote many joint observations which are not yet published.

Miss Sylvia Mossom was responsible for typing the whole book, often from almost illegible manuscript. I am much indebted to her for her skill and patience.

I am also grateful to Miss Helen Wilson for kindly preparing the drawing reproduced (in the third Edition) as Fig. 104. The microphotographs (Figs. 25a and b in the third Edition) were made in the Photographic Department of the National Institute for Medical Research, by courtesy of the Director.

The *British Medical Journal*, *Clinical Science* and the *Lancet* have been so good as to allow the reproduction of certain figures originally published by them.

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CHAPTER ONE

THE TAKING AND STORING OF BLOOD: SURVIVAL OF BLOOD COMPONENTS AFTER TRANSFUSION

One of the earliest transfusions was given to a man who had fallen into a 'Phrensy ... occasioned by a disgrace he received ... in some Amours'. It was hoped that the calf's blood used for the transfusion, 'by its mildness and freshness might possibly allay the heat and ebulition of his Bloud' (Denis, 1667). Even now, blood is sometimes used in this spirit and is given as if it were the elixir of life to patients dying of cancer. However, transfusion achieves its tangible effects by substitution; for example, by supplying red cells which temporarily survive and function in the patient's circulation.

In this chapter the survival of the various components of the blood after transfusion will be considered so that the main therapeutic uses of transfusion can be surveyed. Since stored blood is now more widely used than fresh blood, it has seemed best to consider at each stage what effect storage has upon the constituents of blood, rather than to consider 'stored blood' as a separate subject.

As a preliminary, the taking of blood will be discussed.

Selection of Blood Donors

As a general rule, any adult who is in good health and has not recently had any serious illness is suitable as a donor. Every transfusion service has detailed regulations which slightly modify this general statement. A few of the considerations which determine these rules are discussed here; they may conveniently be divided into those designed to protect the donor and those designed to protect the recipient.

Protection of the Donor

The regular donation of blood makes considerable demands on the iron reserves of the body. Accordingly, those who already have increased demands for iron should not be bled; this includes all pregnant women and those who have been pregnant within the previous year. Previous anaemia of any kind normally disqualifies a person from becoming a

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donor. The question of giving extra iron to regular donors is discussed in Chapter III.

It is usual to question donors specifically about certain serious diseases, for example, cancer, diabetes, epilepsy, goitre, heart disease, hypertension, kidney disease, 'stroke' and tuberculosis; a history of any of these diseases is usually a cause for rejection. Transfusion services are often asked to bleed hypertensives with the object of relieving symptoms. In view of the possibility of precipitating vascular accidents by a sudden lowering of blood pressure, it is wise to bleed such patients only at the express request of their own doctor.

It is advisable to perform a test on all blood donors to make sure that their haemoglobin concentration is not below a reasonable level. A convenient test is to take a drop of blood from the donor and allow it to fall into a selected solution of copper sulphate and thus to determine its specific gravity (Phillips *et al.*, 1945). In Britain the National Blood Transfusion Service (following the lead of the American Red Cross) accepts male donors whose blood contains at least 13.5 g. of haemoglobin per 100 ml. (approximately 90 per cent Haldane), as judged by the fact that a drop sinks in a copper sulphate solution of specific gravity 1.055, and female donors whose haemoglobin concentration is not less than 12.5 g. per 100 ml. (85 per cent Haldane) as judged by the fact that a drop of their blood sinks in a copper sulphate solution of specific gravity 1.053.

Trumbull (1954) recommends that flight crews and those who climb ladders should not return to work for at least 12 hours after giving blood and this seems to be a sensible precaution since it is known that even after the loss of 400 ml. of blood some subjects are liable to faint if they get up suddenly from a sitting or lying position.

Fainting after the loss of blood is considered in Chapter III.

Protection of the Recipient

Some of the unfavourable effects produced by transfusion are considered in Chapter XV, but since some of those due to transmission of disease can be prevented by questioning the donor, they are mentioned briefly here.

Protozoal diseases. The most important is malaria. So far as transfusion services in non-malarious areas are concerned, the blood of those who have had malaria or who have lived recently in endemic malarious areas should be used only for preparing plasma.

Spirochaetal diseases. Any person who is known to have had syphilis is unacceptable as a donor. It is not customary to question donors directly but a serological test for syphilis should be made every time a donor is bled.

Bacteriological diseases. Undulant fever is transmissible and those who have had an attack during the past 2 years should not be used as donors.

Viral diseases. Easily the most important is serum hepatitis. Because it is not known how long a person may remain a carrier of the virus, it has been recommended that anyone with a history of jaundice should be rejected as a donor (W.H.O. Report, 1953). According to McBride and Hervey (1953) approximately 2 per cent of prospective donors give a history of jaundice. Donors should not be bled if they have recently been vaccinated with living virus (e.g. vaccinia).

Transfer of antigens or antibodies. A person who has recently received an injection of horse serum should not be used as a donor because of the risk that his blood may still contain traces of horse serum and so accidentally confer sensitivity on the recipient. Donors who have recently received prophylactic sera or vaccines of various kinds, such as anti-tetanus serum, tetanus toxoid or T.A.B. vaccine, may develop potent haemolytic blood group antibodies of the specificity anti-A, due to the presence of hog pepsin in the prophylactic material; such subjects may thus become 'dangerous universal donors'; this is discussed in more detail in Chapter VIII.

Donors who suffer from very severe allergy are unacceptable as donors because they may transfer their sensitivity to the recipient for a short period (see Chapter XV).

The Withdrawal of Blood

Skin Cleansing

The operator should wash his own hands but should not attempt to 'scrub-up'. The hands should be well dried on a towel and a no-touch technique carefully followed. The proposed site of venepuncture should not be fingered after the disinfectant has been applied.

Although the deeper layers of the skin cannot be sterilized by the application of a disinfectant, the surface of the skin can at least be treated. Seventy per cent ethyl alcohol is still widely favoured (e.g. Price, 1957), although its action is not very rapid. According to Gardner (1948) 70 per cent alcohol takes 2 minutes to disinfect the skin whereas 1 per cent iodine in 70 per cent ethyl alcohol is effective in 30 seconds (see also Story, 1952). Because some subjects get a skin rash after iodine is left on their

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skin, the use of iodine as a routine for donors cannot be recommended, although it can be used without fear of reaction if it is first applied for 30 seconds and then wiped off with 70 per cent alcohol alone.

A satisfactory alternative to iodine is chlorhexidine ('Hibitane') which does not tend to cause skin reactions (see Calman and Murray, 1956). Lowbury *et al.* (1960) found that 2 minutes' treatment of skin with 0.5 per cent chlorhexidine in 70 per cent alcohol was as effective as 1 per cent iodine in 70 per cent alcohol.

Technique of Venepuncture

The veins of the antecubital region are the most suitable. If the donor is right-handed, it is preferable to use a vein in the left arm. The cuff of a sphygmomanometer should be placed round the upper arm and inflated to a pressure of about 60 mm. Hg. If the veins do not then become prominent or if the donor's arm is cold, the cuff should be temporarily removed while the donor warms both his arms in a deep basin of hot water. This procedure is very effective in filling the veins.

Before inserting the transfusion needle, a bleb of 1 per cent procaine should be injected into the skin. For this purpose a very fine needle should be used, e.g. SWG 26 (external diameter 0.481 mm.; American size 25). Procaine is usually supplied in a solution with a pH of about 3 for optimal storage: the injection of too large an amount intradermally has been known to cause necrosis (James, 1958, p. 149).

For bleeding, a convenient size of needle is SWG 15 (external diameter 1.83 mm.; American size 13). The patient's arm should be extended so as to put the veins on the stretch. A vein to choose is one that is fixed. Superficial veins may appear to be easier to enter but when attacked have a habit of slithering away from the needle. The cephalic vein, which lies towards the lateral side of the antecubital fossa, can sometimes be felt and not seen but it is the one to go for when in doubt because it is well fixed and will not evade the needle. Provided that the donor's vein is kept adequately filled it is possible to take 420 ml. of blood in 5 minutes or less, through such a needle. Until recently a considerably larger needle (SWG 13) was often used, but in a proportion of donors, its removal is followed by severe leaking of blood from the vein.

In withdrawing the needle a pad of wool or gauze should be pressed on the site of puncture and the patient should be instructed to keep his arm straight and to raise it whilst maintaining pressure on the pad with the opposite thumb. The practice of retaining the pad in position by flexing the elbow is less satisfactory; not only is less pressure brought to bear on the vein at the site of puncture but pressure may actually be applied proximal to the site of puncture and thus promote bleeding.

(*Fainting during blood donation* is dealt with in Chapter III.)

Air Embolism During Blood Donation

Blood may be allowed to flow into the transfusion bottle by gravity, or its flow may be assisted by having a vacuum in the bottle. The gravity method has the merit of simplicity; however, it is essential to provide an air outlet from the bottle, and it must be remembered that if this outlet becomes blocked a potentially dangerous increase of air pressure may occur in the bottle. If blood continues to flow after the air outlet has become blocked, the pressure of air will increase until it equals the pressure in the vein. It must be remembered that the pressure in the vein will at this time be much higher than normal because of the occlusion by the sphygmomanometer cuff. Therefore if, when the blood stops flowing, the pressure in the vein is decreased by releasing the sphygmomanometer cuff, the pressure in the bottle will be much higher than the pressure in the vein and air will rapidly pass from the bottle up into the donor's arm.

Dr H. Chaplin, Jr., has reported to the author an incident in which a donor received 60-80 ml. of air in this way and suffered a very serious reaction, characterized by pressing substernal pain, choking cough, marked cyanosis and a sense of impending catastrophe. The acute symptoms and the cyanosis lasted 7-8 minutes; after 10 minutes recovery was rapid. In another case in which an estimated 300 ml. of air was injected almost instantaneously into the antecubital vein, the patient became slightly cyanosed and there was an immediate fall in blood pressure. Recovery was complete in 10 minutes (National Research Council, 1951).

It is essential, if the flow of blood ceases during bleeding, to clamp the rubber tubing leading from the needle in the donor's vein before releasing the sphygmomanometer cuff round the arm. Some transfusion centres test the patency of the air outlet from the bottle immediately before venesection.

Embolism by stilette of needle. A remarkable accident was recorded by Hansen (1959). Several days after a blood donation which was marked by some difficulty in obtaining a free flow of blood, the donor complained of pain in the arm and was diagnosed as having aseptic phlebitis. Nearly 4 weeks after donation he complained of dyspnoea on exertion and two days later was found dead in his car. At autopsy there was thrombophlebitis of the distal part of the brachial vein. The stilette of a blood-taking needle was found in the right ventricle; one end had perforated the heart and pericardium posteriorly and there was 200 ml. of blood in the pericardial sac.

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Tetany Following Blood Donation

Tetany is occasionally observed in blood donors (incidence 1 in 1000) and is thought to be due to hyperventilation; it is characteristically observed in nervous subjects. Its manifestations may include carpopedal spasm, laryngismus stridulus and a positive Chvostek's sign. Rapid relief can be obtained by rebreathing from a paper bag, or inhaling 5 per cent carbon dioxide from a cylinder (Frazer and Fowweather, 1942).

False Positive Serological Reactions for Syphilis Following Blood Donation

Following blood donation, flocculation tests for syphilis may temporarily become positive. Thus of 300 healthy sero-negative subjects who gave blood and were tested several times during the following month, almost 10 per cent developed some degree of sero-positivity; most of these subjects had negative serological reactions by the end of 8 weeks (Barnard *et al.*, 1946). The same authors concluded that in subjects giving blood regularly approximately 0.4 per cent would be found to have false positive serological reactions. The tests they used included the Mazzini, Kolmer and Kahn tests.

Use of Cadaver Blood

Tarasov (1960) has reviewed 30 years' experience at the Sklifosovsky Institute in Moscow, involving more than 30,000 transfusions of blood taken from cadavers. Suitable donors are victims of sudden cardiac arrest (e.g. myocardial infarction, hypertensive heart disease, electric shock); victims of accidents involving tissue injuries are not suitable.

In subjects who have died suddenly, blood obtained by venesection flows out in the liquid state; it clots after 20–30 minutes, but 30–90 minutes later it becomes liquid again. This fibrinolysis is seen only in the blood of healthy persons after sudden death. Two to four litres of blood are obtained from the jugular veins of the cadaver and nothing whatever is added. The blood is said to be suitable for transfusion after storage at 4° C. for a period not exceeding 25 days.

Equipment for Collection and Storage of Blood

Blood is still usually taken into glass bottles. The most satisfactory type of taking set then consists simply of a length of tubing connecting the needle which enters the patient's vein to a second needle which is pushed

through a rubber stopper in the neck of the bottle; if non-transparent tubing is used, it should be interrupted at a point near the taking needle by a short length of glass tubing to act as a window. As already mentioned, during the taking of blood an air outlet from the bottle must be provided; this consists of a needle connected to a short length of tubing containing some cotton wool to act as a bacterial filter.

Plastic tubing is now more widely used than rubber tubing in transfusion work. Tubing consisting mainly of polyvinyl chloride is suitable since it can withstand autoclaving. It has the advantage of being transparent and very light in weight; moreover it is so cheap that it can be discarded after use.

Plastic containers for blood, known as plastic bags, have several advantages. They are light in weight and, being flexible, take up the minimum amount of room; they make possible a truly closed technique since no air need gain access to the blood; moreover it is possible to squeeze a mixed sample of blood from the bag into the attached plastic tube and then to isolate the tubing from the bag; in this way a sample of the contents can be examined without contaminating the remainder. Alternatively when the red cells have sedimented, the plasma can be squeezed out of the bag leaving the cells for further storage. Finally it is possible to give blood under pressure, by squeezing the bag, without any risk of air embolism.

It has been claimed that the red cells are better preserved when blood is stored in plastic rather than in glass containers but the differences reported have been small and could well have been due to chance differences between donors and to small systematic errors between different series. In a trial in which red cells from particular donors were stored both in glass and plastic and the survival of the two lots of red cells was then measured in the same donors, it was found that there was no difference between storage in glass and plastic (Dudley *et al.*, 1958).

Some batches of plastic have been shown to have a deleterious effect on red cell preservation (Strumia, Colwell and Ellenberger, 1955; Strumia, Colwell and Dugan, 1959; Donohue and Finch, 1957). Presumably in these cases the plastic contained a toxic substance which leached out during storage but the substance was not identified. Some batches of polyvinyl chloride tubing liberate a substance capable of arresting the isolated rat's heart (Meijler and Durner, 1959). It has been reported that glass bottles may contain a large number of very small particles of glass which might have unfavourable effects on the contents or on the recipient; this is considered further in Chapter XV.

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Anticoagulants

Citrate

The anticoagulant most widely used in blood transfusion is sodium citrate. The safety of citrate for intravenous administration was first demonstrated by Hustin (1914) and very soon afterwards by Agote (1915) and Lewisohn (1915). If the blood is going to be given within a few hours of taking, trisodium citrate is satisfactory, but if the blood is to be stored, a solution containing disodium citrate and dextrose should be used (see below).

It is a common practice to use a solution which contains 2 g. of disodium citrate (and 3 g. of glucose) in 120 ml. of water for mixture with 420 ml. of blood. Assuming that the blood has a PCV of 45 per cent, there will be approximately 230 ml. of plasma and the final concentration of citrate in the citrate-plasma mixture will be 0.57 per cent or approximately 0.022 M. Taking the normal calcium content of plasma to be 10 mg./100 ml. or 0.0025 M, the final calculated concentration in the mixture of plasma and anticoagulant will be approximately 0.0016 M. Although the molar ratio of citrate to calcium is thus more than 10 to 1, it is not uncommon to find clots in blood mixed with this amount of citrate.

Dr J. D. James (personal communication) has compared the effects of using 2.5 g. of disodium citrate (for 420 ml. of blood) with those of using only 2 g. He found that the number of clots in the bottles was distinctly less when 2.5 g. was used. There is no doubt that clots can be avoided almost completely, even with 2.0 g. disodium citrate per bottle, if the blood is mixed with the citrate throughout bleeding and if the bottles are inverted immediately the needles have been withdrawn from them.

If trisodium citrate is used as the anticoagulant it is sufficient to add 60 ml. of a 5 per cent solution for 400–500 ml. of blood.

There are a few circumstances in which it may be desirable to avoid the use of citrate. One possible example is in exchange transfusion in newborn infants. The danger of citrate toxicity can in fact be greatly reduced by using a concentrated suspension of red cells, i.e. by discarding some of the citrated plasma, and by injecting calcium intermittently (see Chapter XVII). An alternative procedure is to take the donor's blood into heparin.

Heparin

If 500 ml. of blood is to be taken from the donor, 15–30 mg. of heparin (approximately 1500–3000 international units (I.U.)) should be used. The heparin should be added to a few millilitres of saline and may be autoclaved in the bottle.

A subject may be heparinized, i.e. given sufficient heparin to render his blood temporarily incoagulable, by injecting intravenously 2 mg. (200 I.U.) of heparin per kilogramme. This is the customary dose to inject into patients who are going to be perfused during the course of cardiac surgery (e.g. Rothnie and Kinmonth, 1960a).

Circulating heparin can be neutralized by injecting protamine sulphate or the newer agent 'Polybrene' (hexadimethrine bromide). The usual dose of protamine sulphate is 1 mg. to neutralize 1 mg. of heparin; for example, to neutralize 5000 units of heparin (50 mg.), 5 ml. of a 1 per cent solution of protamine sulphate would be needed. According to Rothnie and Kinmonth (1960a) after this dosage of protamine it is common to find traces of un-neutralized heparin and this is sometimes the cause of continued bleeding; the same authors recommended 2–4 mg. of protamine for every 1 mg. of the initial dose of heparin. In a further paper they showed that the amount of protamine necessary to neutralize heparin was more variable than the amount of polybrene required and they recommended the use of polybrene in a dose of 1.5 mg. for every 1 mg. of heparin given initially (Rothnie and Kinmonth, 1960b). The rapid injection of protamine or of polybrene produces transient hypotension but this may be avoided by taking not less than 5 minutes over the injection.

EDTA ('Versene' or 'Sequestrene')

The sodium salts of ethylene diamine tetra-acetic acid (EDTA) are powerful chelating agents and prevent coagulation by binding calcium. The binding of EDTA with calcium is more powerful than that of citrate with calcium. In fact, the amount of EDTA required to prevent clotting is only slightly greater than the amount required if one assumes that 1 mole of EDTA binds 1 mole of calcium, the small difference being accounted for by the fact that at blood pH the binding of EDTA and Ca^{++} is not quite complete. Thus in practice 1.1 m-mole EDTA will bind 1.0 m-mole Ca^{++} (Smith *et al.*, 1959).

According to Smith *et al.*, 30 ml. of 1.36 per cent Na_2H_2 EDTA, which provides 1.1 m-mole EDTA, will prevent the clotting of 500 ml. of blood. If one assumes that 500 ml. of normal blood may contain as much as 300 ml. of plasma, which will in turn contain about 0.75 m-mole Ca^{++}, it is evident that the relatively small amount of EDTA provides an adequate safety margin.

'Conversion' from One Anticoagulant to Another

When large volumes of blood have to be introduced rapidly into the circulation, the blood should be well preserved but should not contain any

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appreciable amount of 'free' citrate or EDTA. Large volumes of heparinized blood can safely be transfused but blood stored with heparin alone deteriorates rapidly. The problem can be overcome by storing blood with EDTA or citrate, then, shortly before transfusion, adding first heparin, then sufficient calcium to 'satisfy' all the EDTA or citrate present.

According to Smith *et al.* (1959) EDTA is to be preferred to citrate, mainly because the binding of calcium to EDTA is more exactly predictable, so that the normal calcium content can easily be restored by adding relatively little calcium. To restore the normal calcium level of citrated blood a considerable excess of calcium must be added. A minor disadvantage of citrate is that since it is less effective than EDTA on a molar basis, considerably more sodium must be added to a given volume of blood.

Smith *et al.* (1959) evolved the following solution, 'Edglugate-Mg', for the storage of blood which was to be used in operations involving cardiac 'by-pass':

$Na_2H_2EDTA.\ 2H_2O$	0.410 g.
Sodium gluconate (anhydrous)	0.364 g.
Dextrose (anhydrous)	1.500 g.
$MgCl_2.6H_2O$	0.305 g.
Water to 30 ml.	

for mixture with 500 ml. blood.

Originally, a mixture of trisodium EDTA and disodium EDTA was used to secure a less acid solution but this was abandoned because too much caramelization occurred during autoclaving. The sodium gluconate was added because it was found empirically that this delayed the rate of spontaneous haemolysis during storage. Glucose was added to improve preservation, and $MgCl_2$ to ensure that free magnesium ions were present despite the mixture with EDTA.

A disadvantage of storing blood with this solution is that the plasma potassium rises more rapidly than when blood is stored with ACD (see Chapter XV).

To 'convert' 500 ml. of EDTA blood, 15 mg. of heparin are added, then 1.9 ml. of 10 per cent calcium chloride; (this is greater than the theoretical minimum quantity of calcium, but it is used to compensate for any possible deleterious effect of the raised potassium in the plasma of blood stored for 2 or 3 days).

The preservation of blood stored with various different anticoagulant solutions is discussed later in this chapter.

THE SURVIVAL OF TRANSFUSED RED CELLS AFTER STORAGE

When blood is transfused from one person to another, slightly less than 1 per cent of the donor's red cells disappear from the recipient's circulation on each day after transfusion. Thus when the number surviving is plotted against time, a linear slope is found, cutting the time axis 110–120 days after transfusion (see Chapter V). The survival of the donor's red cells is of great importance in transfusion and is considered in detail in this book. Thus Chapter V is concerned with normal red cell survival, Chapter VI with red cell survival in disease and Chapter XIII with the effect on survival of incompatibility. In this chapter only the effects of storage upon survival will be considered.

Changes Occurring in Red Cells during Storage

Ageing in vitro *and* in vivo

It might be supposed that when blood was cooled to 4° C. the slowing of metabolism would lead to a considerable slowing in the ageing process so that red cells could be stored for a period far longer than their life-span in the body. For example at 4° C. the rate of consumption of dextrose is about 5–10 mg. glucose per litre of red cells per hour (Strumia, 1954b; Chaplin *et al.*, 1954) but at body temperature is in the range 180–500 mg., depending on pH and other conditions (Maizels, 1954; Hughes Jones, Mollison and Robinson, 1957); that is, at 37° C. glucose consumption is at least fifty times more than at 4° C. Nevertheless when red cells are stored at 4° C. in the most favourable medium known (ACD plus adenosine) after 120 days virtually none of them are still viable (Gabrio *et al.*, 1956).

It is now realized, largely as a result of the work of Finch and his collaborators (references below) that the deterioration of red cells during storage is a different process from ageing *in vivo*. For example in stored red cells the content of adenosine triphosphate (ATP) diminishes with time (Rapoport, 1947); *in vivo*, however, as red cells age their content of ATP undergoes little change (Gabrio and Finch, 1954) although the work of Lowy *et al.* (1958) suggests that there may be some fall.

There is evidence that during storage young red cells deteriorate far more rapidly than old red cells. Thus dog red cells with an average age of 5 days were stored for 20 days and transfused; 24 hours later only 10 per cent were left in the recipient's circulation. Dog red cells with an average age of 60 days were also stored for 20 days and found to have a 90 per cent survival 24 hours after transfusion (Gabrio and Finch, 1954). Evidently

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this is the reverse of what would be expected if ageing *in vitro* were the same as ageing *in vivo*.

Assessment of the State of Preservation

The criterion of successful red cell preservation is the normal survival of the red cells after transfusion. When red cells are stored at 4° C. a proportion become incapable of normal survival after transfusion; this proportion increases with time, the rate of increase depending upon the storage medium.

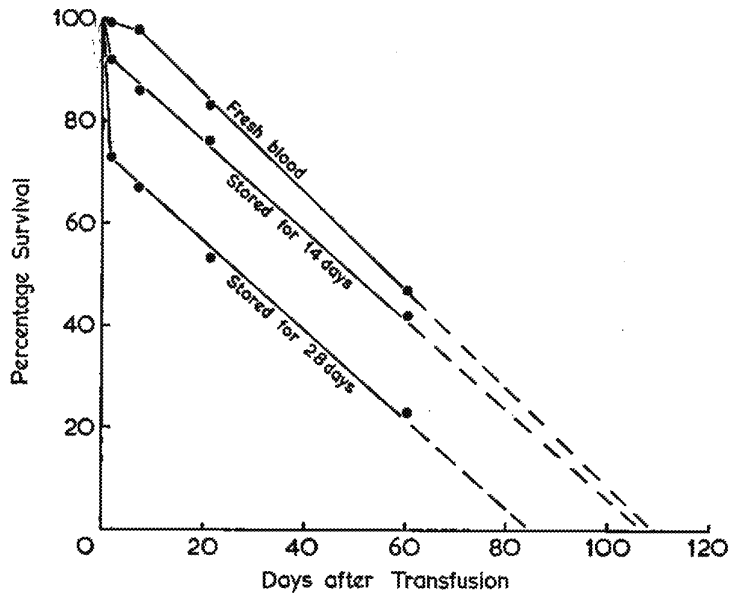

FIGURE 1. Post-transfusion survival of red cells of fresh blood compared with that of red cells stored at 4° C. for 14 to 28 days; when stored red cells are transfused, a proportion leave the circulation in the first 24 hours after transfusion but the rest survive well. (Results obtained by the method of differential agglutination.)

Red cells lose their ability to survive *in vivo* before they haemolyse *in vitro*. For example if red cells are stored at 4° C. for 14 days in a solution of trisodium citrate and sucrose, 99 per cent of them remain intact, but if they are transfused they are rapidly swept from the circulation (Mollison and Young, 1941a; 1942). Substances which retard the rate of haemolysis of stored blood do not necessarily prolong the viability of red cells. Thus

certain derivatives of phenothiazine appeared, from their effects *in vitro*, to be red cell preservatives (Halpern, *et al.*, 1950) but measurements of post-transfusion survival showed that the effects were in fact mildly deleterious (Chaplin *et al.*, 1952).

The osmotic fragility of stored red cells is affected by many factors which do not have any influence on viability. For example, red cells stored at 4° C. in citrate-sucrose solution lose base, due to the osmotic effect exerted by citrate and sucrose; thus the red cells actually become more resistant to hypotonic saline, although judged by post-transfusion survival they are very poorly preserved. Conversely, red cells stored with a relatively large volume of 5 per cent dextrose become swollen, since dextrose penetrates the red cell; such red cells are very 'fragile', judged by lysis in hypotonic saline, but are in fact well preserved (Mollison and Young, 1941a; 1942).

It is known that the post-transfusion survival of stored red cells is closely related to their content of organic phosphates (Rapoport, 1947; Gabrio, Donohue and Finch, 1955). Nevertheless, even when the ATP content of red cells is at least partly maintained during storage by the addition of adenosine at weekly intervals, deterioration is still almost complete by the end of 60–80 days (Mollison and Robinson, 1959).

It seems very likely that loss of lipids from the red cell membrane is one factor which prevents stored red cells from surviving normally after transfusion. Thus it is known that lipids are lost on storage (Lovelock, 1954), and that red cells which have lost lipids by being shaken with alumina undergo rapid destruction on reinjection into the circulation (Harris *et al.*, 1957).

Measurement of Post-Transfusion Survival of Stored Red Cells

When stored blood is transfused, some of the red cells, which may be described as 'non-viable', are removed from the circulation within the first few hours; any red cells left in the circulation by the end of 24 hours usually survive for long periods (see Fig. 1). When the proportion of non-viable red cells in a sample of stored blood is high, indicating a poor state of preservation, some of the red cells are removed within a few minutes (see Fig. 2), the rest being removed over a period of a few hours.

Because of the very rapid removal of a proportion of stored red cells an accurate estimate of the percentage survival can be obtained only by injecting a 'control' sample of fresh red cells, or by estimating red cell volume in some other way. It is not satisfactory just to take a sample 5 or 10 minutes after injection and to regard this as containing 100 per cent of

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the stored cells, since this will lead to an over-estimate of survival. Nor is it satisfactory to take a series of samples during the first hour after injection and to endeavour to estimate '100 per cent survival' by extrapolating back to the time axis, since the early phase of destruction cannot be described by a single component curve; see Fig. 2.

Because the rapid phase of destruction of stored red cells is virtually

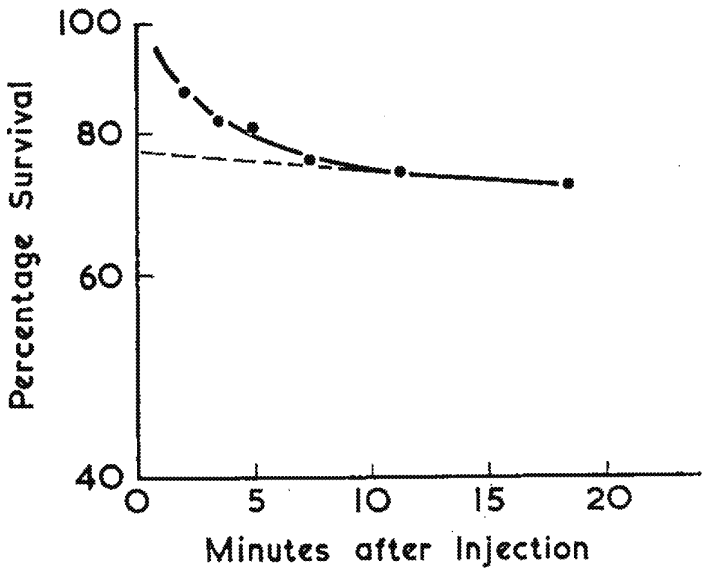

FIGURE 2. Rate of disappearance of ^{51}Cr-labelled stored red cells from the circulation during the 20 minutes following injection. The red cells were from blood which had been stored for 42 days at 4° C. with ACD and inosine. The true percentage survival 24 hours after injection was about 50 per cent.

Each observation is based on a comparison with the survival of fresh red cells labelled with ^{32}P and injected at the same time. If survival had been based on the ^{51}Cr estimates alone, taking samples from 10 minutes onwards and extrapolating the estimates to zero time to obtain a figure for apparent 100 per cent survival, true survival would have been over-estimated by more than 20 per cent.

complete 24 hours after transfusion, an estimate at that time gives a satisfactory measure of post-transfusion survival. It is doubtless advisable to test a second sample (for example at 48 hours) as a check, but there seems to be little point in testing a whole series of samples, since no example is known of a method of preservation which gives good survival at 24 hours and poor survival subsequently.

Use of animals as test subjects. Donohue and Finch (1957) showed that rabbit red cells on storage behaved very like human red cells. For example, when rabbit blood was stored with acid-citrate-dextrose solution, after 35 days the post-transfusion (24 hour) survival of the red cells was 50 per cent, and after 45 days was 20 per cent; these figures are indistinguishable from those found when human red cells are stored. Similarly, when blood was stored with adenosine, the point of 50 per cent post-transfusion survival was reached at 50–55 days when rabbit blood was stored and at 60 days when human blood was stored.

It seems that rabbits could well be used for preliminary tests of new preservative solutions or for such purposes as testing the suitability of new types of containers (see Donohue and Finch, 1957).

The Influence of the Storage Medium

Storage of whole heparinized blood is unsatisfactory, firstly because the blood tends to clot, so that if the blood is to be stored for more than 12–24 hours double the amount of heparin should be used (that is, 30 mg. instead of 15 mg. for 500 ml. of blood), and if the blood is to be stored for more than 48 hours more may be needed. Secondly, red cells deteriorate relatively rapidly in whole heparinized blood. The only measurements of red cell survival *in vivo* appear to be those of Mollison and Young (1942) who stored heparinized blood at +4° C. for 6–10 days. The red cells from approximately 900 ml. of blood were transfused in 1–2 hours to four different subjects, and the concentration of transfused cells in a sample taken immediately afterwards was taken as 100 per cent survival although, as we now know, true survival would probably have been about 80 per cent. At 24 hours survival was estimated to be 76 per cent (? true survival 60 per cent). It may be concluded that one week is far too long a period for which to store heparinized blood before transfusion.

Storage of blood with trisodium citrate alone is unsatisfactory because the red cells deteriorate rather rapidly; see Fig. 3.

The addition of dextrose exerts a strikingly favourable effect on preservation. This was demonstrated conclusively by Rous and Turner (1916); blood was taken from rabbits and stored for 2 weeks in a citrate-dextrose solution; it was then transfused into other animals who had previously been bled; it was shown that the transfusion of these stored red cells prevented the development of anaemia.

Aylward *et al.* (1940) demonstrated that the addition of dextrose to citrated blood decreased the rate of hydrolysis of ester phosphorus during

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storage. Maizels (1941a) confirmed this and suggested that dextrose exerted its favourable effects by providing energy for the synthesis of organic phosphate compounds, particularly diphosphoglycerate and adenosine triphosphate (ATP) which had been shown by previous workers to be the chief organic phosphates in human red cells.

The effect of acidification was investigated by Loutit *et al.* (1943) with the initial object of preventing caramelization in citrate-dextrose solutions

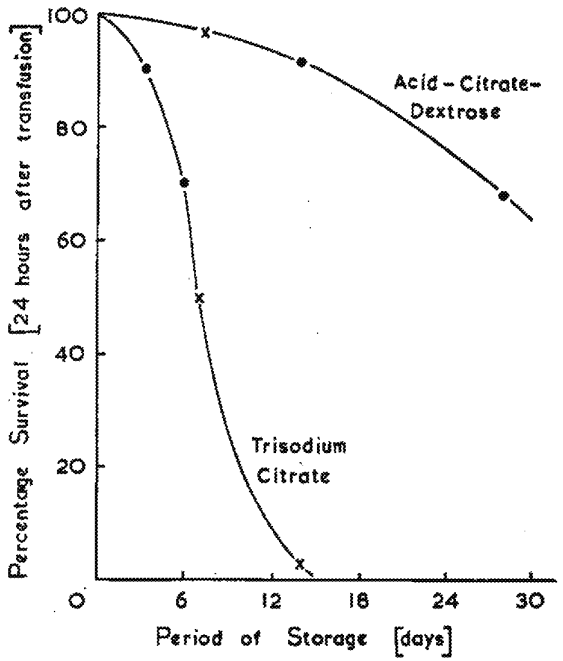

FIGURE 3. Percentage survival, 24 hours after transfusion, of red cells stored for various periods at 4° C., in trisodium citrate or in ACD. Crosses are from data published by Ross *et al.* (1947), and dots from data assembled by Mollison (1951).

during autoclaving. Several citric acid-trisodium citrate-dextrose mixtures were tested and all were found to give better preservation, judged by post-transfusion survival, than trisodium citrate-dextrose alone.

Suggestive evidence of the value of acidification had previously been obtained by Cotter and McNeal (1938), Maizels (1941b) and Wurmser *et al.* (1942), based on changes *in vitro*.

Rapoport (1947) showed that the ATP content of red cells fell more

slowly in ACD solutions than in trisodium citrate-dextrose and that there was a good correlation between the ATP content of stored red cells and their post-transfusion survival.

The post-transfusion survival of red cells stored in ACD is illustrated in Figs. 1, 3 and 4. Fig. 1 shows that the main effect of 14 days' storage is to decrease the percentage of transfused cells which are present in the recipient's circulation 24 hours after transfusion. Red cells which are present at 24 hours survive as well as fresh red cells. Red cells stored for 28 days show a rather different pattern of survival. Approximately 30 per cent are destroyed during the first 24 hours and the remainder survive for a significantly shorter period than fresh red cells. However, since the phase of rapid destruction of stored red cells is virtually complete within 24 hours, the percentage survival at 24 hours is a good index of preservation. The percentage survival at 24 hours of red cells stored in ACD for varying periods of time is illustrated in Figs. 3 and 4.

Fig. 3 contains data for periods of storage up to 28 days. Fig. 4 shows the rapid fall-off in survival of red cells stored in ACD for periods exceeding 28 days.

Various Acid-Citrate-Dextrose Solutions

The solution suggested by Loutit and Mollison (1943) has been in use in Britain ever since; it has the following composition:

Disodium citrate (monohydric)	2 g.
Dextrose (anhydrous)	3 g.
Water	120 ml.

to be mixed with 420 ml. of blood. The solution has a pH of about 5; after mixture with blood, the plasma-citrate has a pH of about 7.1 (and the cells a pH of about 7.0). The relatively large volume of anticoagulant solution is retained in order to limit the amount of blood taken from each donor to 420 ml., in standard bottles having a capacity of 540 ml.

The solution is hypotonic and the red cells swell to about 120 per cent of their normal volume, but this does not seem to be a disadvantage; in fact Loutit (1945) showed that the lower the citrate content the better the preservation. Thus, when the amount of blood was kept at 420 ml., the amount of water in the solution at 120 ml. and the dextrose content at 3 g., 1 g. of disodium citrate was better than 2 g., which was better than 3 g., which was much better than 5 g. Two g. of disodium citrate is adequate to prevent clotting only if the blood is well mixed with the solution during

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collection. Since this is not always the case, some transfusion services prefer to increase the amount of disodium citrate to 2.5 g. (see above).

If disodium citrate is not available, a mixture of trisodium citrate and citric acid can be used: thus the following mixture is equivalent to the disodium citrate-dextrose solution mentioned above:

Trisodium citrate (dihydric)	1.54 g.
Citric acid (monohydric)	0.55 g.
Dextrose (anhydrous)	3.00 g.
Water	120 ml.

This is very similar to a solution widely used in the U.S.A. under the name 'N.I.H. Solution B':

Trisodium citrate	1.32 g.
Citric acid	0.48 g.
Dextrose	1.47 g.
Water	100 ml.

The American Red Cross at present use 110 ml. of this solution to 440 ml. of blood (Dr S. T. Gibson, personal communication). Thus, apart from the use of less dextrose, the American practice is almost identical with the British.

Alsever's solution. Alsever and Ainslie (1941) described a solution containing 0.8 per cent trisodium citrate, 1.9 per cent glucose and 0.42 per cent sodium chloride, 1 volume to be added to an equal volume of blood. The solution was designed to furnish a good yield of dilute plasma and at one time was widely used. Later, the solution was modified by the addition of 0.05 per cent citric acid (see De Gowin *et al.*, 1949, p. 330). The modified solution (with citric acid) was tested by Ross *et al.* (1947) who found that after 28 days' storage red cells had a post-transfusion survival of less than 20 per cent and the solution is not now used for transfusion purposes.

Effect of Reducing the Volume of Preservative Solution

It was at one time believed that dilution of the blood improved preservation. However, when dilution improves preservation it does so indirectly by lowering pH and in mixtures in which the pH is already optimal, dilution is without effect. Thus Loutit (1945) found the following solution to be as satisfactory as the solution in general use:

Disodium citrate	2 g.
Dextrose	3 g.
Water	30 ml.

to be mixed with 510 ml. of blood.

Ross *et al.* (1947) also found that the use of a relatively small volume of anticoagulant solution did not impair preservation. They made the further important observation that the supernatant plasma could be removed from cells stored in an ACD solution without reducing their viability. For example, they took 250 ml. whole blood into 25 ml. of a solution containing 5 per cent disodium citrate and 6 per cent anhydrous dextrose. After 24 hours at 4° C. the blood was centrifuged and the supernatant plasma removed aseptically. The cells were then stored at 4° C. for further periods and transfused without dilution. The survival of such cells appeared to be no different from that of cells stored in the usual way.

As shown by Figs. 3 and 4, the post-transfusion survival of red cells stored at 4° C. diminishes progressively as the period of storage lengthens. In practice there is a certain amount of variation between the red cells of one donor and another, but these differences are seldom large enough to be of practical importance; for example, Dr C. A. Finch (personal communication) has found that red cells stored for 3 weeks in ACD normally have a post-transfusion (24 hours) survival of between 70 and 85 per cent; he found one donor whose cells regularly had a survival of only 60-65 per cent. Evidently it is impossible to guarantee that after a given period of storage red cell survival will always be within certain narrow limits.

From various published observations (Loutit *et al.*, 1943; Ross *et al.*, 1947; Gibson, Evans *et al.*, 1947) it may be concluded that red cells stored in ACD for 14 days usually have a survival of 85 per cent or more and that after 24 days survival is usually 70 per cent or more (see also Donohue and Finch, 1957; and Mollison and Robinson, 1959).

The 'Lesion of Collection'

Gibson *et al.* (1956) considered that the first red cells to be collected were likely to be damaged by being mixed with an excess of acid-citrate-dextrose solution, which is hypotonic and has a pH of only about 5. They labelled the red cells of the first 100 ml. of blood to be collected and found that after 28 days' storage at 5° C. the cells had a post-transfusion survival of 20-32 per cent, whereas the survival of the full 500 ml. was between 44-61 per cent.

If this work should be confirmed one will have to conclude that the damage suffered becomes apparent only after the cells have been stored for some time, because red cells stored in ACD for a day or two have a post-transfusion survival of virtually 100 per cent.

Gibson *et al.* (1957) devised a less acid solution (pH approximately 5.7) consisting of a mixture of trisodium citrate, citric acid and sodium di-

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hydrogen phosphate, which they referred to as 'CPD' (citrate-phosphate-dextrose). The use of a less acid preservative solution has other theoretical advantages besides the possible avoidance of damage during collection of the blood. In ACD blood the ability to utilize glucose falls steadily, because hexokinase activity is destroyed by the fall of pH from about 7.0 to 6.4 which occurs in the first 20 days of storage (Kashket *et al.*, 1957). The use of preservative solutions like CPD which maintain pH near to 7 should help to preserve hexokinase activity. Gibson *et al.* (1957) found that when blood was stored with CPD for 30 days the red cells had a post-transfusion survival of 75 per cent. This figure can be considered only approximate, since red cell volume was not estimated, and it may be too high. As illustrated in Fig. 2, when 100 per cent survival is estimated by extrapolation from samples taken after injection, percentage survival will be over-estimated if the red cells are poorly preserved because some will be lost from the circulation very rapidly.

Before it can be accepted that red cells are better preserved in CPD blood than in ACD blood direct comparisons seem necessary; that is, storage of red cells of particular donors with the two solutions followed by reinjection of the red cells into the same donors.

Storage of Blood with EDTA

Sprague *et al.* (1953) stored blood with a solution containing the disodium salt of EDTA and dextrose and found that the red cells were as well preserved as in ACD (judged by post-transfusion survival).

Smith *et al.* (1959) made tests on blood stored with their solution 'Edglugate-Mg' (see above). After 2 weeks the red cells were about as well preserved as in ACD, but after 3 weeks post-transfusion survival was only about 70 per cent, compared with the expected 80 per cent with ACD.

Effect of Adenosine and Other Purine Nucleosides (see Fig. 4)

Gabrio, Hennessey *et al.* (1955) showed that the ATP content of stored red cells could be restored by incubation at 37° C. with adenosine. A definite effect was obtained with 1.25 μmole (0.34 mg.) adenosine per millilitre of red cells and a maximum effect with 25 μmoles (6.7 mg.). At 37° C., ATP was almost completely restored in 1 hour; at 2° C., the amount restored in 1 hour was about one-fifth.

Restoration of the ATP content was accompanied by an increased post-transfusion survival. Thus in one experiment red cells were first stored for 21 days in ACD, then incubated for 1 hour with adenosine, then stored for a further 21 days at 4° C. Survival was now 87 per cent (average of three

cases). Control cells stored for 42 days without intermediate 'rejuvenation' with adenosine had a survival of only 15 per cent (Gabrio, Donohue and Finch, 1955).

The beneficial effect on stored red cells of incubation with adenosine or guanosine was confirmed by Prankerd (1956); and also by Mollison and

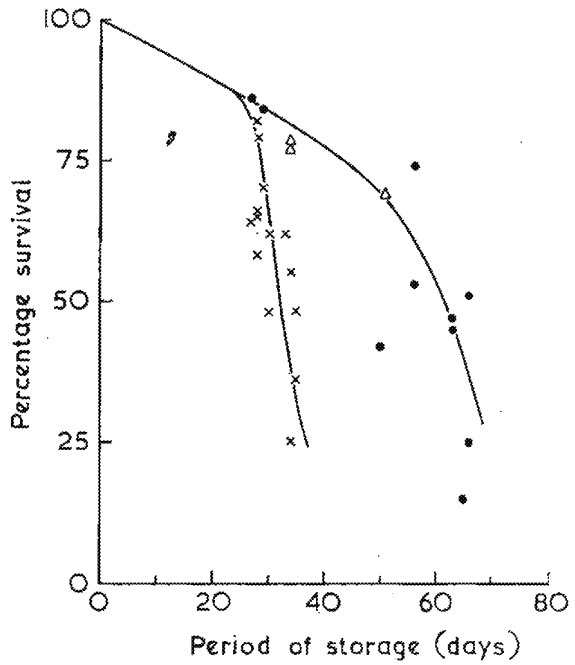

FIGURE 4. Percentage survival, 24 hours after transfusion, of red cells stored at 4° C. for various periods.
× Blood mixed with ACD
● Blood stored with ACD and adenosine
△ Blood stored with ACD alone but incubated at 37° C. for one hour with adenosine immediately before injection.
The lines are arbitrary (from Mollison and Robinson, 1959).

Robinson (1959), who found that in two experiments on samples of red cells stored as ACD blood for 34 days, post-transfusion survival was increased from an average of 40 per cent to one of 78 per cent by incubation for 1 hour at 37° C. with adenosine.

The addition of adenosine at the beginning of the storage period significantly improves the maintenance of red cell viability. Thus, Gabrio,

RCPE0002068_0021

Finch and Huennekens (1956) found that when whole blood was stored without adenosine for 42 days post-transfusion survival fell to 10 per cent, but if adenosine was added at the beginning of the storage period post-transfusion survival after 42 days was 75 per cent. In blood stored with adenosine, post-transfusion survival fell to 50 per cent after 80-90 days. Donohue and Finch (1957) repeated this work in rabbits and obtained almost equally good results.

Donohue *et al.* (1956) and Mollison and Robinson (1959) compared the effect of a single initial addition of adenosine at the beginning of storage with weekly additions during storage and found that weekly additions gave scarcely better results.

Inosine, guanosine, xanthosine and deoxyadenosine are also effective in restoring the ATP content of stored red cells (Gabrio and Huennekens, 1955). Gabrio, Donohue *et al.* (1956) stated that inosine was even more effective than adenosine in restoring the phosphorylated esters of stored red cells, and Gabrio, Finch and Huennekens (1956) concluded that adenosine acted simply as a source of inosine, and that inosine is split by red cells into ribose-5-phosphate which is incorporated into the carbohydrate cycle, and hypoxanthine which remains in the plasma.

Nevertheless, the weight of evidence is firmly against the idea that inosine is as good a red cell preservative as adenosine. In extensive trials of 'ACDI', i.e. ACD to which 1.8 g. inosine had been added for 450 ml. of blood, inosine proved to be a disappointing preservative, results being only slightly better than those observed with ACD alone (Lange *et al.*, 1958). Mollison and Robinson (1959) pointed out that this was partly because comparisons were made after 21 or 28 days of storage when even blood stored with adenosine is not very much better preserved than blood stored with ACD alone and partly because inosine is a less effective preservative than adenosine, an assessment which they supported with some new evidence.

Several workers have found that adenosine is more effective than inosine in raising the ATP content of stored red cells, and have concluded that the purine moiety is used as a substrate for ATP synthesis (Overgaard-Hansen *et al.*, 1956; Hughes Jones, Mollison and Robinson, 1957; Mollison and Robinson, 1959). Moreover it has recently been shown that red cells are highly permeable to adenosine (Whittam, 1960).

If the purine moiety of adenosine, that is adenine, is used as a substrate for ATP synthesis, then adenine itself should be effective in red cell preservation. Nakao *et al.* (1960) showed that when blood was stored with adenine and inosine, red cell preservation, as judged by the ATP level and

osmotic resistance of the red cells was better maintained than when blood was stored with inosine alone. Preliminary observations by Drs E. Simon and R. Chapman in the laboratory of Dr C. A. Finch (personal communication) suggest that the addition of adenine alone to ACD blood has some beneficial effect. Thus, adenine was added in a dose of about 20 mg. (150 μmoles) per 100 ml. of red cells and the blood was then stored for 39 to 46 days. The average post-transfusion survival of the red cells in twenty-four recipients was just over 70 per cent, compared with an average of 41 per cent in eighteen control subjects receiving red cells stored for the same period in ACD alone. The striking thing about this effect is that it can be achieved with a relatively small dose. Thus, the amount of adenine which is effective is about 150 μmoles per 100 ml. of red cells, whereas the most effective amount of adenosine is about 2500 μmoles per ml. of red cells.

In summary, adenosine is almost certainly more effective than inosine in maintaining the viability of red cells during storage, but it seems likely that a mixture of inosine and adenine will prove to be just as effective as adenosine, and certainly less toxic. If it proves possible to obtain consistently satisfactory results by using adenine alone this would have the advantage of diminishing considerably the purine load imposed on the recipient.

Toxicity of purine nucleosides. In animals, the injection of adenosine produces slowing of the heart, impaired auriculo-ventricular conduction and a transient fall in blood pressure (Drury and Szent-Györgyi, 1929; Bennet and Drury, 1931). In man, the intravenous injection of doses between 5 and 100 mg. produces transient faintness, passing off within half a minute; the heart slows and transient heart block may develop (Honey *et al.*, 1930).

If adenosine were to be used as a red cell preservative, these toxic effects would probably not be observed because the compound is rapidly deaminated to inosine when it is added to blood. Rubinstein *et al.* (1956) found that when adenosine in a concentration of 0.4 per cent was added *in vitro* to blood which had been stored at 4° C. for 2 weeks and the mixture was then incubated at 37° C., no adenosine could be detected 2 hours later; if the mixture was left at 4° C., no adenosine could be detected after 19 hours.

Inosine is certainly less toxic than adenosine and injections of 7000 μmoles produce no untoward symptoms though they are followed by an increased excretion of uric acid (Hennessey *et al.*, 1957). This hazard was

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stressed by Rubinstein *et al.* (1959) who stated that 2.5 g. of inosine would yield 1.5 g. of uric acid.

The effects of guanosine were tested by Bennet and Drury (1931). In guinea-pigs it had little influence on blood pressure, compared with adenosine. However, when relatively large amounts of guanosine were injected intraperitoneally there was a severe leucopenia followed by a moderate leucocytosis. Prankerd (1956) transfused a human subject with 500 ml. of blood to which 150 mg. of guanosine had been added; there was no fall in blood pressure and no effect on the electrocardiogram.

Although it appears that inosine and guanosine are relatively non-toxic, more information is needed before they can be recommended for routine addition to stored blood.

Adenine is also probably not very toxic although there does not appear to be any information about tests in human subjects. In rats, doses of 200 mg./kg. daily for a week produced no toxic effects (Brown *et al.*, 1948).

Effect of Storage Temperature on Post-Transfusion Survival

Blood is commonly stored at a temperature of about 4° C. but the reasons for the choice of this temperature are not obvious. Perhaps the most likely explanation is that the intention has always been simply to keep blood as cold as possible without allowing it to freeze. In fact, blood mixed with ACD solution freezes at about −0.5° C. and may be supercooled to −3° C. and maintained indefinitely at that temperature without freezing (Strumia, 1954a).

According to Dorsey (1948; for reference see Meryman, 1956) pure water in small samples (up to 10 ml.) does not freeze at temperatures higher than −3° C.

It might therefore appear that a temperature in the region of 0° C. to −2° C. ought to be used, rather than 4° C. It is rather surprising that so few attempts have been made to discover the optimal temperature for red cell preservation.

Strumia (1954b) claimed that provided a low dextrose concentration is used, storage at 1° C. to 2° C. was superior to storage at 4° C. to 6° C. This conclusion was based on observations of the recipient's bilirubin concentration after transfusion, and the data provide no quantitative information on the relative survival of red cells stored at these two temperatures.

Hughes Jones (1958b) made a careful study of the effect on preservation of storage at temperatures between −10° C. and +10° C. He used the same medium for storage at all temperatures, namely, a solution containing citrate-phosphate-glucose and glycerol. The blood of each of a small

group of volunteers was stored in turn at four different temperatures, namely: −10° C., −2° C., +4° C. and +10° C.; this was a very important point because, as mentioned above, considerable differences are found between donors, and if comparisons based on small numbers are to be valid each donor must serve as his own control. The mean post-transfusion (24 hour) survival after 34 days' storage was: −10° C., 80 per cent; −2° C., 78 per cent; +4° C., 63 per cent and +10° C., 52 per cent.

This work of Hughes Jones seems to show clearly that 4° C. is not the ideal temperature from the point of view of good preservation, although it may still be the best compromise between a temperature which gives good preservation and one which is also safely above the freezing point of blood. The experiment also showed that deterioration was not much more rapid at 10° C. than at 4° C. It would not be safe to conclude that even higher temperatures permit good preservation; Ross *et al.* (1947) found that storage at 25° C. for 4 days in Alsever's solution (see above) reduced the post-transfusion survival of the red cells to 25 per cent.

Blood stored at 37° C. deteriorates very rapidly, only 30 per cent of the cells being viable after 24 hours and virtually none being viable after 48 hours (Jandl and Tomlinson, 1958). The author has found that if blood is incubated at 37° C. for 2 hours immediately after taking it from the donor and is then stored for 28 days, red cell survival is significantly diminished compared with the survival of red cells from the same donor stored for the same period but without 2 hours' incubation (see Table I).

TABLE I

EFFECT OF A PRELIMINARY 2 HOURS' INCUBATION AT 37° C. BEFORE 28 DAYS' STORAGE AT 4° C. ON THE SUBSEQUENT POST-TRANSFUSION SURVIVAL OF RED CELLS

Subject	(*A*) **No preliminary incubation*			(*B*) **Preliminary incubation*		
	Survival† *at*			*Survival*† *at*		
	10 *minutes*	24 *hours*	48 *hours*	10 *minutes*	24 *hours*	48 *hours*
1	94	65	67	92	54	53
2	82	79	76	91	74	74
3	94	82	82	87	75	71
4	80	66	66	78	58	59
Mean	87.5	73.0	72.8	87.0	65.3	64.3

For the 24-hour figures (A vs. B), $t = 3.68$, $p = < 0.05 > 0.01$.

* In order to avoid systematic bias, the red cells of subjects 1 and 2 were stored first by method A, then by method B, and those of subjects 3 and 4 first by method B, and then by method A.

† Survival determined by labelling with ^{51}Cr; results corrected for Cr elution, including an allowance for 'early loss'.

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The details of the experiment are recorded in Table I to call attention to the value of comparing different methods of storage in the same subject. For example, if method B had been used to store the red cells of subjects 2 and 3 and method A for subjects 1 and 4 the conclusion would have been reached that method B gives a better 24-hour survival (average 74.5 per cent) than method A (average 65.5 per cent) – the very reverse of the probable truth.

There seems to be no need to cool blood rapidly after taking it from the donor. Strumia *et al.* (1956) compared normal slow cooling, that is simply putting blood into a refrigerator at 6° C., when it took 12-15 hours to reach refrigerator temperature, with moderately rapidly cooling, that is bringing it to 5° C. within 30 minutes. After 3 weeks' storage, the survival of the red cells appeared to be the same in the two cases. Very rapid cooling, achieved by passing the blood through steel coils, which brought the blood to a temperature of 2° C. in less than 4 seconds, invariably produced damage to the red cells.

Storage below the Normal Freezing Point of Blood

When a solution freezes, pure ice forms and the remaining liquid becomes hypertonic. Lovelock (1953a) has shown that many of the damaging effects of freezing upon tissues are those expected from exposure of the tissues to a hypertonic solution followed (on thawing) by exposure to an isotonic solution. The damaging effect of hypertonicity takes time; thus if tissues can be cooled within a few seconds to a temperature where virtually all the water is frozen, and thawed equally rapidly, serious damage can be avoided.

Very Rapid Freezing

Luyet and Gehenio (1940) showed that if red cells were subjected to ultra-rapid cooling and thawing they could be recovered intact. Meryman (1956) showed that red cells could be successfully frozen and thawed in the same way. Thus the red cells were first mixed with glucose and then sprayed into liquid nitrogen. The resulting droplets were kept at the temperature of liquid nitrogen (—163° C.) and subsequently thawed by being dropped into warm saline. This process has been used for the storage of small volumes of red cells for blood grouping tests. Meryman found that unless glucose was added to the blood before cooling the yield of intact red cells was very small.

Strumia *et al.* (1960 a, b) have carried this work further and shown that better yields of red cells after their rapid freezing and thawing are obtained

if the blood is first mixed with lactose and glucose. The sugars are thought to slow down the formation of ice crystals; the mixture of lactose and glucose is preferable to glucose alone because lactose does not penetrate the red cell membrane and it thus tends to prevent red cell swelling. Strumia *et al.* have studied the effect of very rapid freezing and thawing of blood samples of the order of 5-50 ml. The chief problem is to obtain very rapid heat transfer and the use of thin-walled vessels is essential. They have succeeded in recovering 95 per cent of the red cells intact and have shown that the cells survive very well after transfusion. Attempts are now being made to apply this process successfully to amounts of blood suitable for transfusion; the present indications are that it will be very difficult to avoid haemolysis completely during freezing and thawing.

Slow Freezing after Mixture with Glycerol

An alternative approach to the storage of red cells at low temperatures is to mix them with glycerol before freezing. They can then be frozen and thawed quite slowly without damage (Polge *et al.*, 1949; Audrey Smith, 1950). Lovelock (1953b) has advanced the theory that glycerol, by limiting ice formation, provides a liquid phase in which the salt is distributed as cooling proceeds; thus excessive hypertonicity is avoided.

Sloviter (1951) showed that red cells which had been mixed with glycerol and frozen and thawed could be freed from glycerol by dialysis and were then capable of survival *in vivo*.

Red cells mixed with a glycerol-citrate-phosphate solution (see below) and stored at —20° C. deteriorate only very slowly; after 3 months' storage the post-transfusion survival is only slightly less than that of fresh cells (Chaplin *et al.*, 1954). Even after 18 months' storage more than 50 per cent of the red cells are still viable (Hughes Jones, Mollison and Robinson, 1957. See Fig. 5).

At lower storage temperatures, deterioration is virtually arrested. Thus at temperatures in the range —40° C. to —50° C. there does not seem to be any definite falling off in red cell survival over a period of up to one year or more (Chaplin *et al.*, 1957; Hughes Jones *et al.*, 1957).

Moreover, if red cells which have been stored at —45° C. for 3 months are deglycerolized and stored at 5° C. for 10 days before transfusion, they still have a survival of about 90 per cent; survival is just as good when the red cells are stored at 5° C. in a buffered citrate-dextrose solution as when they are stored in a solution containing protein (Schmidt and Steindfeld, 1960).

If red cells are to be stored at temperatures in the range —40° C. to

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—50° C. they must be mixed with sufficient glycerol to give a final concentration of 4.5 M (approximately 40 per cent w/v) if haemolysis is to be completely prevented (Hughes Jones *et al.*, 1957).

Storage at —79° C. (achieved by adding solid CO_2 to a bath of alcohol) was used in some of the earlier experiments with glycerol-treated red cells and shown to give satisfactory preservation (Mollison *et al.*, 1952; Brown and Hardin, 1953). After a period of storage as long as 21 months

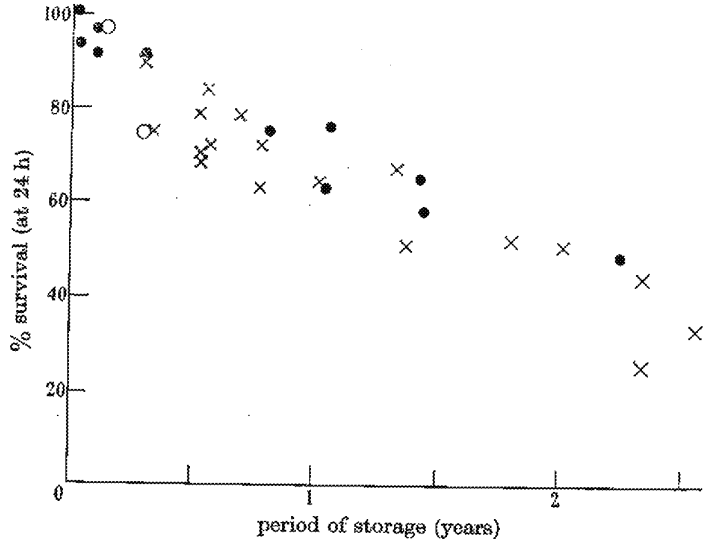

FIGURE 5. Percentage survival, 24 hours after transfusion, of red cells stored at —20° C. for various periods in different glycerol-containing solutions.
● Survival of red cells stored in a solution containing 0.06 M-tripotassium citrate, 0.02 M-potassium dighydrogen phosphate, 0.02 M-dipotassium hydrogen phosphate and 0.005 M-glucose.
× As above but sodium phosphates.
○ As above but sodium citrate and sodium phosphates.

at this temperature, post-transfusion survival may be as good as after storage for only a few weeks (Chaplin, Crawford *et al.*, 1956).

If solutions containing more than about 50 per cent (w/v) glycerol are added to blood, some haemolysis results. The addition of 50 per cent (w/v) glycerol in citrate to an equal volume of blood will give a final concentration of approximately 30 per cent (w/v) in the fluid phase of the mixture, because the glycerol mixes not only with the plasma but also

with the water space in the red cells, amounting to about 65 per cent of their volume.

Thus if a final concentration exceeding 30 per cent (w/v) glycerol is to be attained it is best to add the glycerol in stages; for examples see Hughes Jones *et al.* (1957).

In storage of red cells there are two reasons for avoiding very high glycerol concentrations. First, subsequent washing of the red cells free of glycerol is made harder. Second, exposure of red cells to a final glycerol concentration of 6.0 M (about 50 per cent w/v) interferes with red cell metabolism and diminishes red cell viability (Hughes Jones *et al.*, 1957).

Removal of glycerol from red cells. If red cells which have been stored with glycerol are to be transfused their glycerol content must be reduced to about 2 per cent, as otherwise they will haemolyse on contact with plasma. The removal of glycerol can be accomplished by an intermittent process of washing them in a series of successively lower concentrations of glycerol. Another possibility is to add a very high concentration of citrate to the red cells (Lovelock, 1952). This shrinks the cells and results in the removal from them of a considerable proportion of the glycerol. Thus if the red cells are originally mixed with glycerol to give a final concentration of approximately 20 per cent (w/v) and are then mixed with 30 per cent citrate they can subsequently be resuspended in saline with relatively little lysis (Lovelock, 1952).

If the red cells are equilibrated with a concentration of glycerol exceeding 20 per cent (w/v) and are then washed once in 30 per cent trisodium citrate they cannot subsequently be resuspended in saline without gross lysis. However, if they are washed in a mixture of hypertonic citrate and an appropriate concentration of glycerol it is possible to reduce the number of washes, that is to say, to give less than would be necessary if the cells were simply washed in a series of solutions of successively more dilute solutions of glycerol. Some suitable methods of washing red cells will be found in Chapter XII.

One other way of washing the red cells free from glycerol is by a continuous process in which the concentration of glycerol in the wash solution is progressively reduced, as in the centrifuges described by Brown and Hardin (1953) and Mollison, Lister *et al.* (1958).

Red cells can be glycerolized and deglycerolized in the centrifuge known as the Cohn fractionator and a large number of transfusions of red cells prepared in this way have been given (Haynes *et al.*, 1960).

In most transfusion experiments carried out so far with red cells stored

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in glycerol, the glycerol concentration of the blood at the time of transfusion has been less than 1 per cent. However, it is reassuring to know that Sloviter (1958) has given infusions of as much as 50 g. of glycerol, as a 5 per cent solution in saline, in a period of 2 to 3 hours to human subjects, without any obvious ill effects.

Use of dimethyl sulphoxide. Lovelock and Bishop (1959) found that dimethyl sulphoxide penetrated cells more rapidly than glycerol and that less of the solute was required for complete protection against damage by freezing. Ashwood-Smith (1960) showed that the toxicity of dimethyl sulphoxide was only about half that of glycerol, when injected intraperitoneally into mice; and he also showed that when dimethyl sulphoxide in a final concentration of 15 per cent (v/v) was added to mouse bone-marrow cells and the mixtures were stored at —70° C., the recovered cells were morphologically normal and were capable of synthesizing protein and lipid.

Despite all this favourable evidence, it does not seem that dimethyl sulphoxide has any advantages over glycerol for the preservation of human red cells.

Gas-Transport Function of Stored Red Cells

Valtis and Kennedy (1954) pointed out that the oxygen dissociation curve of stored citrated blood was shifted to the left, suggesting that such blood after transfusion would be, at least temporarily, incapable of releasing as large a volume of oxygen to the tissues as normal blood. They found the changes were nearly maximal after 1 week's storage in ACD and this was confirmed by Gullbring and Ström (1956). The possible clinical significance of these observations is considered in Chapter III.

Other Reversible Changes in Stored Red Cells

When red cells are cooled to 4° C. the rate of consumption of dextrose is greatly slowed and the active transport of sodium and potassium is almost halted. There is thus a tendency for the intracellular and extracellular concentrations to come into equilibrium. In a period of 3 weeks red cells stored in ACD lose almost half their original content of potassium and the plasma concentration rises to about 30 m.Eq./litre (Loutit *et al.*, 1943). These changes are largely reversed within 24 hours of transfusion, although when they are more extreme, as in red cells stored at —20° C. in a sodium citrate-glycerol mixture, they may not be fully reversed for several days after transfusion. Thus Crawford and Mollison (1955) studied the survival

of red cells which, after storage, had a potassium content of only 20-40 m.Eq./litre; in the first 24 hours only half the lost potassium was regained; even after 4 days the cells had still not quite regained their normal potassium content. The red cells at the time of transfusion were shrunken and

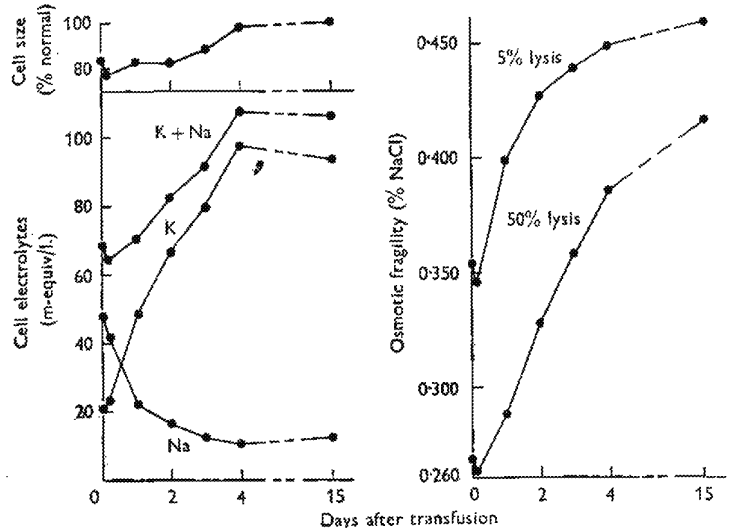

FIGURE 6. Reversal of changes in stored red cells after transfusion. Before transfusion the red cells had been stored for 3 months in a glycerol-sodium citrate-sodium phosphate mixture and had a very low potassium content. After transfusion the normal electrolyte content was not fully restored for more than 4 days (from Crawford and Mollison, 1955).

had a greatly reduced osmotic fragility. These changes also were not fully reversed within 4 days of transfusion; see Fig. 6.

The organic phosphate content of stored red cells is fully restored within 3 hours of transfusion (Gabrio, Stevens and Finch, 1954), as expected from results *in vitro*, see p. 20.

THE SURVIVAL OF TRANSFUSED LEUCOCYTES

Normal Life-span of Leucocytes

Kline and Cliffton (1952) carried out studies in man by injecting ^{32}P which was incorporated into the nucleic acid of the cells; they concluded that the average life-span of leucocytes of all types was 12.8 days, from the time of administration of phosphorus to the time of destruction of the

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cells (approximately 4 days in the marrow and 9 days in the peripheral circulation).

Subsequent work has shown that there is a considerable difference between the life-span of different types of cells. Thus most lymphocytes probably live for a few months (Gowans, 1959) whereas polymorphonuclear neutrophiles probably have a life-span in the blood-stream of only 2 or 3 days, as judged by labelling *in vivo* with $DF^{32}P$ (Athens *et al.*, 1959).

According to Osgood (1954), for every one leucocyte in the blood there are 40–400 in the tissues. It follows that even if leucocytes were unaffected by withdrawal from the body and transfusion into the circulation of another individual, a transfusion of 500 ml. of blood to a normal adult would not be expected to produce any prolonged change in the recipient's total leucocyte count. Nevertheless available observations will be described.

The Survival of Transfused Leucocytes

Weisberger *et al.* (1949) obtained leucocytes from a rabbit by injecting saline intraperitoneally and later irrigating the peritoneal cavity with saline. Heparin was used as an anticoagulant but the leucocytes were promptly washed in Tyrode's solution and were shown to be still capable of phagocytosis and amoeboid movement. The leucocytes were now injected into an ear vein of the same animal; within 3 minutes the animal's total leucocyte count had fallen by about 70 per cent. It appeared that this was a secondary effect, resulting from the rapid disintegration of the transfused leucocytes with liberation of a leucopenia-producing substance.

In further experiments, the leucocytes were tagged with radioactive phosphorus *in vitro*; evidence was obtained that after injection they were trapped in the pulmonary capillaries (Weisberger *et al.*, 1950).

The rapid disappearance of transfused leucocytes from the circulation has also been demonstrated in man. White (1954) tagged leucocytes by mixing them with atebrin which forms a complex with desoxyribose nucleic acid. 500 ml. of blood was taken from donors into ACD to which 50 mg. atebrin had been added. The blood was immediately re-transfused, either to the original donor or, in other cases, to suitable recipients. The tagged cells persisted in the circulation for only 30–90 minutes.

Distinctly longer survival was found by Rosse and Gurney (1959), who used the Pelger-Huët abnormality in leucocytes as a marker. They found 30–50 per cent survival at 6 hours, and 5–10 per cent at 24 hours.

Although it appears to be impossible to produce a prolonged increase in the recipient's leucocyte count by ordinary transfusion procedures, a slightly longer increase has been produced by cross-circulation. Thus Lawrence *et al.* (1945) anastomosed a blood vessel of a normal cat (to be regarded as the donor) to one of a cat rendered leucopenic by previous irradiation. The circulations were allowed to mix until the leucocyte count of the 'recipient' cat had reached a stable level. The cross-circulation was then discontinued. Frequent leucocyte counts were made on the blood of the recipient cat and it was found that virtually all the leucocytes disappeared within a period of some 18 hours following transfusion.

Conversely, Hollingsworth *et al.* (1957) removed leucocytes from a rat by carrying out an exchange transfusion with leucopenic rat blood. The recipient mobilized leucocytes so rapidly that severe leucopenia did not develop during the exchange transfusion, and 30 minutes after the transfusion the leucocyte count was back to normal.

The role of white cells in causing transfusion reactions is discussed in Chapter XV.

THE SURVIVAL OF TRANSFUSED PLATELETS

Normal Life-Span of Platelets

The survival of a subject's own platelets in his own circulation can be estimated after injecting ^{32}P-labelled DFP (di-isopropyl fluorophosphonate). This compound combines with esterases present in platelets as well as in red cells, and if platelets are separated from blood samples taken after the injection of $DF^{32}P$ the rate of disappearance of the labelled population of platelets can be studied because the 'label' is not re-utilized. In four normal subjects the platelets were found to disappear in a linear fashion over a period of 8 or 9 days (Leeksma and Cohen, 1955).

Platelets can also be labelled *in vitro*, by incubating them with $Na_2{}^{51}CrO_4$, although they take up only 10 per cent of the dose used to label them (Maupin and Lovendo, 1959). Studies with ^{51}Cr-labelled platelets have confirmed that the mean life-span is of the order of 8–11 days (Aas and Gardner, 1958; Baldini, Costea and Dameshek, 1960).

Survival Time of Transfused Platelets

In subjects with aplastic anaemia with a very low platelet count, the transfusion of a large amount of fresh blood may produce a temporary increase in platelet count. If this increase is carefully estimated, the survival time of transfused platelets can be deduced. From measurements of this kind Sprague *et al.* (1952) concluded that the platelet survival time was 4–5 days, although in patients who received repeated transfusions, platelet

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survival became progressively shorter, presumably due to the formation of platelet iso-antibodies (Sprague *et al.*, 1952; Stefanini *et al.*, 1952).

When platelets are labelled *in vitro* with ^{51}Cr and injected, either into the donor's own circulation or into the circulation of another subject, the ^{51}Cr counts in the circulation are found to be relatively low immediately after the injection, suggesting that only about 25 per cent of the platelets have survived, but on the following day 60 per cent or more of the injected platelets are found in the circulation; thereafter the platelets disappear over a period of 9-11 days (Aas and Gardner, 1958; Baldini, Costea and Dameshek, 1960).

The phenomenon of temporary sequestration of injected platelets was also observed by Adelson *et al.* (1957). These workers obtained platelets labelled with ^{32}P simply by bleeding patients with polycythaemia vera about 6-7 days after they had received a therapeutic dose (2-5 mc.) of ^{32}P. At this time in the donor's circulation there was relatively little ^{32}P in the plasma and a great deal in the platelets; the platelets were washed repeatedly and then transfused into other subjects. Immediately after transfusion the counts in the blood indicated the presence of only 25 per cent of the injected platelets, but a few hours later the number had risen to approximately 100 per cent. There was then an exponential fall with a half-time of approximately 2 days.

Lewis and Szeto (1959) studied the phenomenon of temporary sequestration of injected platelets by using ^{51}Cr as a label and carrying out surface counting; from this they concluded that the liver and spleen were the sites of sequestration.

Factors affecting the Viability of Transfused Platelets

When bleeding donors for platelet transfusion it seems to be very important to prevent all traces of fibrin formation. Perkins *et al.* (1958) found that the fall in platelet count that occurs when blood is mixed with heparin *in vitro* is greater with small amounts of heparin. They concluded that it was due to clumping and disintegration of platelets rather than to adherence to foreign surfaces, since it was just as great in siliconized glass containers or polythene vials as in uncoated glass containers.

Until recently it was thought that platelet viability was adversely affected by taking the donor's blood into an untreated glass container rather than into silicone-coated glass, or plastic equipment. It is no longer possible to believe that this is of much practical importance in view of results such as those quoted in the previous paragraph and particularly in view of work done by Jackson and Krevans (1960). These authors

carried out massive exchange transfusions in dogs rendered thrombocytopenic by irradiation. Provided fresh donor blood was used, blood taken into glass bottles was as effective as blood taken into plastic equipment in preventing thrombocytopenia. In other experiments they showed that the anticoagulant with which the dog's blood was mixed had no influence on the result, heparin and ACD being just as good as EDTA. They found that the really important factor determining platelet viability was the period for which the blood had been stored. Thus immediately following the transfusion of fresh blood the number of surviving platelets was about 80 per cent of the expected number and 3½ days later 50 per cent were still present. After the transfusion of blood stored for 24 hours the number of surviving platelets fell to 50 per cent in 2½ days. After the transfusion of blood stored for 48 hours the number of platelets in the recipient's circulation immediately after transfusion was less than 50 per cent of the number expected and all platelets had disappeared after a few days. Blood stored for 5-6 days was useless as a source of platelets.

It should perhaps be stressed that these experiments were all carried out in dogs and it is just possible that human platelets may prove to be more readily damaged *in vitro*, for example by contact with untreated glass surfaces, so that after all the use of silicone-coated containers or plastic containers may prove to be desirable in human transfusion practice. Until better evidence has been produced of the value of handling human platelets in this way, it seems reasonable to preserve a sceptical attitude.

Effect of storage. The actual number of platelets in stored blood diminishes quite slowly. For example, Soulier *et al.* (1954) found that blood stored in ACD for 3 weeks might contain 75 per cent of the original number. There was evidence of qualitative changes; for example, on re-calcifying the blood clot-retraction was less than normal.

Such evidence as exists about the effect of storage on human platelets supports the idea that it exerts a very unfavourable effect on viability. Thus Adelson *et al.* (1957) reported that in a single experiment blood stored overnight in a glass bottle at 4° C. provided almost no viable platelets, and Baldini, Costea and Dameshek (1960) found that platelet viability was negligible after 24 hours' storage at 4° C. even when the special dextrose-acetate-saline-gelatin solution of Tullis *et al.* (1959) was used.

Jackson *et al.* (1956) found that when blood stored for 18 hours was used for exchange transfusion in infants there was an immediate fall in platelet count and thereafter a progressive fall for 48 hours, suggesting

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that some transfused platelets survived but with a diminished life-span.

In experiments with rabbit platelets, Baldini, Ebbe and Dameshek (1960) found that the use of a mixture containing adenine and inosine together with phosphate buffer, glucose and plasma gave slightly better preservation than plasma-saline alone but even so platelet survival 24 hours after transfusion was negligible. Platelets frozen in glycerol, thawed and mixed with hypertonic glucose to deglycerolize them, survived better than platelets stored at 4° C. for 24 hours, but even so their survival was very poor.

The role of platelets in transfusion is considered further in Chapter III (platelet transfusions in haemorrhage), in Chapter XV (reactions due to platelet iso-antibodies) and in Chapter XVII (platelet counts following exchange transfusion).

THE SURVIVAL OF TRANSFUSED PLASMA PROTEINS

Transfusions of whole plasma find their main use in the treatment of acute depletion of the plasma volume, and this is considered in Chapter III. The present section is concerned solely with the temporary replacement of particular plasma proteins.

Albumin

If albumin (labelled with ^{131}I) is injected into a normal subject its disappearance can be described by a two-component curve. The rapid component has a half-time of about 0.5 days, and this represents mixing of the injected albumin with the total exchangeable albumin pool in the body; the pool consists of the plasma albumin, amounting to about 130 g., and the extravascular albumin, which is also about 130 g. Thus, 24 hours after an injection of albumin only about 60 per cent is left in the circulation. When the mixture with the total albumin pool is complete, the level of injected albumin falls much more slowly (half-time about 11 days), the rate being determined by normal katabolism (Sterling, 1951).

There are two relatively common conditions in which the concentration of serum albumin is severely diminished, namely, in the nephrotic stage of nephritis and in cirrhosis of the liver.

Nephrosis. In the so-called 'nephrotic stage' of nephritis the patient develops very severe hypo-albuminaemia due to continued loss of albumin in the urine. Other proteins are also lost in the urine in this condition, but albumin, due to its smaller molecular size, is lost predominantly. Infusions of albumin cannot be expected to produce more than a very transient improvement, and any sustained increase in the plasma albumin concentration is out of the question because the rate of excretion in the urine increases as the plasma concentration rises.

In practice the results of albumin infusions are disappointing. For example, Luetscher *et al.* (1949) treated thirteen patients with the nephrotic syndrome; these were relatively favourable cases, being free from marked hypertension or renal insufficiency. Moreover large amounts of albumin – approximately 50 g. per day for 3 to 6 days – were administered. In most cases the total amount of circulating protein increased during the course. However, only in four cases was the protein concentration increased; these four lost their oedema after the injection. In the group as a whole, the plasma protein changes were short-lived. Hardwicke (1954) estimated the overall protein deficiency to be 3-5 kg. in severe cases of the nephrotic syndrome; even if infused albumin were retained in the body and all built into body protein, at least 100 days of administration would be necessary to restore this deficit.

Cirrhosis. In cirrhosis of the liver there is chronic under-production of albumin and the plasma level may be below 1 g. per 100 ml. Infusions of albumin have been used in two main ways: firstly to relieve oedema temporarily, and perhaps get the patient fit for operation; secondly, to maintain the plasma albumin concentration at a more or less normal level for an indefinite period and permit a return to normal activity.

In the treatment of cirrhotic patients with oedema, recently introduced diuretics have proved so effective that infusions of albumin now seem to have little place. A very successful diuretic regime, in which mannitol, chlorthiazide and spirinolactone are combined, has been described by Shaldon *et al.* (1960).

Relatively few attempts have been made to give really large amounts of albumin over long periods. In a small series treated at Birmingham patients were given a 'loading dose' of 700-1000 g. of albumin, and were subsequently given about 50 g. a week. The object of this plan was to raise the plasma albumin concentration to 4 g./100 ml., and this was achieved in ten out of thirteen cases. Seven of the ten patients were kept free from oedema and were able, for a time, to lead practically normal lives. Nevertheless, their total expectation of life may not have been increased.

Dr Patricia Wilkinson and Professor Sheila Sherlock (personal communication) have treated selected patients with cirrhosis, by giving infusions of 50-150 g. of albumin every 2 weeks. Although the

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patients were kept free from oedema, it appears that their state was no better than that of control cases treated with diuretics alone.

Martin (1954a) treated a series of patients with hypo-albuminaemia, several of whom had hepatic cirrhosis, and showed that albumin prepared by the ether-fractionation process of Kekwick and MacKay (1954) could safely be given in doses up to 1 g. per kilogramme body weight, at a rate equal to 15 g. per hour. When this rate was exceeded, symptoms of overloading, consisting of gross venous engorgement of the neck veins, occipital headache and a rising pulse rate were observed.

Congenital analbuminaemia. A family with congenital failure to form albumin was studied by Bennhold and Kallee (1959); infused albumin was found to disappear more slowly than in normal subjects.

Fibrinogen

The rate of disappearance of injected fibrinogen has been studied by giving injections to patients with the very rare condition, hereditary afibrinogenaemia. Gitlin and Borges (1953) studied two cases and found that half the injected fibrinogen disappeared during the first 24 hours, presumably due to mixing with the protein 'pool'; thereafter the fibrinogen disappeared with a half-time of 4 days.

In another case, Ingram *et al.* (1960) studied the survival of fibrinogen, prepared from 'time expired' blood and found that it was indistinguishable from that of fresh blood; i.e. up to half of the injected dose was rapidly lost from the circulation and the remainder disappeared at the rate of about 15 per cent a day.

The Defibrination Syndrome

This syndrome is most commonly associated with accidental retroplacental haemorrhage, retention of a dead foetus *in utero* (for 8 weeks or more) or amniotic fluid embolism of the lungs, but may also be associated with lung or cardiac surgery or with incompatible blood transfusion. One theory is that in these conditions thromboplastic substances are liberated leading to the formation of extensive clots (fibrination) which is followed by fibrinolysis (Schneider, 1956). Another theory is that tissue kinases liberated from damaged tissues activate the fibrinolytic system (see Sharp *et al.*, 1958). In any case the result is severe depletion of plasma fibrinogen and, ultimately, incoagulability of the blood.

According to Sharp *et al.* (1958) the most reliable diagnostic test is to

estimate the reactivity of the plasma to purified thrombin. This may be grossly diminished in cases in which orthodox biochemical methods of estimation suggest that the level of fibrinogen is not dangerously low; that is, it appears that part of the lesion may be an alteration of the reactivity of fibrinogen to thrombin.

It has been suggested that the critical fibrinogen level is 90 mg./100 ml., compared with the mean normal level in pregnancy at term of 300 mg./100 ml. (Hodgkinson *et al.*, 1954).

Once the diagnosis of defibrination syndrome is made, a concentrated solution of fibrinogen should be given intravenously, or, if this is not available, plasma or blood should be transfused. A suitable initial dose of fibrinogen for an adult is 3-6 g. On reconstitution with 200 ml. of distilled water a bottle of the dried material produced in the U.K. contains 200 ml. of a 2 per cent solution, i.e. 4 g.

If plasma is given, 1 litre should be transfused, the concentration of fibrinogen in citrated plasma, reconstituted from the dried product, being about 0.35 g. per 100 ml. (Scott, 1955).

Anti-Haemophilic Factor (AHF)

The transfusion of AHF from one person to another presents special problems because the material is very labile. Thus Pool and Robinson (1959) found that significant amounts were lost during the separation of plasma from red cells immediately after bleeding. Mustard (1957) stressed the importance of preventing any activation of the coagulation mechanism during the taking of blood, and considered that the siliconing of glassware helped to preserve AHF, possibly by diminishing the loss of platelets. Rapoport *et al.* (1959) found that when blood was taken into siliconized bottles comparatively little AHF activity was lost, and they concluded that even after 24 hours' storage at 4° C. at least 60 per cent of AHF was normally present.

Pool and Robinson found that even when plasma was stored at −20° C. there was progressive loss of AHF (half-time of decay about 4 months). It appears that at present the only reliable way of preserving AHF for really long periods is to dry it. Pool and Robinson (1959) tested some commercial lyophilized material and found that its AHF content was about 70 per cent of normal. Kekwick and Wolf (1957) described the preparation of a concentrate of human AHF which contained not less than 85 per cent of the initial activity. The material was lyophilized, and was twenty to twenty-five times as active per gramme of protein as the original plasma.

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As described below, the infusion of AHG (that is, anti-haemophilic globulin, containing AHF) produces only a very transient rise in plasma AHF concentration. If the level is to be raised for a long period, very large amounts of material must be infused. It was this consideration which led Bidwell (1955) to prepare AHF from animal blood, since not only are very large amounts available but the relative quantity of AHF in the blood of some animals is very great compared with the amount in human plasma (Macfarlane *et al.*, 1954). Thus current preparations of bovine or porcine material contain in each gramme of material the equivalent of 2 litres of fresh human plasma. Unfortunately the animal preparations are antigenic and can be used only for periods of about 1 week or 10 days. As a rule a second course of material from the same animal cannot be given.

Transfusions to Haemophiliacs of Materials containing AHF

Transfusions may be given to haemophiliacs either in the hope of arresting haemorrhage or of preventing it. To consider prevention first, if a haemophiliac is to have a tooth extracted it is much easier to minimize bleeding by giving a transfusion before the extraction than by giving a transfusion after bleeding has started. Biggs (1957) has found that the transfusion of 1 litre of fresh plasma in 45 minutes immediately before the tooth is extracted may suffice to reduce post-operative bleeding to a level at which it is no longer troublesome. Such a transfusion sometimes raises the level of AHF by 20 per cent or more. Slightly lower figures were reported by Douglas (1958) who estimated the AHF level after administering fresh frozen plasma to haemophiliacs. The plasma was rapidly separated from fresh ACD blood and stored at −20° C. After administering 1 litre the mean level of AHF was 14 per cent but the level thereafter declined with a half-period of approximately 9 hours.

In order to arrest serious haemorrhage in a haemophiliac it may be necessary to raise the AHF level to approximately 30 per cent of normal (Macfarlane *et al.*, 1954). In practice, in a severe haemophiliac whose blood contains no AHF before treatment, a level of 30 per cent AHF cannot be achieved by the transfusion of fresh human blood or plasma. Pool and Robinson (1959) found that an hour or two after transfusion the AHF level in the recipient's plasma was that expected if one-third of the injected AHF had already disappeared. The remaining AHF disappeared with a half-time of about 10 hours. This agrees almost exactly with the estimate of Biggs (1957) and with that of Douglas (1958) quoted above.

In a severe haemophiliac, a level of 30 per cent of normal AHF can be achieved only by using concentrates either of human or animal material.

Use of concentrate of human AHF. As mentioned above, Kekwick and Wolf (1957) described the preparation of a concentrate of human AHF. The final product was a dry powder which on reconstitution with 100 ml. of distilled water was equivalent to 1000 ml. of fresh human plasma (600–1000 ml. according to subsequent experience). Satisfactory clinical results were observed in six cases. Subsequently this preparation has been used in a considerable number of patients with good therapeutic results, and it seems certain that it is the material of choice for the treatment of serious bleeding in haemophiliacs. Unfortunately the material is difficult to prepare and only very limited supplies are available.

Use of Animal AHG. Two preparations are available, made from bovine and porcine plasma respectively. The bovine material is more likely to contain agglutinins against human platelets and for this reason the porcine material is slightly preferable. Both preparations are highly effective when they are given to a haemophiliac, but, as already mentioned, after about 10 days the recipient becomes refractory, presumably due to development of antibodies against the species protein (one says 'presumably' because in fact precipitating antibodies against the foreign protein cannot be demonstrated by conventional methods). After approximately 10 days not only do the preparations become ineffective but may provoke anaphylactic reactions. If porcine material has been given first and it is necessary to continue treatment beyond 10 days, bovine material can then be substituted and can be given for a further 7 or 10 days. Macfarlane *et al.* (1957) reported some very successful results with these materials, which contained in 200–400 mg. the equivalent of 2500 ml. of fresh human plasma. They found that when the level of AHF in the blood was raised to 30 per cent there was no abnormal bleeding. Because of the antigenicity of the materials they recommended that they should be used only in grave emergencies.

Christmas Factor (Plasma Thromboplastin Component or Factor IX)

About 15 per cent of patients who seem from their clinical history to have classical haemophilia are found on investigation to have a deficiency, not of AHF, but of Christmas factor (the figure of 15 per cent is taken from Pitney and Dacie, 1955). From the point of view of treatment the difference is important because preparations of AHF are of no benefit to patients who lack the Christmas factor. On the other hand, whereas serum contains very little AHF, it may actually be a better source of supply of Christmas factor than plasma, since Christmas factor is present in serum in

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an activated form (Bell and Alton, 1955). Moreover, Christmas factor is less labile than AHF, so that transfusions of stored blood are usually effective.

Other Clotting Factors

In haemorrhagic states clotting factors other than those already described may be deficient. For example, in haemorrhagic disease of the newborn, prothrombin, Factor VII and Factor IX are all deficient. Transfusion will temporarily correct all these deficiencies, although in practice the administration of vitamin K will usually produce a sufficient response to make transfusion unnecessary.

A point that commonly arises in using transfusion to treat haemorrhagic conditions is whether fresh blood should be used. As already mentioned, platelets and AHF deteriorate rapidly on storage. In addition to these, the only clotting factor known to deteriorate appreciably is Factor V (Ferguson and Lewis, 1948; Soulier *et al.*, 1954).

In practice the use of fresh blood is usually indicated because, although the haemorrhagic state may be originally due to a limited deficiency in the haemostatic mechanism, blood loss itself may cause multiple deficiencies.

What is 'Fresh' Blood?

'Fresh' blood is a rather vague term; it presumably should mean blood in which no appreciable deterioration of the relevant constituent has occurred. So far as platelets are concerned, this probably means blood which has not been stored at all. Cross-matching should preferably be carried out first on a small sample before bleeding the donor, so that the blood can be transfused as soon as it has been taken. So far as AHF is concerned, a little more latitude is permissible and blood stored for a few hours is satisfactory.

As described earlier, blood stored in ACD for not more than 14 days is about 90 per cent as good as absolutely fresh blood as a source of viable erythrocytes.

Gamma Globulin

In adults the plasma normally contains about 1 g. of gamma globulin per 100 ml. Five to ten per cent of the total gamma globulin has a relatively high molecular weight (approximately 1,000,000) and separates in the ultracentrifuge with a sedimentation constant of 19*S*; the remainder has a molecular weight of the order of 160,000 and a sedimentation constant of

7*S* (for references see review articles by Kunkel, H. G. and by Porter, R. R. in Putnam, 1960).

When gamma globulin with a sedimentation constant of 7*S* is injected into the circulation it equilibrates with an extravascular pool of about the same size as an intravascular pool. After equilibration the rate of turnover corresponds to a half-life of 21-26 days. Gamma globulin with a sedimentation constant of 19*S* does not equilibrate with an extravascular pool and it has a turnover rate which is at least three times as fast as 7*S* gamma globulin (Cohen and Freeman, 1960).

In newborn infants, born at term, the plasma contains about the same concentration of gamma globulin as in the adult, but during the first month or two of life there is a steady decline, because normally no gamma globulin is synthesized by the infant until it is about 5 or 6 weeks old (Zak and Good, 1959). In premature infants the plasma gamma-globulin concentration at birth may be lower than in mature infants, probably because the placenta is not freely permeable to gamma globulin until after about the thirtieth week of pregnancy (see Chapter XVII). Furthermore, in premature infants the plasma gamma-globulin concentration tends to fall to a lower level than in mature infants because the synthesis of gamma globulin is delayed.

Apart from the question of the first few weeks of life, failure to produce the normal amount of gamma globulin may be encountered in two main forms. Firstly, there is an apparently congenital form, affecting mainly male infants, which is commonly diagnosed in the first 2 or 3 years of life (Bruton *et al.*, 1952; Janeway *et al.*, 1953, Squire, 1960), usually when the infant has suffered from recurrent infections. Secondly, there is an acquired form, which may be met with in either sex at any age. Some cases are associated with multiple myelomatosis or chronic lymphatic leukaemia. Deficiency of gamma globulin may also be encountered as part of a generalized protein deficiency, as in nephritis with proteinuria, but such cases are not usually included in the syndrome of hypogammaglobulinaemia. In hypogammaglobulinaemia there is a deficiency not only of the main antibody protein, that is 7*S* gamma globulin, but also of two other antibody proteins, namely 19*S* gamma globulin (identical with the protein recognized as β_{2M} on immuno electrophoresis) and another antibody protein identified on immuno-electrophoresis as β_{2A} (see Gitlin *et al.*, 1956; Grabar *et al.*, 1958). Since the isoagglutinins are associated mainly with the β_{2M} fraction they are usually absent in hypogammaglobulinaemia.

In children with the congenital form of hypogammaglobulinaemia the level of gamma globulin in the plasma is usually below 50 mg. per 100

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ml. and occasional patients have less than 1 mg. per 100 ml. (Gitlin *et al.*, 1959; Zak and Good, 1959; Kekwick *et al.*, 1961). In adults with the acquired form the level tends to be a little higher (e.g. 12-97 mg./100 ml., Zak and Good, 1959). An upper limit of 200 mg./100 ml. has been arbitrarily adopted in Britain for the purpose of selecting cases for treatment.

It has been found empirically that patients with hypogammaglobulinaemia can be kept relatively free from infections by maintaining their plasma gamma-globulin concentration at about 150 mg./100 ml. by giving regular injections of gamma globulin (Gitlin *et al.*, 1959). It is usual to give an initial 'loading' dose, spread over 5 days, of 2.5 ml./kg. of a 10 per cent solution, and then to give regular weekly injections of 0.25 ml./kg. In infants with hypogammaglobulinaemia, gamma globulin disappears from the circulation a little more slowly (half-time 30 days) than in normal adults; for references, see Burtin, 1958.

The intravenous injection of preparations of gamma globulin should be avoided because this is known to produce unpleasant and even dangerous reactions. The intramuscular injection of gamma globulin very seldom produces any untoward effects.

The rate of 'uptake' of gamma globulin after intramuscular injection was studied by Dupan *et al.* (1959) using ^{131}I-labelled gamma globulin. Twenty-four hours after injection 50–70 per cent was still at the site of injection and 30–60 per cent was left after 2 days. The maximum level reached in the blood was equivalent to the presence of 5–20 per cent of the injected dose.

Use of gamma globulin in prophylaxis against infectious diseases. Pooled gamma globulin from adult donors is a valuable source of antibodies against common viruses. Preparations of gamma globulin have the advantage over normal pooled serum of being far less bulky and of being free from the virus of hepatitis (Ordman *et al.*, 1944; Kekwick and MacKay, 1954). The immunity conferred by an injection of gamma globulin is, of course, temporary and depends upon the amount of antibody injected. As mentioned above, after equilibration the rate of turnover of gamma globulin (7*S*) in adults corresponds to a half-life of 20–25 days. Accordingly, after about 60 days only one-tenth of the injected antibody remains. Ordman *et al.* (1944) found that a dose of gamma globulin which would protect children from measles for periods up to 14 days would not protect them for as long as 7 to 10 weeks. A relatively large dose (450 mg.) of gamma globulin provides some protection for periods up to 6 weeks (see

Kekwick and Mackay, 1954, p. 58). The Ministry of Health (1959) has recommended the following doses of gamma globulin:

For protection against measles in children under 1 year, 250 mg.; 1 to 2 years, 500 mg.; 3 years, 750 mg.

For attenuation of an attack (all ages), 250 mg.

For protection against rubella of a woman exposed to infection during the first 4 months of pregnancy, 1500 mg.

For protection against poliomyelitis in children under 1 year, 500 mg.; 1 to 6 years, 1.0 g.; 7 years and over, 1.5 g.

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CHAPTER TWO

BLOOD VOLUME

Blood volume can be predicted only approximately from measurements of height or weight so that if a precise estimate is needed, it must be obtained by direct measurement. Estimates of blood volume are seldom needed in clinical practice but they are very often needed in clinical research related to transfusion.

If blood were a homogeneous (and colourless) fluid, estimates of blood volume would be very simple: one would need only to inject a given volume of dye and to measure its dilution in the circulating 'blood'. Thus, if 1 ml. of a solution of dye were injected, and its concentration in the blood was found to be 1 per cent of the concentration of the solution injected, the blood volume would be 100 ml. Plasma volume is in fact estimated in just this way, but difficulty arises when an estimate of total blood volume is required. One must then know what fraction of the total blood volume is represented by plasma. It is, of course, easy to determine the relative volume of plasma and red cells in a blood sample obtained from a vein or artery, but there is a great deal of evidence that the ratio observed is not representative of the blood in the body as a whole.

Discrepancy between the 'Venous Haematocrit' and the 'Whole Body Haematocrit' (see Fig. 7)

The expression 'whole body haematocrit' is sometimes used for the percentage of the total blood volume that is occupied by red cells. That is to say, if red cell volume (RCV) and plasma volume (PV) are measured separately, then whole body haematocrit (H_B) $= \frac{RCV}{RCV+PV}$. Almost all workers now agree that in normal subjects H_B is less than the venous haematocrit, or packed cell volume of venous blood, conveniently referred to as H_V. In fact there is good agreement that in normal subjects the ratio $\frac{H_B}{H_V}$ is approximately 0.91.

Two main explanations can be offered for the discrepancy between H_B and H_V: first that the space within which plasma circulates is greater than that in which red cells circulate or secondly that the haematocrit varies in different-sized blood vessels. These two explanations are not, of course, mutually exclusive.

Thus suppose that there are holes in the capillaries large enough for albumin molecules, but not red cells, to pass through, then although the ratio of red cells to plasma within the blood vessels may be uniform throughout the body the ratio of total circulating red cells to total circulating plasma will be lower than the ratio of red cells to plasma within the blood vessels. The main argument usually brought against this view is the fact that when labelled red cells and albumin are injected simultaneously no fall can be observed in the ratio of plasma to cells during the mixing phase (see below). Nevertheless if mixing of injected albumin with plasma

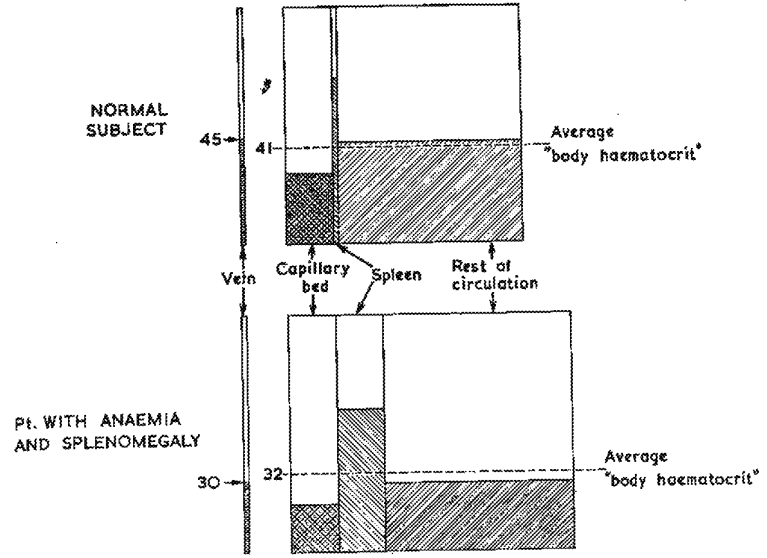

FIGURE 7. Concept of the 'body haematocrit', that is, the fraction of the total vascular bed occupied by red cells (shaded areas). In the normal subject the haematocrit of venous blood is greater than the 'body haematocrit' but in subjects with splenomegaly the 'body haematocrit' may be greater than the venous haematocrit, due to the relatively large amount of splenic blood which has a relatively high haematocrit.

in the extra-capillary space occurred with a half-time of a few seconds it might well be too rapid to be detectable.

An alternative concept is that the very small blood vessels of the body contain relatively more plasma than larger blood vessels. This concept is based largely on the experiments of Fåhraeus (1929) who caused blood to stream through fine glass tubes and noted that when the diameter of the tube was 1.0 mm., the haematocrit of the blood was 40 per cent and when it was 0.05 mm., the haematocrit was only 28 per cent. Fåhraeus also noted that in blood streaming through tubes of a diameter less than 0.1 mm., the velocity of the red cells was considerably greater than that of the plasma. It is now generally accepted that the blood vessels

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are lined with a layer of relatively slowly moving plasma and that the red cells form an axial stream. The concept that the haematocrit is lower in small blood vessels appears to be supported by experiments in which, after the injection of labelled red cells and labelled plasma, the tissues of an animal are analysed to discover the haematocrit. For example, Allen and Reeve (1953) showed in this way that the blood in the liver had 31 per cent less cells than venous blood. The objection to such experiments is chiefly that they do not show exactly where the labelled plasma is, that is, inside or outside the capillaries.

Although the interpretation of the finding that H_B is lower than that of H_V is still not absolutely certain, it must be taken as a fact that the haematocrit of blood taken from an artery or vein is appreciably higher than the proportion of red cells in the total circulating blood. Fortunately, in most cases the body haematocrit can be quite accurately deduced from the venous haematocrit by applying a constant correction. The value of this constant and its degree of variability are discussed later in this chapter.

The effect of ignoring the difference between the body haematocrit and the venous haematocrit can be illustrated by an example:

Suppose plasma volume is found to be 43 ml./kg. and the venous haematocrit to be 45 per cent; then, ignoring the correction mentioned above, blood volume is calculated to be:

$$43 \times \frac{100}{(100-45)} = 78 \text{ ml./kg.}$$

However, if the body haematocrit is taken to be (venous haematocrit $\times 0.91$), then blood volume is calculated as:

$$43 \times \frac{100}{(100-(45 \times 0.91))} = 73 \text{ ml./kg.}$$

Similarly, if red cell volume is found to be 30 ml./kg. and the venous haematocrit to be 45 per cent, blood volume is calculated as:

$$30 \times \frac{100}{45} = 67 \text{ ml./kg.}$$

but, if the venous haematocrit is corrected by the ratio 0.91, blood volume is calculated as:

$$30 \times \frac{100}{(45 \times 0.91)} = 73 \text{ ml./kg.}$$

Thus estimates of blood volume based on the uncorrected venous haematocrit are too high if deduced from plasma volume and too low if deduced from red cell volume.

Although satisfactory estimates of blood volume can be made in most conditions by measuring either plasma volume or red cell volume and applying an appropriate correction to the venous haematocrit, the most reliable estimates are those based on measurements of plasma volume and red cell volume, as Smith, Arnold and Whipple insisted as long ago as 1921.

ESTIMATION OF PLASMA VOLUME

Use of the Dye T-1824

T-1824, 'Evans Blue', has been more used than any other, and the use of this dye only will be discussed. On being mixed with plasma, the T-1824 molecules become attached to albumin (Rawson, 1943); when the concentration of dye is approximately 1 mg./100 ml. plasma, and when the concentration of albumin in the plasma is approximately 3.5 g./100 ml., the proportion of dye molecules to albumin molecules is about one to fifty (Reeve, 1948). Since binding appears to be quite firm at a ratio of one to one (Barnes *et al.*, 1948b), there is always in practice an adequate excess of albumin molecules. At 37° C. the union of T-1824 with albumin is complete in less than a minute, though the reaction is slower at lower temperatures, a point which ought to be kept in mind when preparing standards (Barnes *et al.*, 1948b).

There is no evidence that any dye is lost from the circulation through not being bound rapidly enough to albumin. For example it has been shown that when ^{131}I-labelled albumin and T-1824 are injected they undergo an equal degree of dilution during the first circulation through the heart and lungs (Freinkel *et al.*, 1953) and have similar loss rates during the following hour (Sear *et al.*, 1953).

Some batches of T-1824 contain an appreciable amount of a violet impurity which, on injection, leaves the circulation very rapidly (Cooley, 1954). The use of such preparations must lead to an overestimate of plasma volume.

Injection of Dye

If the dye concentration is to be measured 'directly' (see below), it is sufficient to achieve a concentration of approximately 0.5 mg./100 ml. in the plasma. For example, in an adult with a plasma volume of 3000 ml., 15 mg. of dye would suffice. If the dye is to be extracted, twice this amount should be used.

Care should be taken not to exceed plasma concentrations of 2 mg. of dye per 100 ml. of plasma, for with greater concentrations the patient

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turns blue. Personal experience has shown that concentrations of 1.9 mg./100 ml. do not produce an obvious discolouration (two cases), whereas concentrations of 2.4 mg./100 ml. may turn the patient blue for 4 to 5 days (two cases). One patient in whom the concentration reached 2.7 mg./100 ml. remained blue for 10 days.

One real difficulty in trying to inject a precise amount of T-1824 into a patient is that of seeing the marking on the tip of the plunger, due to the density of the dye. The difficulty can be overcome in one of three ways: firstly, a relatively dilute solution of dye can be used; e.g. 20 ml. of a 0.75 per cent solution can be injected. Secondly, the volume of solution injected can be determined by weighing the syringe before and after injecting the dye. Thirdly, a mark can be made on the plunger of the syringe 1 cm. or so from the tip and a corresponding mark made on the barrel so that, when the syringe contains 20 ml., the two marks are in alignment. The marks can be seen clearly even when the syringe is full of dye.

Whatever syringes are used, they can be calibrated by ejecting the contents of the syringe into a weighed beaker and noting the gain in weight. Then in making the injection into the patient the plunger of the syringe is simply pushed slowly home and no attempt is made to wash in the residual amount of dye.

According to some authors, solutions of T-1824 in normal saline precipitate. However Gibson and Evans (1937a) reported that the light-absorption of a solution of the dye in saline did not change over a period of 10 months, and the author's own experience has been similar; and Wiklander (1956) stored the dye as a 0.3 per cent solution in normal saline and found no precipitation after 1 to 2 years. It is convenient to prepare a sufficient volume of T-1824 in saline for several months' work and to partition the solution into ampoules which are then sealed and autoclaved.

Rate of Mixing

In normal subjects, when dye is injected into an arm vein and samples are withdrawn from another arm vein, mixing of the dye with the circulating plasma usually appears to be complete within 10 minutes (Gibson and Evans, 1937a; Steinbeck, 1950). The rate of mixing of tagged red cells was measured by Berson and Yalow (1952): mixing was 90 per cent complete within the first 60–90 seconds and was apparently complete within 5–8 minutes.

Strumia *et al.* (1958) made observations after injecting T-1824 and

^{51}Cr-labelled red cells and found definite differences in the rate of mixing. Thus, whereas in samples taken from an arm vein 2–3 minutes after injection ^{51}Cr-labelled red cells were almost always fully mixed, mixing of T-1824 sometimes took as long as 9 minutes.

In subjects with cardiac failure mixing is often distinctly slower (Gibson and Evans, 1937a). Moir *et al.* (1956) found that in ten subjects with oedema, mixing took 14 minutes, compared with 9 minutes in normal subjects. (After mixing was complete, the rate of loss of T-1824 was no greater in oedematous subjects than in normal subjects.) In congestive cardiac failure mixing may be very slow. Thus, Reilly *et al.* (1954), using ^{51}Cr-labelled cells, which presumably mixed more rapidly than T-1824, found that mixing sometimes took as long as 30 minutes.

In splenomegaly mixing may take more than 30 minutes; an example is given later in this chapter in discussing the estimation of RCV.

Rate of Disappearance of Dye from the Circulation

In normal subjects about 10 per cent of the injected dye leaves the circulation during the first hour. If several samples are taken and dye concentration is plotted on a logarithmic scale against time, the points fall close to a straight line and this can be extrapolated back to give the dye concentration at zero time (Gregersen and Rawson, 1943). The fit of the points to a straight line is usually satisfactory only up to about 40 minutes after injection; thereafter the rate of loss is usually distinctly less, as illustrated in Fig. 8. About 50 per cent of the dye is present in the plasma 24 hours after injection (Gibson and Evans, 1937a).

In some subjects and particularly in infants it may be impracticable to take more than a single sample.

Noble and Gregersen (1946a) have considered the error of the single sample technique and have concluded that estimates based on a 10-minute sample give a plasma volume which is, on the average, only 1–2 per cent too high. They point out that any error from incomplete mixing tends to counterbalance the error introduced by neglecting the loss of dye during the mixing period.

T-1824 is poorly bound by rabbit albumin and in rabbits overestimates plasma volume by about 8 per cent, compared with the correct estimate given by ^{131}I-labelled albumin (Zizza and Reeve, 1957).

Blood Samples for Estimation of Plasma Volume

In principle it seems desirable to take a series of blood samples: for example, at 15, 25 and 35 minutes after injection of the dye. In practice it is

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doubtful whether the extra trouble is repaid. As a rule the three points do not fall precisely on a straight line, and an arbitrary line has to be fitted to determine the dye concentration at zero time. It is suggested that in subjects in whom the rate of dye loss can be assumed to be normal, either a single sample should be taken 15 minutes after the injection of the dye, or two samples should be taken, at 15 and 30 minutes respectively. The dye concentration at zero time is obtained by multiplying the dye concentration in the 15-minute sample by $\frac{100}{97.5}$, and in the 30-minute sample by $\frac{100}{95}$.

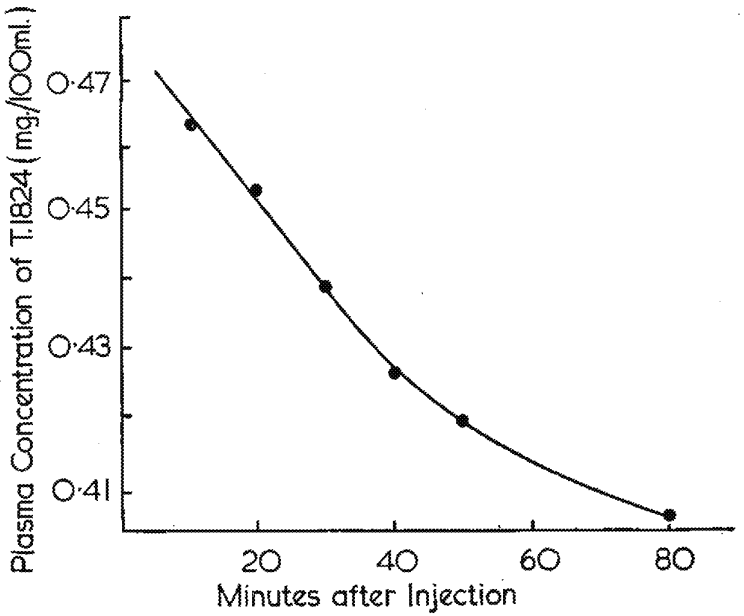

FIGURE 8. Disappearance of T-1824 after injection into a normal subject; the points during the first 40 minutes appear to fall close to a straight line but there is then a break in the curve, indicating that the curve is made up of at least two components.

In principle a correction should be made for alterations in plasma specific gravity between samples. Reeve (1952) has pointed out that when repeated samples of blood are drawn, the samples often show a progressive decline in haemoglobin concentration and packed cell volume, probably because the withdrawal of even quite small amounts of blood initiates a diluting mechanism by which new fluid is added to plasma. Chaplin *et al.* (1953) found a progressive fall in venous haematocrit in eleven out of twenty-eight subjects from whom four samples were withdrawn; this fall usually amounted to less than one haematocrit division and was thought to be in part an artefact due to more pronounced venous stasis in collecting the first sample. It is suggested that in practice an average of all estimates of venous haematocrit should be used and that changes in plasma specific gravity should be ignored since they are scarcely greater than the errors of measurement.

A progressive fall in haematocrit is observed on changing from the upright to the supine position (see below). It is possible that a slight fall in haematocrit during a test of the kind referred to above may be due to the fact that equilibrium had not been reached at the beginning of the test.

If the dye is to be estimated in plasma it is best to wet the syringe with one or two drops of liquid heparin. Some authors prefer to take clotted samples of blood and to estimate the concentration in serum. Serum tends to be less opaque than plasma which is an advantage when the dye concentration is to be measured without extraction; there is satisfactory evidence that the dye concentration in serum is the same as that in plasma (Gregersen and Schiro, 1938).

Estimation of Dye Concentration

The optical density of the post-injection samples is measured in a spectrophotometer at a wavelength of 620 mμ, using pre-injection plasma as a blank. If a photo-electric colorimeter is used, a filter with a narrow band of transmission should be chosen to avoid interference from other pigments, such as haemoglobin; a combination of Ilford filters 803 and 205 is suitable (Mollison *et al.*, 1950).

The dye concentration corresponding to the observed optical density is determined by preparing standards. The first point to emphasize is that these standards must be made up in serum or plasma, since the optical density of the dye in water is some 10 per cent higher than that of the dye in plasma. Ideally, a fresh standard should be prepared at the time of each estimation by adding an appropriate small volume of Evans Blue from the injection solution to plasma taken from the patient immediately before injecting the dye. This can be done with great precision if an 'Agla' micrometer syringe[1] is used. (Barnes *et al.*, 1948a). The use of micropipettes for the purpose is not recommended, since there is a tendency for dye to adhere to the walls of the pipette.

[1] Obtainable from Burroughs Wellcome & Co., London.

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It is important that the reading of the standard should be close to that of the dyed sample from the patient, since the relationship between dye concentration and optical density is not strictly linear. Because of the added labour of preparing a fresh standard at the time of each estimation, there is much to be said for the simpler method of preparing a standard calibration curve for use with a given batch of dye solution. Various dilutions of the dye in plasma are prepared and the optical densities of the samples measured, using dye-free plasma as a blank. In this way a calibration curve can be prepared and can be used throughout a series of experiments. Obviously it is necessary to carry out a check from time to time to ensure that the dye solution has not faded nor the characteristics of the colorimeter altered. In practice, dye solution kept in the dark at 4° C. does not fade appreciably over periods of 10 months (Gibson and Evans, 1937a). The constancy of the response of the galvanometer can be checked by using a grey glass screen of suitable density as an arbitrary standard.

In measuring the amount of dye present in samples taken from the patient there are two main sources of error, namely, haemolysis and varying opacity of the plasma. It should be possible to avoid haemolysis completely; moreover, with the filter recommended, the presence of a small amount of haemoglobin in the plasma will not cause an appreciable error; for example, the presence of 75 mg. of free haemoglobin per 100 ml. of plasma will cause an error of only about 1 per cent.

Plasma opacity is a serious source of error. In most cases trouble can be avoided by making the determinations with the patient fasting. The plasma samples should be measured promptly (within 2 or 3 hours) since opacity may develop on standing (Reeve, 1948). The samples should be inspected with the naked eye before being measured. If there is any suspicion of cloudiness it is best to extract the dye (see below).

Calculation of Plasma Volume

Plasma volume is given by the expression:

$$\frac{\text{Volume of solution injected} \times \text{Concentration of dye in injection solution}}{\text{Concentration of dye in plasma at zero time}}$$

Thus, if 20 ml. have been injected and the concentration of dye in the plasma at zero time corresponds to an optical density of 0.25, and the injection solution, suitably diluted, e.g. 0.02 ml.+3.0 ml. of pre-injection plasma, has an optical density of 0.24, plasma volume is:

$$\frac{20 \times \frac{3.02}{0.02} \times 0.24}{0.25} = 2900 \text{ ml.}$$

Extraction of the Dye from Plasma

In order to overcome errors introduced by plasma opacity and traces of haemoglobin, various methods have been devised for extracting T-1824 from plasma. The most satisfactory principle appears to be to add a suitable detergent to the dyed plasma and then pass it through a column of cellulose pulp; the detergent facilitates the breaking of the bond between the dye and albumin and the dye binds on to the column and is subsequently eluted with acetone (Allen, 1953). In Allen's original method elution is very slow, and it is not easy to obtain complete recovery of the dye, but these difficulties are largely overcome in the modified method of Campbell *et al.* (1958), which, in the author's experience, is the best available method of extracting T-1824 from plasma.

1-4 ml. of dyed plasma are mixed into teepol in Na_2HPO_4 and poured on to a column composed of a particular preparation of wood cellulose; the dye separates as a dark layer at the top of the column. The column is washed free from impurities with sodium phosphate solution and the dye is eluted with acetone-water pH 8; the eluted dye is buffered to pH 6.8 and its optical density determined in a suitable form of spectrophotometer.

Other Methods of Estimating Plasma Volume

It has been shown that ^{131}I-labelled albumin gives the same results as T-1824 (Freinkel *et al.*, 1953; Sear *et al.*, 1953). The loss of ^{131}I-labelled albumin from the plasma is at first as rapid as that of T-1824; for example, in the first hour the loss of both is about 10 per cent (Sear *et al.*, 1953). Subsequently the loss of ^{131}I-labelled albumin is distinctly slower. Sterling (1951) found that the rate of loss could be described by a two-component curve; the rapid component, representing mixture with the total albumin 'pool', had a half-time of 0.5 days; the slow component had a half-time of 10.5 days, and this was thought to represent the half-time of albumin 'turnover'. Garrow and Waterlow (1959) studied the rate of disappearance of ^{131}I-albumin and T-1824. They concluded that both took about 7 days to mix completely; T-1824 appeared to be distributed in a larger space, and this was presumably due to the fact that some of it was taken up by the reticulo-endothelial system. After 7 days, albumin disappeared with a half-time of 21 days, and this is presumably a more reliable estimate than the one quoted above.

In practice, ^{131}I-labelled albumin might seem to be a better label than T-1824 because it does not suffer from interfering factors such as plasma opacity, which may mar the accuracy of the T-1824 method. On the other hand, ^{131}I-labelled albumin is subject to its own errors. Firstly, it

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tends to adhere to glassware, particularly in dilute solutions, so that there may be a greater loss from the diluted standard than from the plasma samples, leading to an underestimate of plasma volume (Reeve and Franks, 1956); secondly, there may be some loosely bound ^{131}I which tends to leave the circulation rapidly, and this leads to an overestimate of plasma volume (Franks and Zizza, 1958).

^{131}I-labelled albumin may be obtained from the Radiochemical Centre, Amersham. To avoid diluting a small amount in a large volume of saline, with consequent risk of loss of protein by adsorption on to glass surfaces, it is convenient to add the labelled albumin to a few millilitres of heparinized plasma obtained from the subject whose plasma volume is to be estimated. Normally an amount of ^{131}I-labelled albumin containing 5 μc. will be ample. The labelled albumin and plasma can be diluted with saline to 25 ml., 20 ml. of which is injected into an arm vein. The procedure is as for T-1824 except that the samples are counted in a well-type scintillation counter. In diluting an aliquot of the injection solution for counting as a standard, sufficient protein will be present if the procedure recommended above has been followed. If not, the injection solution may be diluted in saline to which bovine albumin has been added to give a final concentration of at least 0.05 per cent or alternatively may be diluted in saline containing 1 per cent detergent, as this prevents the adsorption of protein on to glass (see Reeve and Franks, 1956).

Another isotope of iodine, ^{132}I, with a half-life of only 2.3 hours, is available, and can be used to prepare labelled albumin for plasma volume measurement (Veall *et al.*, 1955). Although this isotope has great theoretical advantages, namely, in involving a much smaller radiation hazard and permitting repeated estimates within a short time, the fact that it has to be freshly prepared is in practice a great disadvantage.

Frank and Gray (1953) have described the use of $^{51}CrCl_3$ for estimating plasma volume. They simply injected a solution of the material and claimed that 98 per cent was immediately bound on to the plasma proteins. Unfortunately the compound that they used is difficult to prepare, so that up to the present the method has not been widely used. Small and Verloop (1956) found that $^{51}CrCl_3$, unlike $Na_2\,^{51}CrO_4$, was very readily adsorbed on to glass surfaces; in preparing dilutions *in vitro* for counting, they obtained reliable results only by adding relatively large amounts of 'carrier', that is, non-radioactive chromic chloride.

Several authors have used preparations of dextran with a molecular weight of 100,000 or more for estimating plasma volume. Semple *et al.* (1958), using a special preparation with a molecular weight of 115,000 ('number average'), obtained good agreement with T-1824 in dogs and humans. Jaenike *et al.* (1957), using a preparation with a mean molecular

weight of approximately 200,000, found that ^{131}I-labelled albumin estimates were about 5 per cent higher than dextran estimates in normal subjects but 10 per cent higher in subjects with cardiac failure. The discrepancy in normal subjects might have been due to the presence of some denatured albumin (see above) but the greater discrepancy in cardiac failure suggests that the relatively high estimates given by albumin were due to the greater space in which these smaller molecules were distributed. This conclusion receives support from the observations of Verel (1958) who used a preparation with a mean molecular weight of 400,000 (80 per cent of molecules 200,000–500,000) and found that T-1824 estimates and dextran estimates were essentially the same in normal subjects, but that in cases of cardiac failure and some cases of acute infection dextran gave lower estimates than T-1824.

Estimation of Packed Cell Volume ('Venous Haematocrit')

If an estimate of plasma volume is obtained, blood volume can be deduced from plasma volume and packed cell volume (PCV) of venous blood, provided that the PCV is corrected (*a*) for plasma trapping, (*b*) for the discrepancy between the body haematocrit and the PCV (venous haematocrit). Of these corrections (*b*) is considered in a later section, but (*a*) will be considered here.

When blood is centrifuged, the degree of packing of the red cells depends upon the speed of centrifugation, the radius of the centrifuge, the height of the cell column and the time for which the tubes are spun. Wintrobe haematocrit tubes (10 cm. high) are still widely used, and, as described below, appear to give slightly more accurate results than those obtained by a micro-method. It is customary to centrifuge Wintrobe haematocrit tubes at 3000 rev./min. in a centrifuge with a radial distance from the axis to the bottom of the tube of 15 cm. Under these conditions more reproducible results are obtained if centrifugation is continued for 55 minutes, rather than 30 minutes (Jackson and Nutt, 1951; Chaplin and Mollison, 1952). The amount of plasma trapped in the lower layers of the red cell column is smaller than that in the upper layers (Leeson and Reeve, 1951; Chaplin and Mollison, 1952; Vazquez *et al.*, 1954). Accordingly in applying a correction for trapped plasma, the observed packed cell volume must be taken into account. The appropriate corrections to apply can be deduced from Fig. 9.

Although Chaplin and Mollison (1952) found that neither anisocytosis nor spherocytosis significantly altered plasma trapping, Furth (1956), using ^{51}Cr as a label for plasma, concluded that in a column consisting of red cells from a patient

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with hereditary spherocytosis, the amount of trapped plasma was twice as great as in a column containing normal red cells.

In practice it is difficult to fill haematocrit tubes precisely to the 100 mark. It is more convenient to fill them approximately, then to cover the top of the tube with adhesive tape, or a rubber closure, to avoid evaporation and to read the plasma meniscus after centrifugation. Then if the meniscus is found to be at 101.5, the haematocrit reading is simply corrected by $\frac{100}{101.5}$.

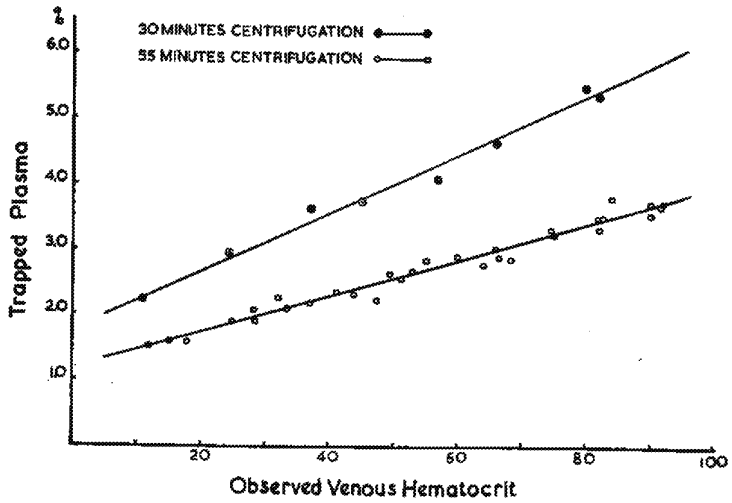

FIGURE 9. Trapped plasma, expressed as a percentage of the packed red cell column, plotted against PCV ('observed venous haematocrit'). The results refer to heparinized blood centrifuged at 3000 rev./min. in Wintrobe haematocrit tubes in a centrifuge of radius 15 cm. The period of centrifugation is shown (from Chaplin and Mollison, 1952).

In the author's experience there are only two serious sources of error in estimating the packed cell volume by this method; firstly, the graduations on the tube may not start exactly at the bottom; when accurate work is being attempted, all tubes must be inspected for this possible fault. (Ideally they should be calibrated; for a method see Mollison (1956, p. 48.)) Secondly, when the leucocyte count is increased, it may be difficult or impossible to decide exactly where the boundary lies between red cells and white cells.

A minor source of error is the degree of oxygenation of the blood. The PCV of a sample which has been shaken till it is bright red will be 0.5 to 1.0 divisions lower than that of an unoxygenated sample. (Personal observations; see also Streeten and Thorn, 1957.) It is convenient to treat all samples alike and not to attempt to oxygenate them.

When selected Wintrobe haematocrit tubes are used, the standard deviation of replicate estimates is found to be about 0.25 (personal observations). This estimate agrees fairly well with the conclusion of Streeten and Thorn (1957) that the S.D. of the MCHC could be kept as low as 0.1.

Another method of determining the PCV is to centrifuge the blood in a capillary glass tube at a relatively high speed, giving a very much greater relative centrifugal force (RCF). RCF increases linearly with the radius of centrifugation, but increases as the square of the speed of rotation. Thus, for example, the standard 'Wintrobe' conditions (radius 15 cm., 3000 rev./min.) give 1500 *g*., whereas a microhaematocrit centrifuge with a radius of only 9 cm. but a speed of 11,000 rev./min., produces 12,000 *g*. In such a centrifuge red cell packing is complete in less than 5 minutes, and the column of red cells is seen to be translucent (Koeppe's criterion), indicating that the column contains little trapped plasma (see Ponder, 1948, p. 56). The more complete packing obtained in such a centrifuge is a theoretical advantage, but in practice this is outweighed by the slightly less good reproducibility of results. The chief difficulties are in getting a flat horizontal seal at the bottom of the tube and of accurate reading of the column. Even when these conditions are satisfied, the S.D. of replicate estimates is about 0.5 (personal observations). Nevertheless, for all but the most accurate work the micro-method is very satisfactory.

Choice of Anticoagulant

Blood may be mixed with any anticoagulant which does not cause shrinking or swelling of the red cells. Thus:

Heparin. 0.1 mg. heparin (= approximately 10 units) will prevent the clotting of 3 ml. of blood, and therefore provide a comfortable excess to prevent the clotting of 1 ml. of blood, and allow for imperfect mixing. It is convenient to prepare a solution containing 10 mg. heparin per millilitre and to pipette 0.2 ml. into a series of tubes (for quantities of blood not exceeding 20 ml.). The tubes are then dried in the incubator (at 37° C.) over a period of 2 or 3 days. They must not be dried at high temperatures.

A very satisfactory method of obtaining blood samples free from any

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trace of clotting is to take them into a syringe wetted with a few drops of heparin. If a solution containing 5000 units of heparin per millilitre is used, even if only a single drop is left in the syringe there will be enough heparin to stop more than 20 ml. of blood from clotting.

If heparinized plasma is to be used for estimates of T-1824 concentration there is the theoretical possibility that there may be enough heparin present to interfere with the measurements, because heparin does absorb some light in the red area of the spectrum (Reeve, 1948). Personal observations have shown that using the red-orange filter described above, and provided that the amount of heparin in different samples is very approximately the same, the error introduced is negligible.

EDTA. The dipotassium salt of EDTA is more soluble than the disodium salt and is therefore to be preferred. In a concentration of 2 mg. per millilitre of blood it will prevent coagulation and will not cause appreciable changes in cell volume. Care must be taken not to use a gross excess; for example, if the concentration of the dipotassium salt is as high as 8 mg. per millilitre of blood, cell volume is reduced by about 5 per cent (personal observations).

Oxalate mixture. As originally described by Heller and Paul (1934), a mixture of three parts ammonium oxalate and two parts potassium oxalate does not cause swelling or shrinking of red cells; 2 mg. of the mixture will prevent the clotting of 1 ml. of blood.

Dissolve 1.2 g. of ammonium oxalate and 0.8 g. potassium oxalate in 50 ml. of distilled water. Pipette 0.5 ml. of the mixture into each of a series of tubes and dry them in the oven at a temperature not exceeding 80° C. Each tube then contains sufficient oxalate to prevent the clotting of 10 ml. of blood.

Influence of Posture on PCV

In subjects who stand up after a period of lying down, plasma volume falls (see below) and haematocrit rises. Conversely, lying down for 1 to 2 hours after walking about produces a fall in haemoglobin concentration and haematocrit of approximately 5 per cent (Walters, 1933; Widdowson and McCance, 1950: see also Fawcett and Wynn, 1960). Some personal observations are shown in Fig. 10.

The subject was a male adult from whom ten venous samples were taken over a period of 2½ hours. The lowest PCV observed (44.0) was after a period of lying down and in a sample obtained without venous stasis; the highest PCV (50.2) was in a sample taken after a period of

standing and after a tourniquet had been placed round the arm for 2 minutes before taking a sample.

The subject had received an injection of ^{51}Cr-labelled red cells (his own) 8 days previously, and the ^{51}Cr count of each sample was determined. If the ^{51}Cr counts were expressed simply in terms of unit volume of whole blood, ignoring the PCV changes, the coefficient of variation was 4.8 per cent; but if PCV was taken into account and estimates were expressed as counts per millilitre of red cells, the coefficient of variation was only 1.3 per cent, that is approximately the variation expected from the counting error (± 1 per cent on 10,000 counts) and the error of

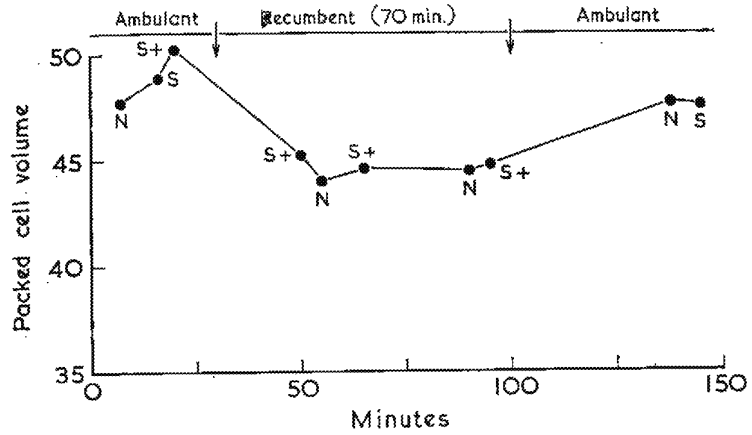

FIGURE 10. Effects on packed cell volume of venous blood of posture and of venous stasis. The subject was ambulant for the first 30 minutes, then recumbent for 70 minutes and finally was ambulant once more. Samples were taken either with no venous stasis (N) or after 1 minute's stasis (S) or after 2 minutes' stasis (S+).

estimating the PCV from duplicate samples (approximately ± 1 per cent); that is, the error expected was $\sqrt{1^2+1^2} = 1.4$ per cent.

Avoidance of Stasis in Taking Blood

In withdrawing blood it is desirable to avoid stasis because when the venous return is temporarily halted, the haematocrit tends to rise; see Fig. 10.

Walsh *et al.* (1953) found that application of a tourniquet to the arm for 1 minute led to an increase in the haematocrit of about 0.4 and application for 3 minutes led to a rise of 3.9 divisions. Berry *et al.* (1950) observed the effect of applying a cuff round the upper arm inflated to a pressure of 60

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mm. of mercury, and obtained the following average figures from a group of thirty-nine subjects. The haematocrit rose from 41.4 to 42.5 at 2 minutes, and to 43.9 at 5 minutes. The rise in haemoglobin concentration was slightly less; the plasma protein concentration rose in 5 minutes from 7.08 g. per 100 ml. to 7.3 g. per 100 ml.

It may be concluded that the application of a tourniquet for a period of less than 1 minute has barely any influence on the values, and it is therefore satisfactory to apply a tourniquet immediately before venepuncture, provided that the tourniquet is released as soon as the vein has been entered. If application of a tourniquet for about 15 seconds does not result in the distension of the vein, it is best to put the subject's hands into warm water rather than to leave the tourniquet on for a longer period. When the hands have been well warmed, it will usually be found that brief application of the tourniquet will fill the veins.

ESTIMATION OF RED CELL VOLUME

The principle of estimating the total circulating red cell volume is similar to that of estimating the plasma volume; that is to say, a measured volume of labelled red cells is introduced into the circulation, and the concentration of the label in unit volume of *red cells* taken from the recipient is then estimated. In practice, the dilution of the label in a sample of whole blood is estimated, and the dilution in unit volume of red cells is calculated from the haematocrit of the sample.

The most convenient method of estimating RCV is to label a sample of the subject's own red cells with ^{51}Cr; ^{32}P is almost as satisfactory.

Red cell volume can also be estimated by transfusing a measured number of serologically distinguishable red cells (Todd and White, 1911; Ashby, 1925). Although this method is undoubtedly capable of yielding reasonably accurate estimates of RCV (see Barnes *et al.*, 1948a, and the first edition of this book), it is too tedious to compete with methods employing radioactive isotopes.

Although many estimates have been made with carbon monoxide (see Sjöstrand, 1948), it appears to be established that the method overestimates RCV by about 12 per cent (Root *et al.*, 1953), because some CO is taken up by muscle (Wennesland, 1957).

Use of Radioactive Chromium

The use of ^{51}Cr for labelling red cells was first suggested by Gray and Sterling (1950). When $Na_2\,^{51}CrO_4$ is added to whole blood, some of the hexavalent Cr is reduced to the trivalent form, presumably by reducing substances in the plasma, because it is found that the uptake of red cells suspended in saline is more complete than that of red cells suspended in plasma (Gray and Sterling, 1950). Similarly, the uptake of Cr by packed red cells is more complete than that of the same red cells suspended in their own plasma (Mollison and Veall, 1955).

Uptake of ^{51}Cr by red cells is more rapid at a relatively low pH, e.g. it is three to four times more rapid at pH 6 than at pH 7.3 (Ebaugh *et al.*, 1953). The present author has found a steady improvement of uptake as pH falls from 8.0 to 6.8 but there seems to be little further improvement below that point (unpublished observations). Uptake of ^{51}Cr by red cells is approximately three times more rapid at 37° C. than at 22° C. (unpublished observations).

Uptake of $Na_2\,^{51}CrO_4$ by red cells is not well understood. In some circumstances a considerable proportion of the Cr is taken up by the red cells within seconds; this 'uptake' is partially reversible as can be shown by transferring the red cells to a solution of non-radioactive chromate (Mollison, 1961).

Suggested method. Radioactive chromium is obtained as sodium chromate with a specific activity of not less than 10 millicuries per milligramme of chromium. It is convenient to divide the stock into a series of ampoules which are then autoclaved. For the measurement of red cell volume it is sufficient to inject about 30 microcuries of ^{51}Cr, although larger amounts of activity are likely to be needed if red cell survival is to be estimated (see Chapter V). ^{51}Cr cannot be stored with citrate solution because citrate tends to reduce hexavalent Cr to the trivalent form (Cunningham *et al.*, 1957).

One method of labelling is as follows: 5–10 ml. of the subject's blood are mixed with one-fifth their volume of acid-citrate-dextrose solution. The blood is centrifuged, most of the supernatant plasma is discarded and an amount of sodium chromate containing 45 μc. of ^{51}Cr is added. The mixture is left at room temperature for about 25 minutes, by which time about 95 per cent of the radioactivity is in the red cells.

A satisfactory method for the rapid labelling of red cells is to wash one volume of cells in about 10 volumes of citrate at about pH 6.9 (e.g. the citrate-phosphate-glucose solution described on p. 67) and then to resuspend one volume of cells in one volume of the citrate solution and incubate them at 37° C. for 10 minutes with $Na_2\,^{51}CrO_4$. During this period over 95 per cent of the ^{51}Cr will be taken up by the red cells (Mollison, 1961).

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At the end of the uptake period the cells are washed twice in sterile saline and finally resuspended in saline to a volume of approximately 25 ml.; 20 per cent of this suspension is injected. A blood sample is collected from the opposite arm not less than 10 minutes after the injection.

In normal subjects, when labelled red cells are injected into an arm vein and blood samples are taken from the same vein or from another arm vein, mixing is virtually complete in about 3 minutes (Strumia *et al.*,

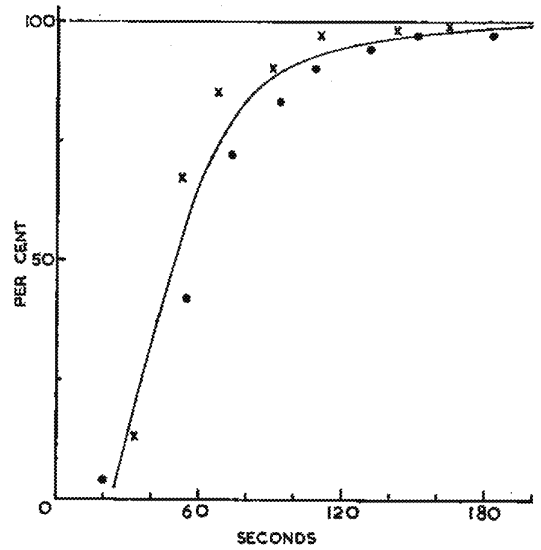

FIGURE 11. The rate of appearance of labelled red cells in samples taken from one superficial arm vein after injecting the cells into a vein in the other arm.
Two different samples of the subject's own red cells were injected, labelled with ^{51}Cr (●) and ^{32}P (×) respectively. One sample was injected about 30 seconds after the other but in each case the times were expressed as seconds from the mid-point of injection to the mid-point of the sample. '100 per cent' = the concentration attained after complete mixing, based on samples taken 10-20 minutes after injection (from Mollison, 1959d).

1958; Mollison, 1959d; see Fig. 11), although in a single subject observed by Strumia *et al.* mixing appeared not to be complete even after 5 minutes.

The interval of 10 minutes is chosen to allow a safe margin in normal subjects, but a distinctly longer interval should be allowed in subjects with congenital cardiac failure in view of the evidence of Reilly *et al.* (1954) that in such subjects mixing may take as long as 30 minutes. In subjects with splenomegaly it is also best to wait for 30 minutes, since mixing may

then take 20 minutes or more (Rothschild *et al.*, 1954). Fig. 12 shows the rate of mixing in a subject whose spleen was subsequently removed and found to weigh 900 g.

When autologous ^{51}Cr-labelled red cells are injected into goats the ^{51}Cr count in peripheral venous blood falls by 20 per cent during the 40 minutes after injection, probably due to slow mixing in the spleen (Anderson and Rogers, 1957).

In estimating red cell volume in oligaemic shock, it is well to remember that few studies have been made of the rate of mixing and that some

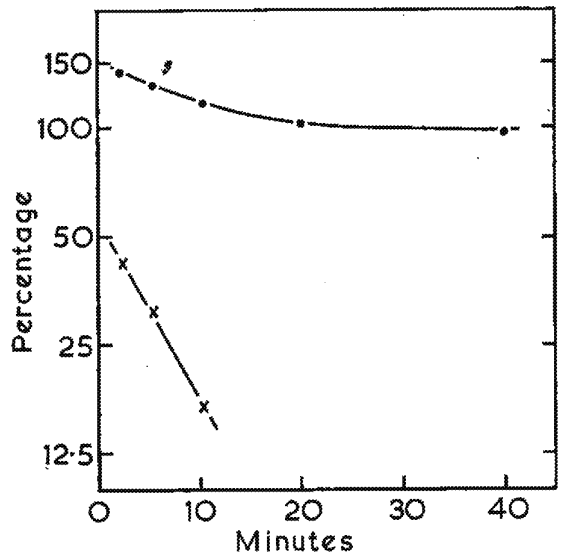

FIGURE 12. Rate of mixing of ^{51}Cr-labelled red cells in a patient with gross splenomegaly; (the spleen was removed a few days later and weighed 940 g.).
The concentration of labelled red cells in venous blood fell progressively for the first 20 minutes after injection but from 40 minutes onwards was stable. The 'rapid component' of the curve has been plotted in the lower half of the figure and it has a half-time of the order of 8 minutes.

authors have found remarkable fluctuations for hours or even days after injury or haemorrhage (see Graber, 1959).

After injecting red cells labelled with chromium there is a scarcely significant loss of radioactivity (about 0.5 per cent) during the following 60 minutes, although by the end of 4 or 5 hours there may be a loss of about 3 per cent (Mollison, 1961).

Use of stored red cells. For emergency use it may be convenient to use stored red cells for estimating red cell volume. Davies and Topley (1959) used red cells from

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blood stored with ACD for not more than 10 days and obtained results which were usually within 5 per cent of estimates made simultaneously with fresh red cells labelled with ^{32}P.

Calculation of red cell volume. Duplicate standards are prepared from the red cell suspension used for injection, by pipetting 0.5 ml. amounts of the suspension into dilute ammonia (see below) and making up the volume to 50 ml. These standards are counted in a scintillation counter together with a sample taken from the recipient after injection; this sample is lysed (e.g. with saponin) before being counted. Before lysing the sample the packed cell volume is estimated in duplicate.

Red cell volume (RCV) is given (in millilitres) by

$$\frac{\text{Total counts injected}}{\text{Counts/minute/ml. in recipient's blood}}$$

For example, suppose 20 ml. of a suspension of labelled red cells have been injected, and that a 1/100 dilution of this suspension has a counting rate of 4500 per minute, and that the sample taken from the recipient 10 minutes after the injection has a counting rate of 2100 per minute, and a packed cell volume of 35 per cent, then:

$$\text{RCV} = \frac{20 \times 100 \times 4500}{2100 \times \frac{100}{35}} = 1500 \text{ ml.}$$

These calculations assume that the volumes of standard and of recipient's blood counted are the same. They also assume that 100 per cent of the injected radioactivity was contained within red cells. In practice it is wise to check this latter assumption by counting a sample of the supernatant from the injection solution, although normally the amount in it should be of the order of 0.2 per cent of the total, and will not be worth correcting for.

Use of Radioactive Phosphorus (^{32}P)

A method of labelling red cells *in vitro* with ^{32}P was first described by Hevesy and Zerahn (1942); some valuable modifications were introduced by Reeve and Veall (1949), including the use of a liquid counting tube (Veall, 1948); and conditions allowing very much more rapid and complete uptake of ^{32}P were described by Mollison, Robinson and Hunter (1958).

Radioactive phosphorus is obtained as a sterile solution of sodium orthophosphate in dilute HCl ('PBS 1' supplied by the Radiochemical Centre, Amersham); the specific activity of this material is greater than 1 mc./μg. This solution is diluted in the citrate-phosphate solution described below so that the radioactivity of the diluted solution is approximately 30 μc./ml. Small amounts of this solution are then placed in ampoules and autoclaved. As described below, a suitable amount for a red cell volume estimation is 3 μc. Therefore a dose suitable for immediate use will be 0.1 ml. of the stock solution containing 30 μc. per ml. A dose suitable for use in a fortnight's time would be 0.2 ml., or in a month's time 0.4 ml. (the half-life of ^{32}P being 14 days).

As described below, the citrate-phosphate solution advised as a diluent contains only the equivalent of 40 μg. of P per ml., which is too small to affect the uptake of ^{32}P.

^{32}P may also be obtained as a sterile solution in isotonic saline with 100 μg./ml. of phosphate added as 'carrier' ('PBS 2' supplied by the Radiochemical Centre, Amersham). This solution can be diluted in exactly the way described for the preparation without carrier; since about 30 ml. of diluent will be used for each millilitre of stock ^{32}P, the P concentration will be effectively that of the diluting solution.

When ^{32}P is added to heparinized blood and the mixture is incubated at 37° C. for about 1 hour, 25-30 per cent of the ^{32}P enters the red cells (Hevesy and Zerahn, 1942; Reeve and Veall, 1949), but if the red cells are first washed in a citrate solution buffered to about 6.9, and are then incubated with ^{32}P, they take up over 90 per cent in 25 minutes (Mollison, Robinson and Hunter, 1958). The method is as follows: 5-10 ml. of blood are taken from the subject into a syringe wetted with heparin. The blood is transferred to a sterile container and centrifuged. The supernatant plasma is aspirated and discarded; usually, in heparinized blood, the leucocytes adhere together in a plaque, and if possible this should be removed with the plasma. The packed cells are resuspended in 15-20 ml. of the following solution:

Trisodium citrate	3.0 g.
$NaH_2PO_4.2H_2O$	0.015 g.
Glucose	0.2 g.
Water to 100 ml.	

This solution, referred to in this section as 'citrate-phosphate', initially has a pH of 7.4, but after autoclaving at 20 lb./sq. in. for 30 minutes, pH falls to 6.9.

The cell suspension is centrifuged and most of the supernatant removed,

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so that the 'strength' of the remaining cell suspension is about 50 per cent. The ^{32}P (3 μc.) is now added, and mixed with the cells, and the container is placed in a water-bath at 37° C. After 25 minutes the cells are washed twice in ice-cold saline and finally resuspended in a volume of about 25 ml.

Chaplin (1952) recommended the addition of some of the subject's own plasma, in a final concentration of 1 per cent to the saline used for washing the red cells and for resuspending them, although he stated that he had no proof of the efficacy of this step. The addition of plasma seems unnecessary as a routine for Mollison *et al.* (1958) who used unbuffered normal saline for washing and resuspending the red cells, after labelling with ^{32}P by the rapid method described above obtained very close agreement with simultaneous estimates made with ^{51}Cr-labelled cells.

The container with the labelled red cells is kept in ice-water until everything is ready for the injection. Then 20 ml. of the mixture are injected and the rest retained for preparing a standard and checking the amount of activity in the supernatant solution, which should normally be of the order of 0.5 per cent.

If these directions are followed the actual amount of radioactivity injected will be of the order of 2 μc.

After the cells have been reinjected into the circulation, ^{32}P escapes from them at the rate of approximately 10 per cent in the first hour. The most accurate estimate of the ^{32}P counts at zero time is obtained by taking a series of blood samples (three or four) at intervals during the period between 10 and 60 minutes after injection, plotting the estimates on semi-logarithmic paper, fitting the best straight line and extrapolating this line to the time axis (see Fig. 13). The number of counts in each sample must be expressed in relation to the packed cell volume of the sample, i.e. as counts per minute per millilitre of red cells, to correct for slight changes in the packed cell volume of different samples.

The rate of loss of ^{32}P from the red cells is slightly influenced by the conditions under which they are labelled. Thus, when red cells are labelled by incubating ^{32}P with whole heparinized blood, the subsequent rate of loss is about 9 per cent in the first 60 minutes after reinjection, and the amount left at 24 hours is about 48 per cent (see Fig. 13). When red cells are labelled after being washed in citrate-phosphate as recommended above, about 10 per cent is lost in the first 60 minutes after reinjection, and the amount left at 24 hours is about 35 per cent. If red cells are washed in a more acid solution of citrate (pH 6.4) before being incubated with ^{32}P, the rate of loss of ^{32}P *in vivo* is more rapid still and unreliable estimates of red cell volume are obtained (unpublished observations).

If it is inconvenient to take several blood samples from the patient and a slightly less precise result is acceptable, a single sample can be taken 10 minutes after injection and corrected by some appropriate factor such as $\frac{100}{98.5}$ to allow for the small loss of phosphorus in the first 10 minutes.

Chaplin (1952) estimated the error of the ^{32}P method by making duplicate estimates, at an interval of 1 week, in ten subjects. He found that the coefficient of variation was 1.6 per cent when three blood samples were taken (at 15, 30 and 45 minutes) and was 2.3 per cent when the estimates were based only on the 15-minute sample.

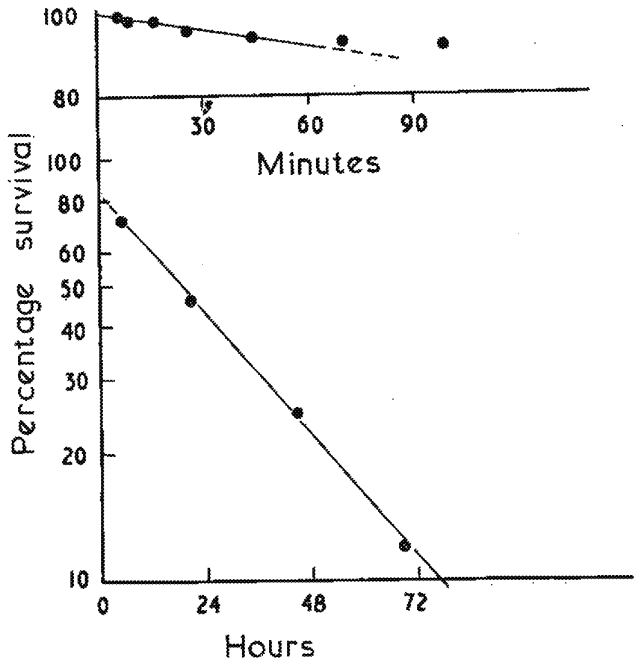

FIGURE 13. Percentage survival of ^{32}P in the circulation following the injection of ^{32}P-labelled red cells; (the red cells were labelled by incubating heparinized blood with ^{32}P).

About 10 per cent of the ^{32}P is lost in the first 60 minutes but thereafter the rate of loss diminishes. At 4 hours about 70 per cent of the ^{32}P is left and from then onwards the half-time of elution is about 24 hours.

After withdrawing blood for the determination of packed cell volume, each sample is haemolysed by the addition of saponin and then poured directly into a Geiger-Müller counter designed to take liquid samples

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(Veall, 1948). This type of counter requires 10 ml. samples; if only smaller samples are available, they must be diluted.

In preparing a dilution of the injection solution to count as a standard, it must be remembered that the counting rate is affected by the specific gravity and, to a lesser extent, by the chemical composition of the solution. Thus if the injection solution is diluted in water it will give a slightly higher counting rate than if it is diluted in blood. For most purposes it is sufficiently accurate to prepare the standard in phosphate solution and apply a correction factor of 0.98 (personal observations).

When duplicate standards are prepared (as they always should be), discrepancies between them are sometimes found. Wiklander (1956) found that this was often due to leucocytes which had taken up ^{32}P and remained as particulate matter in the injection solution. As described above, an attempt should be made to remove as many leucocytes as possible from the blood before labelling with ^{32}P. The present author has found that an equally serious source of error is incomplete lysis of the red cells, and that this can be overcome by making up the standards in 0.04 per cent ammonia rather than water. As shown by Barer and Gaffney (1957), the greater clarity of solutions obtained by adding red cells to dilute alkali rather than to distilled water is due to the dissolving of stroma.

Decontamination of counting cups. Difficulty is sometimes experienced in getting rid of traces of radioactivity from containers used for the counting of radioactive solutions. A highly efficient cleaning solution may be prepared as follows: Cover the bottom of a beaker with crystals of potassium permanganate; fill the beaker rapidly with concentrated sulphuric acid. Filling must be rapid as if only a small volume of acid is poured on at first heat cannot be dissipated rapidly enough and the mixture may explode. The solution is highly corrosive and must be kept very carefully. Containers which are to be cleaned should be rinsed once with the fluid and then flushed with *cold* water.

Agreement between ^{32}P and ^{51}Cr Estimates

Using the rapid uptake method for ^{32}P described here, almost exact agreement with the ^{51}Cr method was found in making red cell volume estimates in seven subjects (Mollison, Robinson and Hunter, 1958) and the coefficient of variation between the two methods was only about 1 per cent. The causes of previously reported minor discrepancies were discussed by the same authors.

Radiation Dosage from ^{32}P and ^{51}Cr

The dose of ^{32}P recommended here for a single estimate of red cell volume in an adult is 2 μc., that is, less than 0.05 μc. per kg. This gives a total absorbed dose of 0.05 rads, that is, the equivalent of a single day's exposure at the maximum permissible rate to an occupationally-exposed worker (Veall and Vetter, 1958, p. 143).

The radiation dosage from ^{51}Cr is considered in Chapter V.

Red Cell Volume Deduced From Plasma Volume and Venous Haematocrit

Gibson *et al.* (1946) estimated red cell volume (RCV) in forty normal subjects, using ^{55}Fe-labelled red cells from donors previously injected with ^{55}Fe. Plasma volume (PV) and venous haematocrit (H_V) were estimated in the same forty subjects and red cell volume was deduced from these measurements. The ratio (A)

$$\frac{\text{RCV estimated with labelled red cells}}{\text{RCV deduced from PV and } H_V}$$

appeared to be a constant with a value of 0.845. Barnes *et al.* (1948a), using the Ashby method to determine true red cell volume, found the ratio to be 0.88. Finally, Reeve and Veall (1949), using a ^{32}P method, found the ratio to be 0.87.

Gibson *et al.* also calculated the body haematocrit (H_B) in their cases, i.e.

$$\frac{\text{Red cell volume}}{\text{Total blood volume}}$$

They found that in their series, over a venous haematocrit range of 38.5 to 48.1, the ratio (B)

$$\frac{H_B}{H_V}$$

was a constant with a value of 0.91.

It has been shown that expressions (A) and (B) cannot both be constants (Mollison *et al.*, 1950); in fact, expression (A) is not a constant but varies with venous haematocrit, whereas expression (B) $\frac{H_B}{H_V}$ is a constant.

Evidence that in adults the value of this constant is approximately 0.91 is considered later in this chapter. Thus body haematocrit (H_B) can be deduced from the observed venous haematocrit (H_V) by first correcting for trapped plasma (see Fig. 9) to give the true volume of packed cells in

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venous blood and then correcting this value by 0.91. Red cell volume (RCV) can then be deduced from plasma volume (PV), using the formula:

$$RCV = PV \times \frac{H_B}{100 - H_B}.$$

RESULTS OF BLOOD VOLUME DETERMINATIONS

Normal Plasma Volume

Some estimates of plasma volume in normal adult males and in children are shown in Table II. The plasma volume of women tends to be slightly lower than that of men. Thus, von Porat (1951) found mean values of 44.8 ml./kg. in women and 46.1 ml./kg. in men, and Wadsworth (1954)

TABLE II

PLASMA VOLUME IN NORMAL SUBJECTS

Authors	*No. of subjects*	*Identification in Fig. 14*	*Average venous hmt.*	*Average plasma volume (ml./kg.)*
		Adult males		
Gibson *et al.* (1946)	40	(5)	42.5	47.8
Gregersen (see Noble and Gregersen, 1946b)	51	(7)	45.0	45.0
Henschel *et al.* (1947)	32	—	46.3	45.0
Reeve and Veall (1949)	13	(6)	42.5*	46.6
		Children		
Russell (1949)†	64	(4)	43.0	48.1

* This figure was obtained by multiplying the observed values by 0.95. The other four estimates of venous haematocrit are presumed to be uncorrected for trapped plasma.

† Data relating to children weighing 10–30 kg.

found the mean plasma volume of eight normal women to be 43.1 ml./kg. The difficulty of predicting plasma volume accurately from body weight in any given subject is discussed below.

Relation between Plasma Volume and Packed Cell Volume (Venous Haematocrit)

In estimating plasma volume in a series of newborn infants it was noted that plasma volume (expressed as ml./kg. body weight) diminished steadily as the venous haematocrit rose over the range 40–70 per cent (Mollison *et al.*, 1950). This is illustrated in Fig. 14. The regression line was

calculated from data relating to thirty-four normal newborn infants and the slope was found to be highly significant.

It follows that to compare the relationship of plasma volume to weight in adults and newborn infants one must choose subjects with similar haematocrit values. For example, the average venous haematocrit of the thirty-four normal infants referred to above was 60 per cent and the average plasma volume was 41.3 ml./kg. This is close to the estimates of Gibson (1939b) for the plasma volume of adults with comparable venous haematocrit values (see Fig. 14). In Fig. 14 various other estimates of

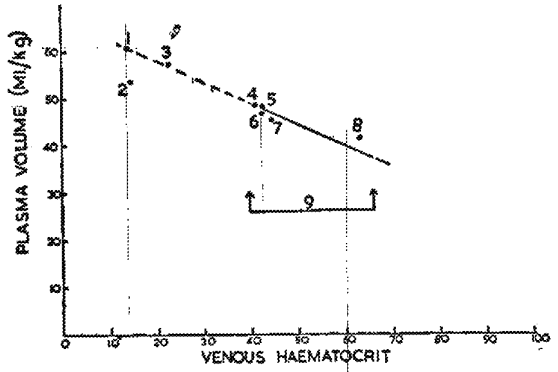

FIGURE 14. Relationship between plasma volume (in ml./kg.) and venous haematocrit. Points 1, 2 and 3 are derived from data in Table II; points 4, 5, 6 and 7 from data in Table III. Point 8 has been calculated from data published by Gibson (1939b); further details were given in the first edition of this book. The solid line whose limits are indicated by the arrows (9) is the regression line from the data of Mollison *et al.* (1950) for the plasma volume of 34 normal infants. (In this Figure and in Figs. 15 and 17, all estimates of venous haematocrit have been multiplied by 0.95 to correct for trapped plasma, although later work has shown that this is not the most accurate correction.)

plasma volume have been plotted to demonstrate the relationship of plasma volume to venous haematocrit over a wide range.

Estimates of the plasma volume in severe anaemia which are plotted in Fig. 14 are taken from the data set out in Table III. Although it appears that there is a tendency for plasma volume to fall as the venous haematocrit rises, it must be emphasized that many exceptions occur, notably in polycythaemia vera.

Normal Red Cell Volume

Gibson, Peacock *et al.* (1946), using red cells labelled with radio-iron, found that red cell volume in normal adult males (average haematocrit

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42.4) was 29.7 ml./kg. Reeve and Veall (1949), using ^{32}P-labelled red cells, found an almost identical figure (30.0 ml./kg.). Using a similar technique, it was found that in newborn infants with comparable haematocrit levels red cell volume in ml./kg. was almost precisely the same, i.e. 29.2 ml./kg. (Mollison *et al.*, 1950). In normal newborn infants with an average venous haematocrit of 56.8, red cell volume was much higher (see Table IV).

In the series of infants it was observed that although the relationship between venous haematocrit and red cell volume was substantially linear

TABLE III

PLASMA VOLUME IN SEVERE ANAEMIA

Group	*Author*	*Identification in Fig. 14*	*No. of cases*	*Average venous hmt. (×0.95)*	*Average plasma volume (ml./kg.)*	*S.D. (ml./kg.)*
Hypochromic anaemia; observed hmt. below 30 per cent	Abstracted from data of Gibson (1939b)	(3)	9	22.5	57	12.4
Pernicious anaemia; observed hmt. below 20 per cent	From data of Gibson (1939a)	(2)	8	14.4	54	10.0
Miscellaneous anaemias; observed hmt. below 20 per cent	Present author (unpublished)	(1)	8	13.8	60.5	4.9

N.B. The factor of (×0.95) used to correct for plasma trapping in this table is now known to lead to an over-correction, particularly for samples with relatively low packed cell volumes.

up to haematocrit values of 55 per cent, as previously reported by Hahn and Bale (1942), above that level red cell volume rose disproportionately. The relationship between red cell volume and venous haematocrit is indicated by the line in Fig. 15. In adults with venous haematocrit values exceeding 75 per cent, the disproportionate increase in red cell volume, compared with the increase in venous haematocrit, is even more striking, as shown by the estimates of Nylin *et al.* (1950) in subjects with congenital heart disease. If the curved line shown in Fig. 15 is continued, it fits their data satisfactorily.

The red cell volume of normal women, in relation to body weight, is lower than that of men; thus Wadsworth (1954) found 23.4 ml./kg.,

TABLE IV

RED CELL VOLUME IN NORMAL SUBJECTS

Authors	*Subjects*	*No. of subjects*	*Average venous hmt.*	*Average red cell volume (ml./kg.)*
Gibson, Peacock *et al.* (1946)	Adult males	40	42.4	29.7
Reeve and Veall (1949)	Adults	13	42.5*	30.0
Mollison *et al.* (1950)	Selected infants with hmt. values within the normal adult range	9	42.6*	29.2
Mollison *et al.* (1950)	Normal newborn infants	28	56.8*	41.8

* Observed venous haematocrit × 0.95.

Hedlund (1953) 24 ml./kg. and Berlin *et al.* (1951) 27 ml./kg. Similarly, in relation to surface area, Hedlund found the red cell volume of seven

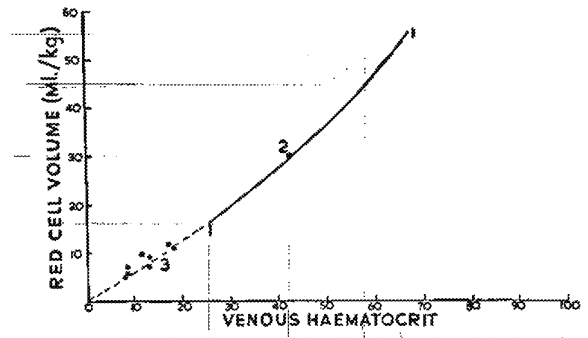

FIGURE 15. Relationship between red cell volume (in ml./kg.) and venous haematocrit. The slightly curved line (1—1) is taken from the data of Mollison *et al.* (1950) and is based on observations on 42 newborn infants. Point 2 is from the data of Reeve and Veall (1949). The constellation of points (3) is composed of estimates of red cell volume in anaemic adults, made by the author.

normal women to be 868 ml./sq. metre, compared with 1131 ml./sq. metre in thirty-five normal men.

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The Relationship between Body Haematocrit and Venous Haematocrit

When total circulating red cell volume and plasma volume are estimated in the same subject, the whole body haematocrit (H_B), that is to say,

$$\frac{\text{Red cell volume}}{\text{Red cell volume} + \text{Plasma volume}}$$

can be compared with the venous haematocrit. To allow for differences in conditions of centrifugation, observed values for venous haematocrit must be corrected for trapped plasma. It has been reported that the ratio

$$\frac{\text{Body haematocrit}}{\text{Venous haematocrit}}, \text{ or } \frac{H_B}{H_V}$$

remains constant over the venous haematocrit range 9–82 per cent, with a value of 0.91 and a standard deviation of 0.026 (Chaplin *et al.*, 1953). This is illustrated in Fig. 16.

It can be shown that if there is an extra-capillary space into which plasma but not red cells can penetrate and this space remains constant, it will not cause an alteration in the body haematocrit/venous haematocrit ratio at different levels of haematocrit. Suppose this extra space is V_2, and V_1 is the space in which the red cells circulate, then when the haematocrit inside space V_1 is 10 per cent, H_B will be $10 \div (V_1 + V_2)$ and when the haematocrit inside space V_1 is 80 per cent, H_B will be $80 \div (V_1 + V_2)$.

On the other hand, if a certain proportion of the plasma label were rapidly lost from the circulation, leading to a systematic overestimate of plasma volume, the effect on the estimate of body haematocrit would vary with venous haematocrit as pointed out by Chaplin *et al.* (1953).

The value of 0.91 for the ratio $\frac{H_B}{H_V}$ was also obtained by Gibson *et al.* (1946) in normal subjects. As pointed out by Chaplin *et al.*, the exact figure for the ratio is a little arbitrary since it depends, for example, upon the interpretation of the disappearance curve of the dye or other substance used for estimating plasma volume, Similarly, Donohue *et al.* (1955) claimed that if venous samples were taken without employing any stasis, slightly lower haematocrit values were obtained and, consequently, a slightly higher body haematocrit/venous haematocrit ratio (approximately 0.92).

As mentioned above, the S.D. of the $\frac{H_B}{H_V}$ ratio was found by Chaplin *et al.* to be 0.026 in subjects with widely varying haematocrits and this agrees well with the S.D. of 0.019 observed by Hicks *et al.* in subjects with

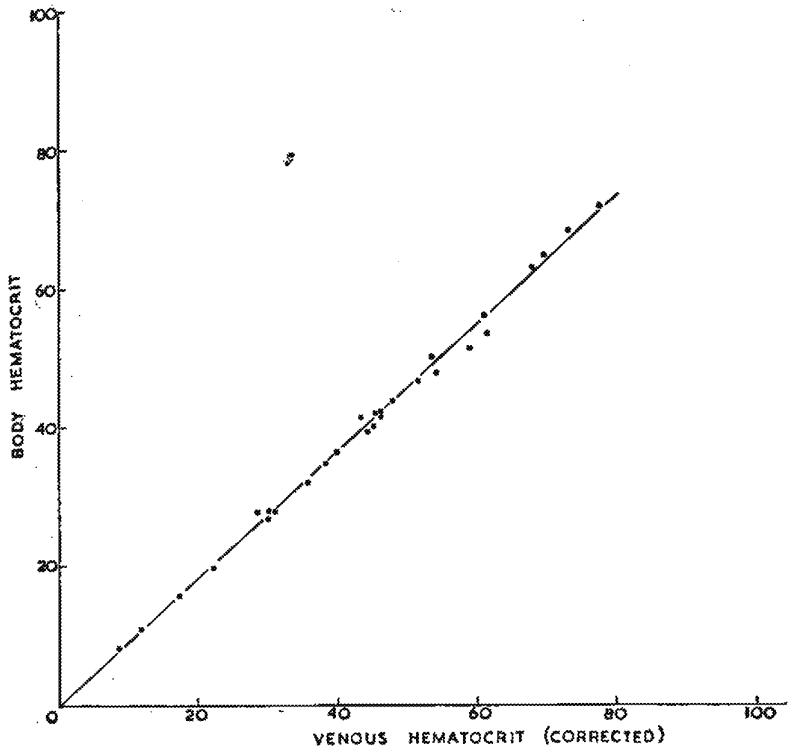

FIGURE 16. Relationship of body haematocrit, estimated from separate measurements of red cell volume and plasma volume, to venous haematocrit (corrected for trapped plasma) (from Chaplin *et al.*, 1953).

a narrower haematocrit range and with the S.D. of 0.023 observed in splenectomized dogs (see Reeve, 1957).

Hope and Verel (1955) noted that the blood in the brachial artery tends to have a slightly lower haematocrit than the blood in the antecubital vein; they found that the ratio of body haematocrit to venous haematocrit might be lower than normal in subjects with severe anaemia, in whom the

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ratio of body haematocrit to arterial haematocrit was still within normal limits.

Conditions in which the Body Haematocrit : Venous Haematocrit Ratio Differs Significantly from Normal

Verel (1954) found that in subjects with severe anaemia and splenomegaly the body haematocrit : venous haematocrit ratio was raised, and might in fact be greater than 1.0. This was confirmed by Rothschild *et al.* (1954) and by Fudenberg *et al.* (1961), who gave the following figures for the body haematocrit : venous haematocrit ratio:

Normal subjects	0.896
Patients with moderate splenomegaly (to umbilicus)	0.986
Patients with marked splenomegaly (to pelvic brim)	1.053
Patients with massive splenomegaly (to right flank)	1.156

In patients whose spleen had been removed the ratio was approximately 0.88.

Fig. 7 shows how the body haematocrit : venous haematocrit ratio is altered by splenomegaly.

It is to be expected that in polycythaemia in which the venous haematocrit is already very high splenomegaly will have relatively less effect, and when the degree of splenomegaly is only very slight the body haematocrit : venous haematocrit ratio may be within normal limits (Chaplin *et al.*, 1953).

The body haematocrit : venous haematocrit ratio tends to be low in congestive cardiac failure (Rothschild *et al.*, 1954) and in oedema secondary to steroid therapy (Fudenberg *et al.*, 1961).

The body haematocrit : venous haematocrit ratio was reported to be 0.87 in newborn infants by Mollison *et al.* (1950). The authors pointed out that they had made no correction for any loss of injected dye during the first 10 minutes after injection, and that failure to take this into account might have led them to overestimate plasma volume slightly, and thus to underestimate the true body haematocrit : venous haematocrit ratio which may have been nearer 0.89. It is possible that the underestimate of the ratio may have been even greater, because it is possible that the rate of loss of T-1824 is greater in newborn infants than in adults. Reeve (1957) has pointed out that under some conditions T-1824 does overestimate plasma volume, and that this probably accounts for the very low ratio (0.85) previously reported in rabbits (Armin *et al.* (1952)).

Value and Limitations of the Body Haematocrit : Venous Haematocrit Ratio

There is no doubt that under most conditions the ratio $\frac{H_B}{H_V}$ remains approximately constant. For example, it was found to remain constant within the first 6 hours after injury in humans (Davies and Topley, 1959), and also following acute massive haemorrhage in dogs (Reeve *et al.*, 1953). Under most circumstances, then, a reliable estimate of blood volume (BV) can be obtained from an estimate of total red cell volume (RCV) or plasma volume (PV) by assuming that the body haematocrit (H_B) is equal to the venous haematocrit × 0.91 and using the formula:

$$BV = RCV \times \frac{100}{H_B}$$

or,

$$BV = PV \times \frac{100}{100 - H_B}.$$

As already mentioned, the only conditions in which these formulae are known to be unreliable are in patients with oedema or with splenomegaly.

Normal Total Blood Volume

The blood volume of normal adult men was found to average 77.5 ml./kg. by Gibson *et al.* (1946); this compares closely with Reeve and Veall's (1949) estimate of 76.6 ml./kg. The blood volume of normal adult women is somewhat lower in relation to body weight, e.g. Wadsworth (1954) reported a figure of 66.5 ml./kg. All these estimates are based on combined measurements of red cell volume and plasma volume.

The difficulty of predicting the blood volume of any particular individual was re-emphasized by Hicks *et al.* (1956). The mean total blood volume of sixty-three normal subjects was found to be 5040 ml. (S.D. 784 ml.). The relation between blood volume and weight was calculated for the whole group and from the resulting regression equation the S.D. was found to be 650 ml.; for height the prediction was a little better (S.D. 535 ml.) and for height and weight together was slightly better again (483 ml.). The best prediction was obtained by taking into account fat distribution and girth as well as height and weight, but even then the S.D. was 383 ml.; that is to say, the prediction was only twice as good as that of assuming that all normal blood volumes are 5 litres.

Edwards and Whyte (1960) have made the point that when blood volume is predicted from weight the error is distinctly less when the

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regression equation relating the two is used than when blood volume is calculated simply as so many millilitres per kilogramme. Thus in their series estimates based on body weight had an error of about 19 per cent when using the regression equation and 30 per cent when using simply the average volume for weight.

Allen *et al.* (1956) estimated the blood volume of a group of Chinese subjects and concluded that accurate predictions could be made from a formula in which the subject's height was cubed. Thus, for males the blood volume in litres was given by: $0.417\ H^3+0.0450\ W-0.03$, where H was the height in metres and W the weight in kg. (for women the expression was $0.414\ H^3+0.0328\ W-0.03$). Allen *et al.* also concluded that the lower blood volume of women in relation to weight was due to the larger amount of fat in women, this tissue containing relatively little blood. They found the mean density of men to be 1.067 and that of women 1.040, the density of adipose tissue being 0.948. They also concluded that the blood content of the lean body mass was 92 ml./kg. and that of fat 11-22 ml./kg.

As mentioned, Allen *et al.* made their measurements on Chinese subjects, and it seems that, unfortunately, the formulae obtained may not apply closely to subjects with rather different physical characteristics. In a series of English subjects tested by the author, in which red cell volume and plasma volume were estimated separately, and the results compared with predictions from the formula of Allen *et al.*, occasional discrepancies of up to 1 litre were observed. Baugh *et al.* (1958) found that estimates in Eskimos differed on the average by 0.7 litres from the figures predicted from the formulae of Allen *et al.*

Muldowney (1957) has claimed that RCV can be very accurately predicted from lean body mass, as estimated from total body water, 95 per cent of his estimates being within 75 ml. of the figure predicted from the formula: Red cell mass = (32.74 × lean body mass)+155.17.

Variations in Blood Volume in Health

Effect of Changes in Red Cell Volume

As already mentioned, at haematocrit levels below 55 per cent the relation between total red cell volume and packed cell volume (venous haematocrit) is approximately linear. Plasma volume tends to increase as the haematocrit falls, although the rate of increase of plasma volume is less than the rate of decrease of red cell volume, so that blood volume tends to fall slightly as haematocrit falls (see Fig. 17, and see also Whyte, 1956).

Above a haematocrit of about 55 per cent RCV rises disproportionately to haematocrit (see Fig. 15). Since in this haematocrit range plasma volume apparently continues to decrease only linearly (see Fig. 14), blood volume rises disproportionately (see Fig. 17).

Measurements in newborn infants with haematocrits ranging from twenty to seventy support the idea that blood volume increases as haematocrit rises (Mollison *et al.*, 1950).

Differences between infants and adults. It has already been pointed out that although the normal newborn infant has a higher blood volume than the normal adult, the difference appears to depend almost entirely upon the different levels of the venous haematocrit; some figures are given in Table V. In premature infants blood volume at birth seems to be distinctly

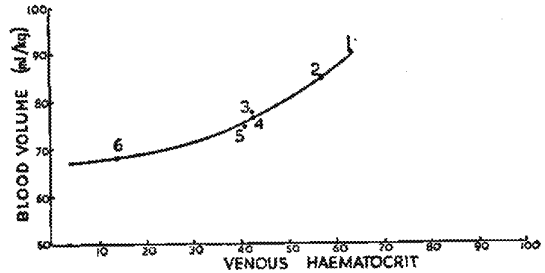

FIGURE 17. Relationship between total blood volume (in ml./kg.) and venous haematocrit. Points 2, 3 and 4 are based on combined red cell volume and plasma volume measurements (2, Mollison *et al.*, 1950; 3, Gibson, Peacock *et al.*, 1946; 4, Reeve and Veall, 1949). Points 1, 5 and 6 have been calculated from plasma volume and body haematocrit, deduced from venous haematocrit (1, Gibson, 1939b, as used in Fig. 14; 5, Russell, 1949, as used in Fig. 14; 6, unpublished observations).

greater in relation to body weight than in mature infants (Schulman and Smith, 1954b; Sisson *et al.*, 1959).

Using the estimates of plasma volume and venous haematocrit of Russell (1949) for a group of sixty-four infants and young children weighing 10 kg. to 30 kg. and calculating total blood volume from the formula given above, it can be concluded that infants of this weight have an average blood volume of approximately 75.4 ml./kg. (average venous haematocrit ($\times 0.95$) = 40.8). Calculations from Russell's data for ten infants aged 3-11 months with an average venous haematocrit ($\times 0.95$) of 39.2 per cent, give a value of 71.5 ml./kg.

Effect of posture and of rest in bed. Plasma volume is affected by a change in posture; on changing from the supine to the upright position fluid leaves

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the blood vessels of the legs under the influence of the increased intravascular pressure (Waterfield, 1931). Twenty to 30 minutes' standing, after 30 minutes lying down, produces a decrease in plasma volume of about 300 ml. (Thompson *et al.*, 1927). Further data may be found in the

TABLE V

BLOOD VOLUME OF NEWBORN INFANTS AND ADULTS: ESTIMATES BASED ON SEPARATE MEASUREMENTS OF PLASMA VOLUME AND RED CELL VOLUME

Subjects	*Average venous hmt.* (×0.95)	*Blood volume* (*ml./kg.*)
Newborn infants: Normal	59.7	84.7
Selected*	42.6	77.1
Normal adults	42.5	76.6

* Infants with haematocrit values within the normal adult range.

papers by Walters (1933), Widdowson and McCance (1950) and Fawcett and Wynn (1960).

Lying in bed for 3 weeks produces a fall in plasma volume amounting to about 500 ml. in normal males; this is accompanied by a scarcely significant fall in red cell volume and thus the haematocrit rises (Taylor *et al.*, 1945).

Pregnancy. Plasma volume starts to increase early during pregnancy, probably before the twelfth week, and reaches a peak between 30 and 60 days before term. At this time it is increased to about 65 per cent above its normal value (Thomson *et al.*, 1938). The contention that a decrease in plasma volume begins during the last 30 days of pregnancy was not confirmed by White (1950). Normal non-pregnant values are reached by the end of the second week after delivery.

Roscoe and Donaldson (1946) confirmed many of these findings; they found that between the thirty-third and thirty-eighth weeks of pregnancy blood volume was some 25 per cent greater in absolute terms than in the non-pregnant woman and was some 20 per cent greater when related to surface area.

During pregnancy red cell volume increases by about 200 ml., but, since plasma volume increases to a much greater extent (approximately 1600 ml., according to Thomson *et al.*, 1938), haemoglobin and haematocrit values fall. Roscoe and Donaldson found the mean venous haematocrit at 33–38 weeks to be 36 per cent, compared with 42 per cent in the non-pregnant woman.

Although it is still widely accepted that anaemia develops during normal pregnancy, there is evidence that normal, or almost normal, haemoglobin values can be maintained in almost all subjects by the administration of iron (see Chapter IV). Estimates of the changes in red cell volume during pregnancy in women receiving adequate doses of iron are not yet available.

Variations in Blood Volume in Disease

In *polycythaemia vera* the blood volume is greatly increased. When groups of cases are considered there is a relationship between haematocrit and blood volume. Thus Abbatt *et al.* (1954) found that blood volume was increased by 43 per cent in five patients with haematocrits between 45 and 58 per cent and was increased by 84 per cent in three patients with haematocrits between 77 and 82 per cent. There were some striking discrepancies; one patient with a haematocrit of 57 per cent had a blood volume 85 per cent above normal and one patient with a haematocrit of 72 per cent had a blood volume which was within the normal range. A partial compensatory decrease in plasma volume was seen in only six out of sixteen subjects. In five subjects plasma volume was normal and in five more was actually increased.

Prentice *et al.* (1952) and Wilson and Boyle (1952) also found that the haematocrit was often a bad guide to the red cell volume in polycythaemia.

Secondary polycythaemia. In congenital cyanotic heart disease Prader *et al.* (1949) found plasma volume to be reduced to 39 ml./kg., compared with their normal figure of 47 ml./kg.; the average haematocrit in their series was 68 per cent. Measurement of red cell volume (^{32}P) and plasma volume (T-1824) in similar cases showed that red cell volume was enormously increased and plasma volume distinctly reduced. Following operation for pulmonary stenosis, which results in the establishment of an adequate blood flow, a great reduction in red cell volume (^{32}P) can be demonstrated (Clay *et al.*, 1951).

'Polycythaemia of stress.' It has been claimed that in some subjects, as a response to stress, the plasma volume falls so that although the total circulating red cell volume is not increased the PCV is above normal limits. In three cases of this kind investigated by Crawford and De Gruchy (1958) PCV was between 52 and 60 per cent but total circulating red cell

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volume was 27-34 ml./kg. (within normal limits) and total blood volume only 58-65 ml., that is below normal limits.

Pernicious anaemia. Hallberg (1955) measured the blood volume in pernicious anaemia by Sjöstrand's carbon-monoxide method; although for reasons given earlier this method is subject to a systematic error, it doubtless enables satisfactory comparisons to be made between different cases in which the method is used.

Hallberg found that on the average total blood volume was reduced by about 10 per cent, the reduction in red cell volume not being fully compensated by an increase in plasma volume. With treatment there was a tendency for plasma volume to fall and red cell volume and blood volume to rise. However, in individual cases the blood volume was very variable at the beginning of treatment so that the changes in the haemoglobin concentration did not accurately reflect the rate of red cell regeneration. It thus appears that in pernicious anaemia, as in polycythaemia vera, the haematocrit does not always give a reliable indication of red cell volume.

In thyrotoxicosis total red cell volume is high in relation to lean body mass, and in *myxoedema* it is low (Muldowney *et al.*, 1957).

Dehydration and salt loss. Dehydration, due simply to deprivation of water, causes no appreciable alteration in plasma volume (Nadal *et al.*, 1941). Black *et al.* (1942) also found no haemoconcentration in four men who abstained from water for 3 to 4 days and lost 4 to 7 per cent of their body weight. They suggested that this was because the reduction in water content of the body was shared by all the tissues.

By contrast, when salt is lost from the body, plasma volume shrinks. A rise of ten divisions in the venous haematocrit was noted by Nadal *et al.* (1941) in some instances. It appears that in these cases the salt (and water) lost from the body comes from the plasma and extracellular fluid only.

Cardiac failure. All published reports agree that in congestive cardiac failure the plasma volume, measured with T-1824, is increased (e.g. Gibson and Evans, 1937b; Hedlund, 1953). Ross *et al.* (1952) suggested that in congestive failure T-1824 is lost rapidly from the circulation after injection so that plasma volume is overestimated. Some evidence in support of this is provided by comparative estimates of plasma volume in cardiac failure using Dextran with an average molecular weight distinctly greater than that of albumin (see above). Nevertheless, Hedlund (1953) measured plasma volume (T-1824) and red cell volume (^{32}P) in patients

with congestive failure and found that the body haematocrit : venous haematocrit ratio was normal, suggesting that plasma volume was being correctly measured.

One obvious difficulty is that of expressing results in terms of body weights in patients who may have lost flesh but gained much salt and water. However, there seems no doubt that in many patients with congestive failure blood volume is increased in relation to weight even when no correction to 'dry weight' is made; e.g. Reilly *et al.* (1954) found in such cases that blood volume was raised by 20 per cent in relation to wet weight and 30-35 per cent in relation to dry weight.

In *chronic nephritis* Harris and Gibson (1939) found that plasma volume tended to vary directly with the serum albumin concentration, but their figures show a very big scatter and do not demonstrate the relationship conclusively. In nephrotic syndromes blood volume was lower than normal, when 'normal' was predicted from the patient's height. In nephritis with congestive failure, blood volume was slightly increased.

Effect of posture on plasma volume in oedematous subjects. Fawcett and Wynn (1960) showed that the changes in plasma volume accompanying changes in posture are greater in oedematous subjects than in normal subjects. Thus in patients who assumed the upright position after 12 hours lying in bed, plasma protein concentration rose by 15 per cent and haematocrit by 12 per cent. To avoid the influence of these extreme changes the authors suggested that patients should as a routine be seated for at least 15 minutes before having blood samples taken.

Severe starvation. During severe starvation blood volume falls slightly in absolute figures but increases greatly in relation to body weight. Thus the data of Henschel *et al.* (1947), when re-calculated so as to estimate true blood volume from plasma volume and venous haematocrit, yield the following figures: in thirty-one adults with an average weight of 69.9 kg. and an average venous haematocrit of 46.4, blood volume was initially 5.10 litres, or 73.6 ml./kg. After 24 weeks' severe starvation, body weight had fallen to 53.4 kg. and venous haematocrit to 36.6; blood volume was now 4.88 litres, that is, a slight fall in absolute terms, but in terms of body weight an increase to 91.4 ml./kg. The present author (1946) also found that except in grossly oedematous subjects, blood volume was increased in relation to body weight in severe starvation.

Thus in starvation, despite a fall in serum protein concentration, plasma volume does not diminish in proportion to body weight. It has been

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pointed out that an increase in the extracellular fluid volume of the body is one of the most striking features of starvation and that the plasma shares in this general oedema (McCance and Widdowson, 1951).

Acute reduction in blood volume due to haemorrhage, burns, etc. is dealt with in the following chapter.

CHAPTER THREE

HAEMORRHAGE AND TRANSFUSION

The first transfusion with some claim to be considered beneficial to the patient was performed in a case of post-partum haemorrhage. Although the patient received only 8 ounces in 3 hours, 'she felt as if *life* were infused into her body' (Blundell, 1829). It is in cases of acute haemorrhage that transfusion still produces its most dramatic effects.

The natural restoration of blood volume, after acute haemorrhage, is a slow process. If only 1000 ml. or even 1500 ml. of blood have been lost, the body can temporarily adjust itself to the lowered blood volume and blood pressure can be maintained by vaso-constriction until plasma volume is restored. When greater amounts of blood are lost, blood pressure is no longer maintained and the supply of oxygen to vital centres is no longer adequate. In experimental animals (splenectomized dogs) a sudden haemorrhage, equivalent to 40 per cent of the original blood volume, carries a mortality of 50 per cent if untreated (Rawson *et al.*, 1959).

To those who practise transfusion it is thus of first importance to be familiar with the signs produced by haemorrhage and also to be familiar with the mechanisms by which the body attempts to compensate for blood loss. Normally, the picture of acute haemorrhage in man is confused by other signs connected with the cause of the haemorrhage; for example, by pain. Moreover, it is seldom possible to form any precise estimate of the amount of blood that has been lost. However, studies of the effect of sudden loss of blood, produced by the venesection of volunteers, have provided valuable information about the effects which haemorrhage alone produces in man.

The Vaso-Vagal Attack

Before discussing other effects of blood loss, one response, 'the vaso-vagal attack', must be described. This may be induced in certain subjects by the mere sight of blood being taken from another person and can be provoked in all subjects by withdrawing a sufficient quantity of blood.

The faint, or 'vaso-vagal attack' appears to be a hypothalamic response. Its features are as follows: the subject starts to sweat and turn pale; the skin feels cold, the pulse slows strikingly and the heart rate may fall to 30

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per minute; the blood pressure may become unrecordable; there may be vomiting and involuntary defaecation. When taking blood from donors, warning of oncoming fainting is often given by a fall in flow, as Harvey (1628, Chapter XII) noted. As fainting develops the subject feels weak and objects start to appear dim. Loss of consciousness may follow and convulsions are occasionally seen.

It appears that these effects are produced in two ways: firstly, by the action of the autonomic system, causing slowing of the heart, vomiting and sweating and, perhaps most important, dilation of the arterioles, leading to a sudden fall in blood pressure (Barcroft *et al.*, 1944); and secondly, by an output of posterior pituitary hormone, causing blanching of the skin and inhibition of urine secretion.

Fainting commonly occurs in subjects who have lost blood; moreover some of the signs produced by severe haemorrhage, such as a fall in blood pressure, also occur in the vaso-vagal attack. It is therefore important to be familiar with the vaso-vagal attack and to appreciate that an apparently grave clinical picture can be produced by emotion alone. In patients who display these signs but who have not lost blood, rapid recovery occurs when the patient is placed in the supine position and tilted so that the head is lower than the rest of the body. The slow pulse rate (30-60 per minute) in the vaso-vagal attack is the most useful single sign in differential diagnosis.

As already mentioned, the vaso-vagal attack or 'faint' frequently occurs in patients who have lost blood. Estimates of the frequency with which fainting occurs in blood donors vary according to the definition of the term 'faint'. Thus in the Medical Research Council's inquiry (1944) 'fainting' was defined as the manifestation of any of a series of signs or symptoms such as pallor, sweating, dizziness or nausea. In this sense of the word some 5-6 per cent of donors fainted. Poles and Boycott (1942) found that in their first 10,000 cases only 2.8 per cent of donors fainted, but they applied the term only to those who lost consciousness or could not stand or sit without doing so.

Poles and Boycott found a definite relation between the incidence of fainting and the amount of blood donated. While only 3.8 per cent of a series of 341 donors who gave 440 ml. fainted, the incidence rose to 8.5 per cent in 410 donors who gave 540 ml. of blood. Barcroft *et al.* (1944) reported that fainting occurred in eleven out of twenty-eight normal males who lost 800-1000 ml. of blood and in fifteen out of twenty-nine who lost 1000-1200 ml. Ebert *et al.* (1941) observed loss of consciousness in five out of six volunteers from whom 780 ml. to 1200 ml. of blood were withdrawn in 6-13 minutes. Finally, Howarth and Sharpey-Schafer (1947), who withdrew up to 1500 ml. of blood from normal volunteers, noted that fainting could be produced in all subjects provided that enough blood was withdrawn within a limited time.

HAEMORRHAGE

Circulatory Effects

The loss of 430 ml. of blood in 4 minutes usually produces only trivial effects on the circulation. As a rule there is no change in blood pressure or pulse rate, though the venous pressure may fall slightly and take more than 30 minutes to regain its initial level (Loutit *et al.*, 1942).

Wallace and Sharpey-Schafer (1941) studied twenty-eight subjects from whom amounts of blood varying from 465 to 1150 ml. of blood were rapidly withdrawn. In twenty-two cases blood pressure did not change significantly, even though in five cases, 1050 to 1150 ml. of blood had been removed, but in several of these subjects it was noted that a sudden change of position, from lying down to sitting up, produced a fall in blood pressure and was sometimes accompanied by loss of consciousness. This was also observed in two of the cases observed by Ebert *et al.* (1941). This phase may be regarded as one of partial compensation in which the subject feels all right so long as he rests. The next grade of response is one in which blood pressure is not maintained even when the subject is supine. Among Wallace and Sharpey-Schafer's subjects there were eight in whom blood pressure remained low for 24 hours after bleeding.

Howarth and Sharpey-Schafer (1947) noted that persons who had lost some 1500 to 2000 ml. of blood had a low right auricular pressure and a low cardiac output despite some increase in heart rate. These patients were pale, cold and clammy and sometimes displayed air hunger. This, of course, is the classical picture of haemorrhage and of 'wound shock', and since 1917 it has been appreciated that this condition is associated with a reduced blood volume.

Hypertensive response. Although a fall in blood pressure is the commonest response to haemorrhage, young adults who suffer only a moderate haemorrhage may exhibit transient hypertension (Grant and Reeve, 1951). In these patients transfusion is usually unnecessary. Of a series of fifty-two injured subjects who had a sustained systolic blood pressure above 140 mm. Hg, only two required transfusion and then only after blood loss at operation (Howard *et al.*, 1955).

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In children the hypertensive response may be associated with severe injuries and be suddenly followed by a profound fall in blood pressure (annotation in *Lancet*, 1955, **ii**, 1023).

Effect of anaesthesia. The circulatory effects produced by acute haemorrhage differ in anaesthetized subjects; for example, venesection of approximately 1500 ml. may be followed by only small changes in pulse and blood pressure (de Wardner *et al.*, 1953).

The Role of Haemorrhage in 'Wound Shock'

Investigations carried out during the 1914-18 war showed that in 'wound shock' blood volume was reduced and that the severity of the clinical picture was roughly paralleled by the degree of reduction in blood volume (Keith, 1919; Robertson and Bock, 1919). Although the syndrome of 'irreversible shock' is still not well understood, clinical investigations during the Second World War confirmed the observation that the effects of haemorrhage account adequately for the main phenomena of 'wound shock' (see for example Grant and Reeve, 1951). Thus in the management of injured patients a major problem is the assessment of the degree of haemorrhage; the value of different methods will be briefly considered.

The size and nature of the wound are the most valuable guides. It is seldom possible to make any accurate estimate of the amount of blood lost externally, but the size of the wound in limb injuries is fairly well correlated with the degree of blood loss. Thus Grant and Reeve (1951) found that for each area of tissue damaged corresponding to the size of the patient's hand, a loss of 10 per cent of the patient's blood volume should be assumed. It should be remembered that swelling surrounding the area of the wound is due to loss of blood into the tissues and thus often indicates a gross reduction in circulating blood volume.

Patients with wounds of the extremities involving large muscles may require very large transfusions. Thus Prentice *et al.* (1954) found that such patients frequently needed the transfusion of an amount of blood equal to their initial blood volume; they considered that the continued loss of blood into the tissues was important in bringing about the extensive loss of blood from the circulation. Clarke *et al.* (1955) found that a single injury to the thigh might be associated with a blood loss of 2.5 litres or even more.

Perforating wounds of the chest or abdomen are usually associated with considerable haemorrhage into the pleural or peritoneal cavities.

Systolic blood pressure. When the amount of blood loss cannot be estimated reliably from inspection of the wound, the next most reliable sign of a reduction in blood volume is a lowered systolic blood pressure. Grant and Reeve's (1951) findings were as follows: in previously untransfused subjects a systolic blood pressure of 100 mm. Hg or more indicates a blood volume of at least 70 per cent normal; a systolic pressure of over 140 mm. Hg, a blood volume of at least 80 per cent normal; a blood pressure of less than 100 mm. Hg, a blood volume of less than about 70 per cent normal. On the whole, these figures agree well with the observations published in 1919 by Robertson and Bock and by Keith, and with more recent observations, such as those of Noble and Gregersen (1946b). These investigators also emphasized that in severe injuries without external blood loss, there is a tendency to underestimate the amount of blood that has been lost into the tissues.

Signs of vasoconstriction in an injured subject should suggest concealed haemorrhage. Perhaps the most reliable sign of peripheral constriction is the temperature of the nose: if the nose is cold to the touch in a person lying in reasonably warm surroundings, peripheral circulatory failure may be presumed. Pallor alone is not uncommon in those in whom blood loss is negligible, but a short period of rest in the head-down position, coupled with warming, should restore some colour to the face. If signs of vasoconstriction persist in a person who may have suffered an internal haemorrhage, the effect of transfusion should be tried.

A Note on the Term 'Shock'

The term 'shock' is ambiguous. As Grant (1954) pointed out, it is often used to imply simply that the patient is pale and cold and so seems ill, irrespective of pulse rate and blood pressure. Perhaps the term is most widely understood in medical circles as the clinical syndrome of peripheral circulatory failure whose chief signs are a thready pulse and cold, clammy extremities. This syndrome embraces conditions of diverse aetiology which may conveniently be divided into two groups: those associated with oligaemia and those associated with atony of the blood vessels.

Transfusion is of great value in oligaemic states but is of negligible value in conditions of vascular atony associated for example with severe pneumonia or diphtheria. Transfusion has been tried in the state of collapse that may accompany coronary thrombosis but is evidently of no value (Epstein and Relman, 1949).

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Blood Loss at Operation

There is some evidence that even operations associated with very little apparent blood loss are accompanied by a decrease in circulating red cell volume. Thus Davies and Fisher (1958) in a series of nine patients undergoing meniscotomy found that on the average red cell volume decreased by 7 per cent in the period between the day before operation and 3 days after operation. In the following week there was a further fall of 2 per cent.

Measurement of blood volume changes following other operations confirms that the decrease is greater than expected from the amount of blood lost externally. For example, Wiklander (1956) found that the average total blood loss associated with partial gastrectomy was about 1400 ml.; of this about 650 ml. was lost at operation; a further 300 ml. was lost from the circulation in the next 24 hours and a further 450 ml. in the week after that.

The loss determined by weighing swabs at operation was usually only about 70 per cent of that determined from estimates of blood volume. This agrees well with the conclusion of Cáceres and Whittembury (1959) who found that the weighing of sponges at operation underestimated blood loss by 25 per cent, due partly to evaporation of fluid from the sponges before weighing and partly to bleeding into tissues which was not allowed for. The authors concluded that the relation between the true reduction in blood volume and the apparent reduction, as estimated from weighing swabs, was constant enough to enable satisfactory estimates to be made, i.e. apparent loss plus 25 per cent.

Acute Loss of Plasma without Red Cells

Although burning is the classical cause of acute plasma loss from the circulation, there are a few other conditions which have been shown to be associated with plasma loss. Perhaps the most important of these is acute obstruction of the small intestine. When strangulation of the small intestine occurs a large amount of blood may be trapped in the obstructed loop. Even in the absence of strangulation, simple obstruction accompanied by distension leads to a considerable diminution of plasma volume, although the mechanism of this is not well understood. In crushing injuries there may also be a considerable local loss of plasma.

In severe burning there is an almost immediate reduction in plasma volume, due to loss of plasma into the burnt area. Subsequently there is a further loss which continues for a period of about 24 hours.

Changes in plasma volume following burns, produced experimentally,

were measured by Keeley *et al.* (1939). Within a matter of minutes a fall in plasma volume amounting to 27 per cent to 36 per cent of the initial volume was observed; following this initial fall there was a further gradual decrease. It was noted that plasma protein concentration fell slightly after burning and this was doubtless due to the entrance of protein-free extracellular fluid into the blood stream.

As in the acute reduction in blood volume due to haemorrhage, there is an initial phase of compensation. In this phase, blood pressure may be maintained though the pulse volume is likely to be poor and the skin cold. After a few hours blood pressure falls and the general condition deteriorates. It is very important to begin transfusion at the earliest possible moment, before the blood pressure has fallen. The amount of plasma to be transfused is considered later in this chapter.

Loss of red cells from the circulation in burns. In severe burns there is an immediate loss of red cells, due directly to the heating of the cells to temperatures above 50° C. (Shen *et al.*, 1943). In addition there is an unexplained and largely unpredictable loss of red cells from the circulation during the period between 12 and 48 hours after injury in burns involving more than 20 per cent full-thickness skin loss (Topley and Jackson, 1957).

Spontaneous Restoration of Blood Volume

The rate of restoration of blood volume after haemorrhage was studied by Ebert *et al.* (1941) who found that after the loss of about 1000 ml. of blood adults required some 36 hours to restore blood volume. During that period no appreciable number of red cells was replaced, so that the lost red cells and plasma were replaced by plasma alone. Fig. 18 shows the rate of fall of haematocrit in one of the cases investigated by Ebert *et al.*; in this instance, blood volume was not completely restored within 36 hours.

These data have been supplemented by observations made in wounded soldiers by Grant and Reeve (1951). By estimating the haemoglobin concentration of blood a few hours after injury, and by estimating blood volume, and by assuming a haemoglobin concentration before wounding of 16 g./100 ml., they were able to calculate the amount of fluid that had been added to the circulation during the first 12 hours after wounding. Their results were as follows: subjects who had lost 20 per cent or less of their blood, mean haemoglobin on admission 15.2 g./100 ml.; subjects who had lost 21 per cent to 40 per cent of their blood, mean haemoglobin on admission 13.8 g./100 ml., corresponding to the addition of approxi-

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mately 660 ml. of fluid in 12 hours; subjects who had lost more than 40 per cent of their blood, mean haemoglobin on admission 11.8 g./100 ml., corresponding to the addition of nearly 1 litre of fluid during the 12 hours after injury.

In general, then, the greater the initial haemorrhage the lower the haemoglobin concentration. Nevertheless in civil practice there is the additional factor of uncertainty whether the patient was anaemic before

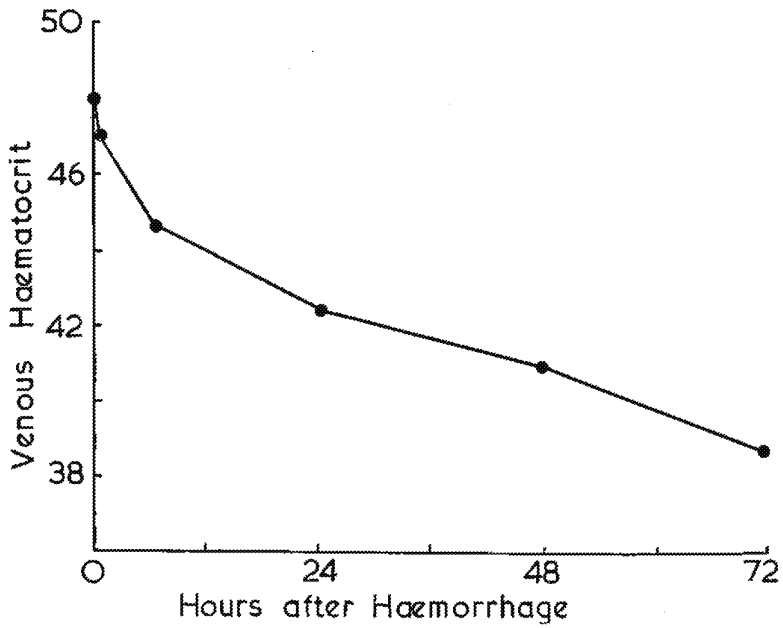

FIGURE 18. Rate of fall of haematocrit in a healthy male adult after the sudden loss of 1070 ml. of blood (data from Ebert, Stead and Gibson, 1941).

the haemorrhage; it is thus unwise to place much reliance on the haemoglobin concentration.

It appears that the rate of restoration of plasma volume is limited by the rate at which protein is added to the plasma. The rate at which water can be made available is not the controlling factor, since in moderately severe dehydration, plasma volume is not reduced (see Chapter II). Moreover, in volunteers bled of a litre or more there must be ample reserves of water (and salt) and yet plasma volume is not restored for some 36 hours or more. In these cases there is only a slight drop in serum protein concentration

after the haemorrhage, and the concentration then shows little change though plasma volume is increased. Evidently a small amount of protein-free fluid is first added and then the rate of addition of fluid is controlled by the rate at which protein is added to the circulation. It follows that, after haemorrhage, plasma volume can be only very temporarily restored by infusions of saline.

If large amounts of saline are given they can only serve to swell the extravascular fluid. The syndrome of 'post-operative drowning' due to the giving of large amounts of intravenous saline during operation was recognized long ago (Grünbaum and Grünbaum, 1911).

The situation is modified in patients who have lost salt; for example, patients with gastro-intestinal haemorrhage who have been vomiting. Such patients are likely to be salt-deficient and plasma volume cannot be fully restored until extra salt has been administered.

Haemoglobin Regeneration after Normal Blood Donation

Wadsworth (1955) found that in normal healthy subjects bled of about 400 ml. of blood (8 per cent of their blood volume) there was a small but significant increase in the reticulocyte count, with a peak on the ninth day after bleeding. The haemoglobin level was lowest 1 or 2 weeks after bleeding; thereafter there was a rapid increase and pre-donation levels were reached 3 to 4 weeks after bleeding (Fig. 19).

Wadsworth emphasized that after a relatively small haemorrhage the changes in haemoglobin concentration were only a little greater than those expected from random variation. From an examination of daily venous blood samples taken from subjects who had not been bled, he concluded that only changes greater than 1.5 g./100 ml. could be considered significant. That is to say, in terms of the Haldane scale, differences greater than 8 per cent. This is similar to the conclusions of Cotter *et al.* (1953) who found that two haemoglobin readings taken on the same day were unlikely to differ by more than 6 per cent and two samples taken on different days were unlikely to differ by more than 9 per cent.

Moore (1948) has stressed the importance of blood donation as a source of iron loss. Menstruation is often considered very important as a cause of loss of iron from the body, but Moore points out that not more than about 650 ml. of blood are lost in a year by the average woman. A person who gives two blood donations a year loses a considerably greater amount.

Laurell (1947) investigated the serum iron concentration and the iron-binding capacity of the serum of blood donors some 2 months after they

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had last given blood. Most of the subjects had donated about 2 litres during the previous year and about 1 litre before that. Haemoglobin values, red cell counts and serum iron values were all slightly subnormal; the iron-binding capacity of the serum was significantly increased.

Hagberg *et al.* (1958) confirmed that in donors who had given 5-6 units of blood (each of 350 ml.) in the previous year or two serum iron was diminished and total iron-binding capacity increased, and concluded that if no supplementary iron was given the interval between the bleedings of healthy male donors should be 3 months.

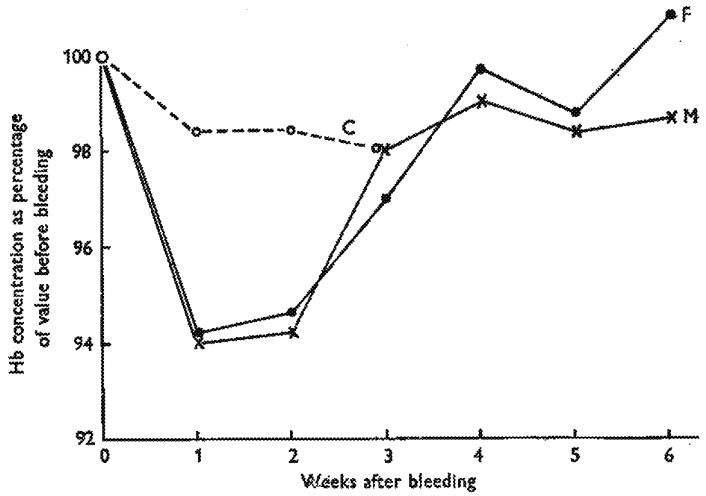

FIGURE 19. Mean haemoglobin concentration in seven women (F) and seven men (M) at weekly intervals after being bled of about 8 per cent of their blood volume. The figure also shows the change in the mean haemoglobin concentration of four men and four women who were not bled (C) (from Wadsworth, 1955).

Heistö and Foss (1958) found that even after a single donation of 450 ml. iron absorption after a test dose was sometimes increased, and they suggested that extra iron should be given after each blood donation, even the first. There is general agreement that those who give blood regularly should be provided with extra iron: for example, 0.4 g. (equivalent to 6 grains) of ferrous sulphate daily for 30 days following each donation.

Iron as a Limiting Factor in Haemoglobin Regeneration

Finch *et al.* (1950) have demonstrated the importance of iron as a limiting factor in haemoglobin regeneration after haemorrhage. 500 ml.

of blood were withdrawn each week for 7 weeks from four healthy males with supposedly normal iron stores. The venous haematocrit fell on the average from 44.5 to 33.6 and did not return to its original level for a further 2 months. These changes were not prevented by the administration of iron to the subjects some time before venesection. The fall in venous haematocrit was greater in two males whose iron stores had been depleted by previous numerous phlebotomies. By contrast, the fall in venous haematocrit was smaller in two subjects who received 0.6 to 1.2 g. of ferrous sulphate daily during the period of weekly venesections. After four bleedings, each of 500 ml., the haematocrit fell from 43.7 to 36.6, but then, despite further weekly bleedings, there was a tendency for the haematocrit to rise; after 3500 ml. has been withdrawn in 7 weeks the haematocrit was 39 per cent and the serum iron concentration was only very slightly reduced. Similarly in subjects with haemochromatosis, with greatly increased iron stores, the haemoglobin concentration may be kept within normal limits despite the removal of as much as 1 litre of blood a week. Thus, Davis and Arrowsmith (1952) studied one patient from whom 50 litres of blood was removed in 62 weeks without the production of anaemia.

The Circulation in Severe Anaemia

From the phase of low venous pressure and low cardiac output following haemorrhage, the subject may pass into a new phase, aptly described by Howarth and Sharpey-Schafer (1947) as the 'hyperkinetic phase'. The subject still looks ill but now the limbs are warm, venous pressure is raised and cardiac output increased. It seems clear that this phase begins only after blood volume has been partially restored, and it is to be regarded as a syndrome of compensation for anaemia. Indeed the same picture is seen in patients suffering from chronic severe anaemia (Sharpey-Schafer, 1944). Sharpey-Schafer (1945) has stressed the risks of transfusing patients who are in this phase, and these risks are discussed in the following chapter.

It might seem that in practice there was little risk of confusing the picture of acute haemorrhage with that of compensation for severe anaemia, and in general this is true. If a patient has recently had a large external haemorrhage or has been seriously injured (see below), it is almost certain that venous pressure will be low and that transfusion will be well tolerated. However, the sort of patient who causes difficulty is one who is having repeated haematemesis. In such patients only careful examination will reveal the state of the circulation. Howarth and Sharpey-Schafer (1947) have stressed the value of using the jugular venous pressure as a clinical

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guide. If jugular venous pressure is raised, transfusion must be given with the greatest caution.

RESTORATION OF BLOOD VOLUME BY TRANSFUSION

Following acute haemorrhage, blood volume can be restored by a transfusion of blood, serum, plasma or albumin; or by infusing a plasma substitute (see below).

The ability of plasma and serum to restore blood volume after haemorrhage has been clearly demonstrated in animals. For example, Magladery *et al.* (1940) bled dogs of 50-60 per cent of their blood volume and found that transfusions of serum or plasma were approximately as effective as blood in restoring the animals.

Plasma and serum increase plasma volume according to their protein content. In calculating the size of the transfusion that a patient needs, the volume of citrate solution mixed with the blood or plasma must be ignored. Thus 540 ml. of citrated blood contain 420 ml. of blood and 120 ml. of citrate-dextrose solution. If a patient is calculated to have lost 2000 ml. of blood, he needs five, not four, bottles of citrated blood to restore his blood volume.

The importance of the protein content was well shown by some experiments of Heyl *et al.* (1943). They bled normal male subjects of an amount equal to 10-20 per cent of the blood volume in a period of 10-15 minutes, and then injected 25 per cent human albumin. They found that plasma volume was increased by an amount much greater than the volume of solution injected. Thus on the average each gram of albumin drew into the circulation 17.4 ml. of fluid in an hour. Mild degrees of dehydration did not prevent fluid from being drawn into the blood stream.

Minimum acceptable haemoglobin level. In acute experiments in dogs Case *et al.* (1955) found that when the packed cell volume was lowered from 49 to 32 there was no depression of ventricular function but that when the packed cell volume was lowered to between 24 and 31 there was a definite depression, probably because at this level coronary vasodilatation was already maximal. Clinical impressions suggest that in injured patients the haemoglobin should not be allowed to fall below 9 g./100 ml. (Grant and Reeve, 1951). Evidently this is a minimum. The ideal should be to replace approximately as much whole blood as the patient has lost.

A further reason for using blood rather than plasma in patients who have been injured is that the patient is less likely to develop a severe degree of anaemia subsequently.

Defective gas-transport function of stored red cells. The oxygen dissociation curve of red cells stored in citrate is 'shifted to the left', suggesting that after transfusion the red cells will, at least temporarily, be incapable of releasing as large a volume of oxygen to the tissues as normal red cells. In blood stored in ACD the changes are maximal after 1 week's storage. *In vivo* the changes are largely reversed by the end of 24 hours after transfusion (Valtis and Kennedy, 1954).

Valtis and Kennedy (1954) pointed out that under some circumstances the transfusion of stored blood might actually make a patient worse for a few hours. The transfused blood, by expanding the blood volume, would lessen the oxygen-carrying power of unit volume of the patient's own blood and the transfused red cells would give up oxygen much less readily than normal cells. Thus the net effect might be that after transfusion the patient's blood would release less oxygen to the tissues than previously. This state of affairs would last for only a few hours but it does seem possible that the immediate effects of the transfusion of stored blood are very little different from those of the transfusion of plasma. This is not an argument for using plasma in the treatment of acute haemorrhage, for within a few hours the patient transfused with blood will be much better off.

Initial Assessment of Severity

Occasionally the extent of blood loss is obvious, as in a patient who has sustained an injury involving a large blood vessel and arrives with blood-soaked dressings. More often, in injured subjects, the extent of the oligaemia must be deduced from the severity of the injuries or the state of the patient's circulation. Some methods for estimating the degree of oligaemia according to the extent of the injuries have already been described, and it will suffice to repeat here that in a patient with severe injuries it should be assumed that, however well the patient may appear, immediate transfusion is needed.

In assessing the state of the circulation, no single clinical sign can be relied on in assessing the extent of oligaemia (Fisher, 1958), but measurement of the systolic blood pressure is perhaps the most useful guide; if it is below 70 mm. Hg and there are signs of marked circulatory failure, such as severe pallor, a very rapid heart rate, great restlessness, etc., immediate transfusion is essential if life is to be saved.

If the degree of hypotension is less severe (systolic pressure 80-100 mm. Hg) and the injuries do not seem grave and the patient has a slow pulse he may be placed in the head-down position, i.e. with the foot of the bed

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raised 6 inches on blocks. If within 10-15 minutes the systolic blood pressure has not risen to above 100 mm. Hg, transfusion should be started.

The Rate of Blood Transfusion

In treating oligaemia of any kind, prompt, rapid transfusion is essential. For example, in dealing with a severe case of burns, if plasma is not immediately available, it is much better to start a transfusion of blood than to wait for plasma to arrive. Similarly, though blood is best for treating a case of severe post-partum haemorrhage, if the blood pressure is low plasma should be given immediately whilst waiting for the blood to be matched.

Again, though the giving of ice-cold blood may cause local discomfort and venespasm, these are trifling considerations in dealing with a severe case of oligaemic shock. The blood should be given straight out of the refrigerator if necessary.

As a rule the first 500 ml. should be given in 5 to 10 minutes; the second 500 ml. should be given equally rapidly in most cases. These rates of transfusion cannot be attained by simply allowing the blood to run in under gravity through the standard giving needle lying in a peripheral vein. Ways of increasing the rate of flow are discussed later in this chapter.

Intra-arterial transfusion. Some surgeons have formed the impression that in subjects exsanguinated to the point of death a transfusion given intra-arterially may save life when the intravenous route would be unavailing. It has been suggested that the advantage may lie in the fact that the coronary circulation is filled directly when blood is introduced into the arterial side of the circulation.

The intra-arterial route was compared with the intravenous route by Case *et al.* (1953). In dogs who were bled to hypotensive levels the coronary flow and arterial pressure responded as rapidly and to the same extent whether the blood was given intravenously or intra-arterially. It was emphasized that in previous work there had been a tendency to give blood more rapidly by the intra-arterial route and that this, in some cases, accounted for the apparent superiority of the intra-arterial route. The authors pointed out that in fact it must be easier to give blood faster by the intravenous route because of the lower pressure in the venous system. They concluded that the only theoretical advantage of intra-arterial infusion was when, due to cardiac arrest, or almost completely ineffective contraction, the blood could not be passed from the venous side to the arterial side. They considered that this possible advantage would be offset by the extra delay in setting up the intra-arterial infusion and the risk of gangrene to the extremities.

How Long to Continue Transfusion

A rise in systolic pressure to 100 mm. Hg is not an indication for stopping the transfusion. A relatively small transfusion given to an injured person may produce considerable clinical improvement without fully restoring blood volume. Evans *et al.* (1944) made serial estimations of plasma volume in injured subjects with signs of peripheral failure. They noted that after a small transfusion, say 500 ml. of blood, blood pressure might rise and, superficially, the patient appear much improved. Nevertheless, blood volume remained low for as long as 4 days. During the intervening period the patient might easily pass into shock again. They estimated that their subjects should have received 1000 to 1500 ml. of blood initially. Grant and Reeve (1951) suggested that the patient's blood volume should be raised to at least 80 per cent of normal before operation was begun. Thus, transfusion should be continued not only till the systolic pressure rises to above 100 mm. Hg, but also until there are signs of the relief of the peripheral circulatory failure; that is to say, until the pulse becomes fuller and the extremities warmer. The warmth of the patient's nose is the most reliable guide to skin blood flow. Unless the blood volume has been raised to well above the critical level, transfusion should continue during operation.

Failure to Respond to Transfusion

If an injured person whose blood pressure is low fails to respond to transfusion, it is usually for one of three reasons. Firstly, the patient may still be losing blood, so that transfusion is not succeeding in restoring blood volume to an adequate level. Secondly, there may be infection (especially gas gangrene). Thirdly, transfusion may have been begun too late and the patient may be in the condition which has sometimes been described as 'irreversible shock'. If the patient does not respond after a reasonable amount of blood or plasma has been transfused, operation should not be further delayed, since it may help to deal with the first and second causes (Grant and Reeve, 1951).

Overloading of the Circulation

Although the usual tendency probably is to underestimate blood loss and although there is evidence that it is difficult to overload the circulation of a patient who has lost blood through injury (see Grant and Reeve, 1951),

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cases have been described in which overloading, characterized by engorgment of the neck veins and moist sounds on auscultation, has been produced in patients following too large or too rapid transfusion (Downs, 1958). It is relatively easy to overload the circulation of patients whose hypotension is due to infection.

Transfusion in Burns

As a general rule, children with burns of 10 per cent or more of their body area should receive intravenous therapy; in adults burns of less than 15 per cent of the surface area can safely be treated by oral therapy alone (Bull, 1954).

In calculating the area of the burn, the following figures for the relative areas of the different parts of the body are helpful (Berkow, 1931):

Head	6 per cent	6
Trunk and neck (back and front together)	38 per cent	38
Upper limb (hand alone, 2.5 per cent)	9 per cent × 2	18
Lower limb: thigh alone, 9.5 per cent leg alone, 6.5 per cent foot alone, 3.0 per cent	19 per cent × 2	38
		100

The figures are slightly different in young children, in whom the surface area of the head is relatively larger and that of the lower limbs relatively smaller. For example, in an infant aged 3 years the surface area of the head is about 15 per cent of the total surface area.

According to Bull (1954) in adults for every 10 per cent of the body surface burnt about 1 litre of plasma will have to be transfused. Of this amount, half should be given in the first 8 hours or so after burning, and the whole within 24 hours.

Estimation of the venous haematocrit is only an approximate guide to the reduction in plasma volume. The average normal venous haematocrit is given by Wintrobe (1946, p. 73) as 42 per cent for females, 47 per cent for males and 35–37 per cent for infants and children between 3 months and 10 years. However, when one considers that the normal haematocrit values of males are scattered over the range 40–54 per cent, one can appreciate the limitations of any formula for plasma dosage based on normal values.

In any case if the venous haematocrit is determined soon after burning the value may only be slightly raised. This must on no account be taken as a reason for cautious transfusion if a large area of the body surface has been burnt.

A further point mentioned by Bull (1954) is that due to the destruction of red cells in patients with severe burns the reduction in blood volume is greater than is indicated by the rise in haematocrit.

Although the venous haematocrit is only a rough guide to the severity of the plasma loss it is a very useful guide to serial changes in plasma volume; Bull (1954) suggests hourly determinations as a guide to the rate of transfusion.

As soon as the venous haematocrit has been reduced to, say, 45 per cent, there is much to be said for giving a transfusion of blood as well as plasma to patients with burns involving more than 20 per cent of the body surface. As mentioned above, not only is there an immediate loss of red cells, due to direct damage by heating, but there is a further loss in the next 48 hours which is only partly accounted for by bleeding into the area surrounding the burn. Topley and Jackson (1957) carried out a trial of early versus late transfusion in patients with full-skin-thickness burns involving 10–40 per cent of the body surface. 'Late' transfusion (after anaemia had developed) gave as good results as 'early' transfusion (given immediately after admission). Of course, transfusion here refers only to the giving of whole blood; all patients received sufficient plasma to restore blood volume.

Technique of Rapid Transfusion

The bore of the transfusion needle. The bore of the needle is a very important factor in limiting the rate of flow. Discussion of results is complicated by the fact that American gauge numbers are approximately one less than their British equivalents. Another important point is that gauge numbers are linked to external diameter and that for a given external diameter the internal diameter of American needles is distinctly greater than that of British needles. Some data are given in Table VI.

There is agreement that for rapid transfusions, the internal diameter of the needle should be not less than about 1.1 mm. For example, Walter *et al.* (1955) found that with an American gauge 21 needle (internal diameter 0.51 mm.), even with a head of pressure of 40 inches, the rate of flow of ACD blood was only 7 ml. per minute. With an American gauge 17 needle (internal diameter 1.51 mm.), the flow was 56 ml. a minute.

Chaplin and Chang (1955) measured the time taken for 100 ml. of citrated blood (haemoglobin 12.6 g./100 ml.) under a head of pressure of 32 in. (130 cm.) to flow through needles of various bore sizes. They found

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that with a needle of internal diameter 1.37 mm. the time was 1.2 minutes; with an internal diameter of 0.84 mm., the time was 3.6 minutes; with an internal diameter of 0.69 mm., the time was 7.6 minutes; with an internal diameter of 0.51 mm., the time was 16.0 minutes; and with an internal diameter of 0.33 mm., the time was 70.0 minutes. Times were approximately 10 per cent longer when blood with a haemoglobin concentration of 14.3 g./100 ml. was used.

TABLE VI

GAUGE NUMBERS OF BRITISH AND AMERICAN NEEDLES TOGETHER WITH INTERNAL AND EXTERNAL DIAMETERS

Needle gauge		*Diameter (mm.)*		
		Internal		*External*
British ('S.W.G.')	*American*	*British*	*American*	
26	25	0.24	—	0.47
25	24	0.28	—	0.51
24	—	0.30	—	0.55
—	23	—	0.33	0.58
23	—	0.35	—	0.61
—	22	—	0.41	0 63
22	21	0.40	0.51	0.71
21	20	0.52	0.59	0 81
20	19	0.55	0.69	0.91
19	18	0.66	0.84	1.05
—	17	—	1.07	1.17
18	—	0.75	—	1.22
—	16	—	1.19	1.30
17*	—	1.05	—	1.42
—	15	—	1.37	1.47
16	14	1.20	—	1.63
15†	13	1.34	—	1.83
13‡	11	1.70	—	2.34

* also known as 15/10
† also known as 19/10
‡ also known as 24/10

Methods of Applying Positive Pressure

In patients with peripheral circulatory failure due to oligaemia, the veins may be constricted, so that the rate of flow of the transfusion may be too slow even when the bottle of blood is suspended as far above the level of the bed as the transfusion set will permit; see, for example, Humble and Belyavin (1944). The transfusion of blood or serum may itself induce venous spasm because plasma (or serum) may contain vasoconstrictor substances (see Chapter XV). The fact that transfusion of serum might induce vasoconstriction was mentioned by Pugsley and Farquharson (1944) who recommended that the infused fluid should be warmed and a large vein selected.

Venous spasm is less of an obstacle in arm veins for the muscular wall of the vein is thinner in arm veins than in leg veins. Since it is also far easier to introduce a needle into the arm veins than into the leg veins without cutting the skin, the arms are in general to be preferred. Local warming of the arm, with hot water bottles, helps to relieve venous spasm.

When the need for rapid transfusion is great, positive pressure will have to be applied. The best method is to use some form of rotary pump which compresses the tubing of the transfusion set and drives the blood onwards. One of the earliest pumps of this kind was the Jouvelet pump (see for example Melrose and Shackman, 1951). Rotary pumps designed for rubber tubing are not as a rule suitable for plastic tubing, for which special rotary pumps have been devised (obtainable from Messrs. Capon Heaton, Birmingham).

If no rotary pump is available, and the need for rapid transfusion is great, air can be pumped into the transfusion bottle. The method has been used very widely in the past, and is safe enough in very experienced hands. Unfortunately, if it is allowed to become a standard method in a hospital it will inevitably be used by less experienced people, and there is then the risk of massive air embolism. If the transfusion is left unattended, even for a minute or two, and the bottle empties during that period, enough air may enter the patient's veins to produce rapid death.

Transfusion in Relation to Platelet-Deficiency

There is strong evidence that viable platelets rather than platelet material must be transfused to obtain an haemostatic effect. In dogs rendered thrombocytopenic by irradiation, the lymph becomes rich in red cells which have leaked out of small blood vessels; in these circumstances the transfusion to the dog of platelets from fresh blood has an immediate haemostatic effect whereas the transfusion of lyophilized platelets, resuspended in saline, is completely ineffective (Woods, *et al.* 1953; Jackson *et al.*, 1959).

As described in Chapter I, blood stored even for 24 hours at 4° C. contains relatively few viable platelets. Despite this, there are reports suggesting that transfusions of platelets derived from blood stored for up to 48 hours control bleeding (e.g. Tobin and Friedman, 1960). Blood stored for more than 2 days is relatively ineffective. Thus in a trial in

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patients with acute leukaemia associated with severe thrombocytopenia and bleeding, the effect of fresh blood and blood stored for 2 to 9 days was compared in the same cases. Precautions were taken to prevent either the patient or the doctor from knowing whether the blood was fresh or stored. In five out of six patients in whom transfusion produced either a diminution or cessation of bleeding, fresh blood had been used.

In patients with idiopathic thrombocytopenic purpura, transfused platelets are rapidly destroyed (Sprague *et al.*, 1952). In one patient mentioned by Krevans (1961), following the transfusion of 270 ml. of platelet-rich plasma with a count of almost 8 mill/c.mm., almost all the platelets were removed within 24 hours.

A haemorrhagic state following massive transfusion. Petechiae and spontaneous haemorrhage into the gut or urinary tract may be observed in patients who have been given very large transfusions. Krevans and Jackson (1955) observed haemorrhagic phenomena in eleven out of fourteen adults transfused with more than 5 litres of blood within 48 hours, but in none of thirteen adults transfused with less than 5 litres. In the patients transfused with more than 5 litres the platelets usually fell to less than 50,000 per c.mm. In another series severe bleeding was observed in thirteen adults who received 5 litres or more of stored blood to replace blood lost at operation (Stefanini *et al.*, 1954). The authors concluded that the excessive bleeding was due mainly to platelet deficiency, stored blood being a poor source of viable platelets.

Later, Jackson *et al.* (1956) reported that all of twenty-one patients who received more than 7 litres of stored blood developed abnormal bleeding and that platelet counts remained low for 3-5 days after transfusion. It was clearly shown that the main factor was the use of stored blood containing non-viable platelets; exchange transfusion of dogs with fresh blood did not produce thrombocytopenia.

Generalized haemorrhage has been produced experimentally in dogs by giving very large tranfusions of fresh blood but no evidence was obtained of any loss of red cells from the circulation until the volume of blood transfused reached 150-200 ml./kg., equivalent to a transfusion of the order of 10 litres to a human adult with an initially normal blood volume (Huggins *et al.*, 1956).

Plasma Substitutes

The good effect of transfusion in haemorrhage is due mainly to the sustained increase in blood volume which it produces. Bayliss (1919) showed that a fluid which contained colloids having an osmotic pressure similar to that of plasma proteins could be used as a substitute for blood.

Many substances have been tried as plasma substitutes but not one of these has entirely fulfilled the theoretical requirements. For example, gum arabic, introduced by Bayliss (1919), later fell into disrepute because it was found to be stored in the tissues. The main positive quality required of a plasma substitute is that of not leaving the circulation rapidly after infusion. The main negative qualities required are lack of toxicity and failure to induce sensitization; it is also desirable that the substance should not be stored in the body for long periods.

Dextran

Dextran has many of the properties of the ideal blood substitute. Although it is stored in the tissues for many weeks after infusion, but the quantities remaining are very small (Bull, Ricketts *et al.*, 1949). Evidence has been produced that dextran is metabolized in the body (Gray, 1953). No harmful effects were demonstrated during the first clinical trials (Grönwall and Ingelman, 1944; Bohmansson *et al.*, 1946), although subsequent work has called attention to the occurrence of occasional untoward reactions (see below).

Dextran molecules are long chains of glucose units. The average molecular size can be reduced by partial hydrolysis and it is possible to prepare dextran solutions with a defined range of molecular size.

Dextran which is prepared in Britain has an average molecular weight of 150,000-175,000.

The figure of approximately 150,000 is a weight average (M_W); in materials like Dextran preparations in which there are molecules of widely varying sizes, it makes a good deal of difference whether the average molecular weight is based on proportion by weight of each molecular 'species' or the proportion by number of each 'species'. Thus, for example, the number average molecular weight (M_N) of British clinical Dextran is approximately 90,000. For a further explanation of this point, see Squire *et al.* (1955), p. 16.

Material prepared according to the British specification contains a proportion of molecules of a molecular weight of less than about 70,000 which are rapidly excreted in the urine. According to the specification not more than 25 per cent of the injected Dextran should be excreted in this way. The remaining material is lost only slowly from the circulation, about one-third of the Dextran in the plasma at any given time being removed during the subsequent 24 hours. After dextran infusions the plasma protein concentration is temporarily depressed and the sum

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(plasma protein concentration+plasma dextran concentration) tends to remain constant (Bull, Ricketts *et al.*, 1949).

The presence of dextran in the plasma causes rouleaux formation and this may interfere with the interpretation of compatibility tests (see Chapter XII). In the author's laboratory samples of serum from eight patients who had received one to three bottles of (British) Dextran were tested; five samples, all with dextran concentrations above 1.3 g./100 ml., caused strong rouleaux formation. Before beginning an infusion of dextran into a patient, a sample of serum should be obtained and kept, in case blood has to be transfused later on.

Dr A. L. Stalker (personal communication) has observed severe intravascular 'sludging' in rabbits following the intravenous injection of clinical Dextran prepared to the British specification (average molecular weight (M_w) approximately 150,000). Large rouleaux were demonstrated in circulating blood and there was extensive trapping of red cells in small vessels. In sacrificed animals focal necrotic lesions were found in the liver and myocardium. These changes were not observed in animals transfused with clinical Dextran with a molecular weight of the order of 75,000. (These observations were made with the ear-chamber technique.)

Retention of Dextran in the Circulation

Dextran produced in the U.S.A. has a lower molecular weight than the British material (approximate M_w, 75,000; M_n, 50,000). Accordingly less is retained in the circulation and more is excreted in the urine. Evidently this material produces a less sustained effect on plasma volume; it is not known whether the absence of material of relatively high molecular weight makes it in practice a safer product. In patients who have received 1500 ml. of American Dextran no significant cross-matching difficulties have been encountered (Dr H. Chaplin, Jr., personal communication).

It is evident that conclusions reached about the effects of one type of dextran may not apply to another. For example, Armstrong *et al.* (1954) gave infusions of two types of dextran — 'Intradex' and 'Macrodex' — to subjects bled of 240–500 ml. of blood. Twenty-four hours later they found that the subjects who had received 'Intradex' had retained 71 per cent of the dextran whereas those who had received 'Macrodex' had retained about 42 per cent ('Intradex' is a British product, 'Macrodex' a Swedish product, probably resembling the type of dextran at present manufactured in the U.S.A.).

Armstrong *et al.* also compared the effects of dextran and saline respectively on the restoration of blood volume after haemorrhage. In subjects bled of about 400 ml. who received no treatment, the haematocrit fell only

slowly during the next 24 hours, indicating a slow restoration of plasma volume. Even at 48 hours plasma volume was not fully restored. When 500 ml. of saline was infused immediately after venesection, the results were very much the same. However when 500–1000 ml. of dextran ('Macrodex' or 'Intradex') were infused, there was an immediate fall in the haematocrit and there were insignificant changes during the following 48 hours, suggesting that plasma volume had been restored.

Køster *et al.* (1957) gave infusions of 'Macrodex' (said to have a mean molecular weight of 91,000 — presumably a weight average) to normal subjects, and estimated plasma volume with ^{131}I-labelled albumin. 500 ml. of a 6 per cent solution were infused in 1 hour; after a further hour plasma volume was still increased by about 560 ml., and after 4 hours by 450 ml. When 500 ml. of a 10 per cent solution of dextran was infused, plasma volume 1 hour later was increased by 870 ml., and 4 hours later by 690 ml. Køster *et al.* calculated that 1 g. of dextran retained 18 ml. of fluid in the circulation.

These observations are in substantial agreement with those of Wilkinson (1956), who infused 1 litre of 6 per cent dextran ('Dextraven') into each of six subjects in 25–63 minutes and produced an immediate increase in plasma volume of about 800 ml., accompanied, incidentally, by transient vaso-motor disturbances.

Dextran appears to be a satisfactory substitute for plasma even when it is used in relatively large amounts in the treatment of burns. For example, Bull and Jackson (1955) observed that in patients with burns who were treated with dextran rather than plasma, the results were as good as in patients treated with plasma alone, provided that the total amount of dextran given to any one patient was limited to an amount equal to the patient's own plasma volume.

Despite this experience, it must be remembered that the use of dextran alone to replace large volumes of lost plasma involves failure to replace various plasma constituents such as coagulation factors and, until more experience has been gained, it seems best, under ordinary circumstances, to limit the amount of dextran given to any one patient to 1 litre.

Allergic reactions following dextran infusions. When dextran is administered for therapeutic purposes, it is very unusual to encounter reactions of an allergic type but such reactions have been seen quite frequently in volunteers receiving dextran for experimental purposes. For example, Heistö and Lund (1953) gave dextran from eight different batches to a total of twenty-nine healthy volunteers. 1000 ml. were administered in

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1 hour and fifteen of the subjects developed reactions. In most cases the reaction took the form of flushing of the face and neck coming on between a few minutes and 1 hour after starting the infusion. Headache, malaise, a rise in temperature and vomiting were frequent. Reactions were much commoner with dextrans produced with a particular strain of bacteria. It is thought that, in manufacture, this particular strain has been avoided in all countries since the beginning of 1954.

Although reactions of an allergic type seem to be less common with the type of clinical dextran which is now being used, it must be mentioned that Kabat and Berg (1953) have shown that many types of clinical dextran are capable of acting as antigens in man and that small quantities of anti-dextran may be found in the serum of individuals who have not previously received dextran infusions.

Kabat *et al.* (1957) found a significant correlation between initial skin sensitivity to certain native dextrans and the incidence of systemic allergic reactions, and also between the occurrence of precipitating antibodies in the serum and the incidence of reactions.

Abnormal bleeding following dextran infusions. An apparently unrelated untoward effect following large infusions of dextran is the occurrence of bleeding. In tests on eleven healthy volunteers the least amount of dextran that produced a prolongation of bleeding time was 1500 ml. given in a single infusion. In many subjects prolongation of bleeding time developed only after larger quantities had been given: for example, in one subject only after 6500 ml. had been given over a period of 5 days. The increase in bleeding time was no longer present 24 hours after the infusions (Carbone *et al.*, 1954). In a further study it was shown that the prolonged bleeding time was not due solely to dilution, since when an equivalent degree of haemodilution was produced by infusions of intravenous albumin, prolonged bleeding did not develop (Adelson *et al.*, 1955).

In an extensive study Langdell *et al.* (1958) compared the effect on bleeding time of several preparations of dextran. Subjects received an infusion of 1000 ml. at the rate of 20 ml. a minute, and the bleeding time was estimated at intervals during the following 9 hours. When dextran with a relatively low molecular weight (50,000-75,000) was used, bleeding time was prolonged (more than 10 minutes) in 30-50 per cent of subjects. When dextran with a molecular weight of approximately 180,000 was used, bleeding time was prolonged in 50-70 per cent of subjects, and when dextran with a molecular weight of approximately 260,000 was infused, bleeding time was prolonged in eight out of eight subjects. In a control

series of subjects receiving 5 per cent albumin, bleeding time was prolonged in only 2.5 per cent of cases. There was apparently no correlation between the increase in blood volume and the prolongation of bleeding time, and the maximum incidence of a prolonged bleeding time was 3-9 hours after the infusion. Nevertheless, there may, of course, have been a relationship between the dextran concentration in the blood and the prolongation of bleeding time, since there is much better retention of dextran when a high molecular weight preparation is used.

As a result of their investigations the authors concluded that the prolonged bleeding was due to interference with platelet activity.

Out of seventy-seven subjects in the series with a prolonged bleeding time, only two developed spontaneous bleeding or bruising, and this evidence agrees substantially with the rather infrequent occurrence of haemorrhagic phenomena in patients receiving large infusions of dextran.

McKenzie and Langlands (1956) observed massive haematuria and bleeding from the gums in a patient with nephrotic nephritis treated with infusions of 10 per cent, salt-free, dextran. The haemorrhagic signs developed after the patient had received a total of 270 g. of dextran (162 g. in the previous 48 hours). The signs subsided over the course of 2 weeks.

Polyvinylpyrrolidone (PVP) has been extensively used as a plasma substitute on the Continent of Europe, but in Britain and the U.S.A. there has been some hesitation in using it, mainly because it is not metabolized in the body. As usually prepared, the product is not so well retained in the circulation as the type of dextran at present available in Britain. For example, Armstrong *et al.* (1954) compared the retention of PVP and 'Intradex' in the circulation at 24 hours; that of PVP was approximately 48 per cent and that of 'Intradex' approximately 71 per cent.

Gelatin has been shown to be quite well retained in the circulation (Ravdin, 1952) and has the attractions of being non-toxic and of being completely metabolized in the body. However, gelatin preparations of a molecular size large enough to be well retained in the circulation do not remain fluid at low temperatures, and this has been generally considered a serious disadvantage in a preparation which is essentially needed for emergency conditions.

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CHAPTER FOUR

TRANSFUSION AND ANAEMIA

As a general rule, transfusion should be used as a method of treating anaemia only when the anaemia cannot be cured by the administration of iron, vitamin B_{12} or other haematinics, or if an immediate increase in red cell volume is judged to be essential. (The separate question of the treatment of acute haemorrhage has been dealt with in the previous chapter.) It must never be forgotten that transfusion carries risks which are large compared with those of conservative treatment.

Anaemias which cannot be cured by the administration of haematinics are those due predominantly to failure of red cell production, such as aplastic anaemia, and those due predominantly to red cell destruction, for example, paroxysmal nocturnal haemoglobinuria.

Examples of circumstances in which immediate transfusion may be required, even though the anaemia is not due to one of the above causes, are as follows: anaemia produced by haemorrhage which is likely to recur; for example, that associated with repeated haematemeses; severe anaemia in a patient who has to undergo an emergency operation, or in a pregnant women near term, or in a patient who develops a severe infection, such as pneumonia.

Anaemias Due Predominantly to Under-Production of Red Cells

Examples of this type of anaemia are: aplastic anaemia, various types of leukaemia, and anaemias associated with chronic nephritis, hepatic cirrhosis and severe chronic infection.

In an anaemia of this kind, provided that there is no active red cell destruction, transfusion produces a prolonged increase in packed cell volume. Thus, Fig. 20 (lower curve) shows the changes in PCV which followed the transfusion of 700 ml. of packed red cells to a patient with chronic nephritis. This patient was in a stationary phase, and it was shown that transfused red cells survived normally in his circulation. The fact that the PCV remained above the pretransfusion level for 3 months after transfusion suggests that red cell production was not depressed by the transfusion.

Haemolytic Anaemias

Two main types of haemolytic anaemia may be recognized: the first is due to the production of 'faulty' red cells, incapable of a normal survival,

for example, hereditary spherocytosis; in patients with a haemolytic anaemia of this kind transfused red cells usually survive normally and transfusion therefore produces a relatively sustained increase in red cell volume.

The second type of haemolytic anaemia is due to a destructive process which brings about the removal of red cells from the blood stream in a random fashion. Normal red cells are destroyed about as rapidly as the patient's own red cells except in rare cases in which destruction is due to an auto-antibody with blood-group specificity (see Chapter VI).

In anaemias in which transfused red cells are rapidly destroyed, transfusions produce only the most transient benefit, and should be avoided as far as possible. Apart from their poor effect, transfusions may stimulate the formation of iso-antibodies and make the future selection of compatible donors more difficult. Haemolytic anaemias associated with auto-antibodies of the 'warm' type almost always respond to steroids, and transfusion should be reserved for emergency.

In *chronic leukaemia* with splenomegaly there are often signs of a mild haemolytic process. Berlin (1951) showed that the survival of transfused red cells was diminished and that, on the whole, the larger the spleen, the shorter was the survival. Hagen and Watson (1950) found that the transfusion requirements of patients with chronic leukaemia might be considerably reduced after splenectomy, and this measure appears to be worth while when it becomes difficult to maintain a satisfactory haemoglobin concentration by transfusion. 'Surface counting' after the injection of ^{51}Cr-labelled red cells, may help to decide whether splenectomy is worthwhile (see Chapter VI).

In *acute leukaemia* remission sometimes follows blood transfusion. Bessis (1948), in reviewing the literature, noted that there was a correlation between the total amount of blood transfused and the duration of the remission. He concluded that there was an anti-leukaemic substance in normal blood and that replacement transfusion, using large amounts of blood, might produce better results than simple transfusions with relatively small amounts. In thirty-eight cases treated by replacement transfusion he observed clinical remissions in thirty cases and of these, six had 'complete marrow remission' (Bessis, 1949).

The technique was to 'heparinize' the patient, by injecting a dose of 2 mg./kg. (10 mg. = 1000 international units), and to withdraw the blood through a polythene catheter passed into an arm vein. By passing the catheter up the vein for a sufficient distance the use of a pressure cuff round the arm was avoided. The blood was withdrawn either by taking it into evacuated bottles, or by the use of a special

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pump. After withdrawal of about 500 ml. of blood, an equivalent quantity of fresh blood was transfused into the opposite arm. The intermittent withdrawal and transfusion was continued until some 15 litres had been replaced in a period of 2-4 hours.

The original method of Bessis involved using the blood of twenty to thirty donors and since there are more convenient and more certain ways of inducing remissions in leukaemia it has not been widely used.

In a later study Wetherley-Mein and Cottom (1956) compared the effects of fresh and stored blood in leukaemia and found that a prompt fall in total white count was much commoner after transfusions of fresh blood.

When transfusions are needed in leukaemia, it is often because the patient has a relatively low platelet count and is bleeding. As mentioned in Chapters I and III, under these conditions fresh blood is indicated because it is greatly superior to stored blood as a source of viable platelets.

Anaemia Produced by a Form of Haemorrhage Likely to Recur

Patients who have developed severe anaemia as a result of recurrent haemorrhage have to be transfused, unless it is reasonably certain that there will be no further haemorrhage. It is most undesirable to allow such patients to become severely anaemic and for this reason patients who have recently had haematemeses and whose haemoglobin is as low as 7-8 g./100 ml. (45-55 per cent on the Haldane scale) should be transfused. If the haemoglobin falls to 4.5 g./100 ml. (30 per cent) or thereabouts, the patient may pass into the 'hyperkinetic' phase described by Howarth and Sharpey-Schafer (1947), and it may then be extremely hazardous to give a transfusion. The method of transfusing such patients is described below.

Pre-Operative Transfusion

As mentioned in the previous chapter, there is evidence that when the PCV falls below about 30 per cent, corresponding to a haemoglobin concentration of about 10 g./100 ml., there is some interference with cardiac function. Therefore, before surgery is undertaken the haemoglobin should be raised above this level, even if only trivial haemorrhage is expected. Before major surgery it seems desirable to raise the haemoglobin level to within the normal range; that is to say, above 12.5 g./100 ml. in women, or 13.5 g./100 ml. in men.

It should be stressed that pre-operative transfusions can often be avoided by determining the patient's haemoglobin concentration well in

advance of operation, and, if anaemia is found, attempting to treat it by conservative measures.

Anaemia in Pregnancy

As described in Chapter II, plasma volume increases considerably in pregnancy. Until comparatively recently it was believed that red cell volume did not increase proportionately so that some anaemia was inevitable. It is now thought that anaemia can be largely prevented by the administration of an adequate amount of iron to the pregnant woman so that in most cases normal (non-pregnant) haemoglobin values are maintained (Benstead and Theobald, 1952). According to Fisher and Biggs (1955), only a small proportion of pregnant women (10 per cent) remain anaemic despite the administration of iron by mouth.

It has been suggested that if tests are made at about the thirtieth week of pregnancy rather than just before term, even when iron administration has been adequate, haemoglobin values will be found to be slightly below non-pregnant values (Morgan, 1961).

Severe anaemia in pregnancy not due to iron deficiency, is usually due to folic-acid deficiency, and some authors advocate the routine administration of folic acid (5 mg. a day) as well as iron to pregnant women. There are of course some anaemias, for example anaemia associated with pyelitis of pregnancy, which do not respond to any haematinic agents.

In all cases in which the haemoglobin concentration cannot be raised by the administration of haematinic agents the woman must be transfused during the last few weeks of pregnancy to ensure that the haemoglobin level is not below about 10 g./100 ml. at the onset of labour.

Post-partum anaemia. Women who are moderately anaemic at the time of labour and who lose more than the average amount of blood during delivery may be found a few days later to have a haemoglobin concentration as low as 9 g./100 ml. In some hospitals it is customary to treat such patients with a transfusion, often of only a single bottle of blood. This practice is open to considerable criticism. A transfusion of a single bottle of blood raises the haemoglobin concentration by a relatively small amount (approximately 10 per cent in a woman) and carries small but definite risks.

If a patient is judged to require only a single bottle of blood, the chances are that she does not require transfusion at all.

Transfusion for a Degree of Anaemia Endangering Life

In patients with very severe anaemia transfusion may easily overload the circulation and precipitate cardiac failure; therefore, before deciding

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on transfusion, the possibility of treating the patient conservatively should always be carefully considered.

Two complications which may make immediate transfusion necessary are haemorrhage and pneumonia.

Methods of transfusing very anaemic patients (or any patient with incipient cardiac failure) are described later in this chapter.

Effect of Transfusion on the Bone Marrow

Whereas anaemia stimulates erythropoiesis, plethora depresses it. Robertson (1917) gave repeated transfusions to normal rabbits and showed

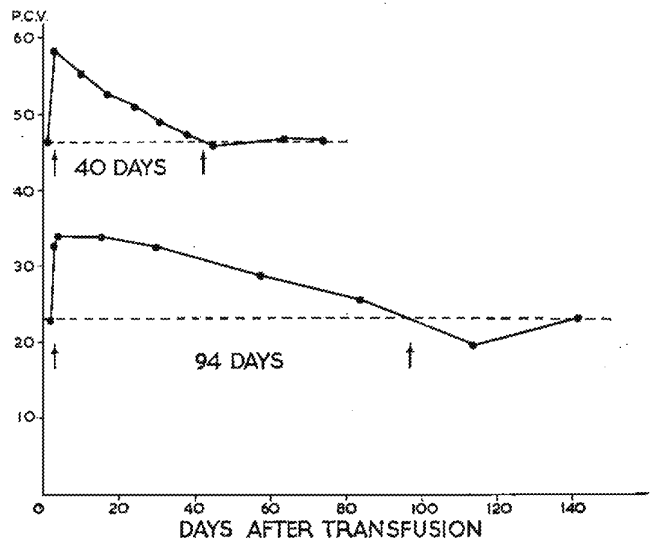

FIGURE 20. Changes in packed cell volume (PCV) following transfusion. Upper curve: a group of normal subjects (data from Pace *et al.*, 1947). Lower curve: a single subject with chronic nephritis. In both curves the arrows mark the departure from, and return to, a base line (from Mollison, 1954).

that the reticulocytes started to diminish as plethora developed: in established plethora, reticulocytes almost disappeared from the blood. Boycott and Oakley (1933) confirmed these observations and showed that the reticulocyte count of animals could also be diminished by placing them in an atmosphere containing 65 per cent oxygen. Similarly, in man, the continuous administration of oxygen reduces the output of the bone marrow (Tinsley *et al.*, 1949). Pace *et al.* (1947) gave transfusions of 1000

ml. of red cells to a series of normal subjects and, on the average, raised the packed cell volume from 46.5 per cent to 58.5 per cent. Forty days later the packed cell volume had returned to the pre-transfusion level (see Fig. 20, upper curve); if red cell production had remained normal the packed cell volume would have remained above the pre-transfusion level for 110–120 days. Further evidence of suppression of red cell production was provided by a fall in the reticulocyte count following transfusion.

In some haemolytic anaemias the suppression of red cell production by a transfusion may be beneficial. Thus Dacie (1948) showed that if a sufficient volume of normal red cells was transfused to a patient with nocturnal haemoglobinuria the number of the patient's own cells in the circulation fell to a low level and haemoglobinuria temporarily ceased.

Deductions about Survival of Transfused Red Cells from Changes in PCV Following Transfusion

It was thought at one time that the survival time of red cells could be deduced by producing a plethora, either by transfusion or by transient exposure to low oxygen tension, and then noting the time taken for the haemoglobin concentration to return to its normal level. It is now realized that this calculation assumes that red cell production does not diminish under these circumstances. If it does diminish, the time for which the haemoglobin concentration remains elevated will be less than the mean life time of the red cells (see upper curve in Fig. 20).

The effect of diminished red cell production after transfusion can be most easily appreciated if the extreme case is considered, in which production ceases altogether. The population of red cells would then diminish in a linear fashion, reaching zero at about 115 days. Reference to the upper curve in Fig. 20 shows that if the initial slope of the curve immediately after transfusion is prolonged to the time axis it does in fact intersect it at about 140 days, suggesting that production is almost arrested by transfusion.

Fig. 21 shows some observations in a patient with aplastic anaemia. The slope of fall of the PCV, if extrapolated, would have reached zero 50 days after transfusion; thus it could have been concluded that the average red cell life span was 50 days or less. Estimates of survival of the patient's own red cells and of transfused red cells showed that the life span was in fact about 50 days.

As a rough guide, then, if the changes in PCV or haemoglobin concentration following transfusion are plotted, and the initial slope, when extrapolated, intersects the time axis at 115 days or more, it does not

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prove that survival is normal; but if the time axis is intersected at some earlier time, such as 50 days, it can safely be deduced that the survival of the transfused red cells is reduced.

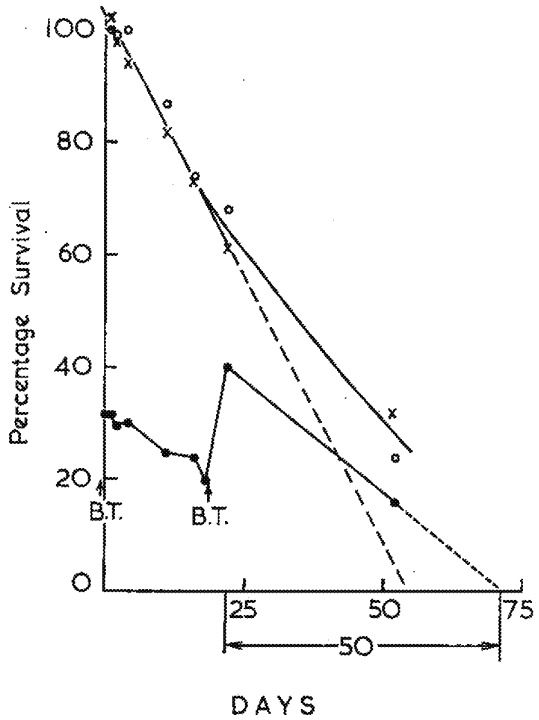

FIGURE 21. Red cell survival and changes in PCV following transfusion in a patient with aplastic anaemia.
×—× Survival of patient's own red cells
○—○ Survival of transfused red cells
The survival results are similar and extrapolation of the initial slope of the curve gives an estimate of mean life-span of approximately 50 days.
●—● PCV.
After each blood transfusion (B.T.) PCV fell steadily; if the slope is extrapolated it cuts the base line after about 50 days.

Transfusion as a Means of Supplying Iron

Transfusions of blood supply the recipient with a certain amount of iron, each 100 ml. of blood containing some 50 mg., almost all of which is present in the form of haemoglobin. When the red cells break down, this iron is retained in the body and can be used by the recipient for the formation of new haemoglobin. In hypochromic anaemia the patient has depleted iron stores and his total deficiency of iron is usually of the order of 2000 mg. Thus to supply sufficient iron by transfusion alone, about 4000 ml. of whole blood would be needed. It is obviously wrong to attempt to supply the iron in this way, since almost all cases can be cured by giving iron by mouth. Even more rapid results can be obtained by giving iron parenterally, though this is seldom necessary.

'Transfusions' by Routes other than the Blood Vessels

Intraperitoneal Transfusions

Hunter (1884) gave large intraperitoneal transfusions of rabbit blood to other rabbits, and showed that the recipient's red cell count was raised for approximately 21 days. Similar observations were made by Sansby (1925), who concluded that the injected red cells reached the circulation and survived for a considerable period.

Hahn *et al.* (1944) gave intraperitoneal injections of red cells tagged with radio-iron, to dogs. They showed that the red cells moved promptly into the circulation via the lymphatics. Nevertheless the uptake was not complete 1 week after the injections. McKee and Stewart (1950) used a similar technique to study the uptake of red cells from the peritoneal cavity of dogs with ascites and found that about 50 per cent entered the circulation in 72 hours.

In man, the uptake of red cells injected into the peritoneal cavity has been demonstrated, either by using differences in MN groups (Clausen, 1938) or by using donors with elliptocytosis (Hedenstedt, 1947). Quantitative observations were made by Pritchard and Weisman (1957), using ^{51}Cr-labelled red cells. In subjects who received an intraperitoneal injection of their own labelled intact red cells, either obtained from a vein or, in two cases, from the peritoneal cavity (following a ruptured ectopic gestation), labelled cells appeared in the circulation within 24 hours and reached a maximum at 3–11 days after injection. At the peak, the amount in the blood reached 65 per cent of the maximum expected if all the red cells had been taken up. This figure agrees quite well with that of 60 per cent found by Stohlman and Schneiderman (1956) in dogs, but is lower than that found by the present author in two (human) cases. The first recipient was a newborn infant with severe spina-bifida, from which it died at the age of 3 months. Two injections of ^{51}Cr-labelled red cells were given from compatible donors. The first injection was of 2 ml. and the second of approximately 20 ml. Following the smaller injection there was

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a delay of about 24 hours before any labelled cells appeared in the blood stream and even after 48 hours only 5 per cent had been taken up; during the following week about 50 per cent of the cells appeared in the blood stream. Following the larger injection, uptake began earlier and was subsequently more rapid and more complete, 86 per cent of the red cells reaching the blood stream within 6 days; see Fig. 22.

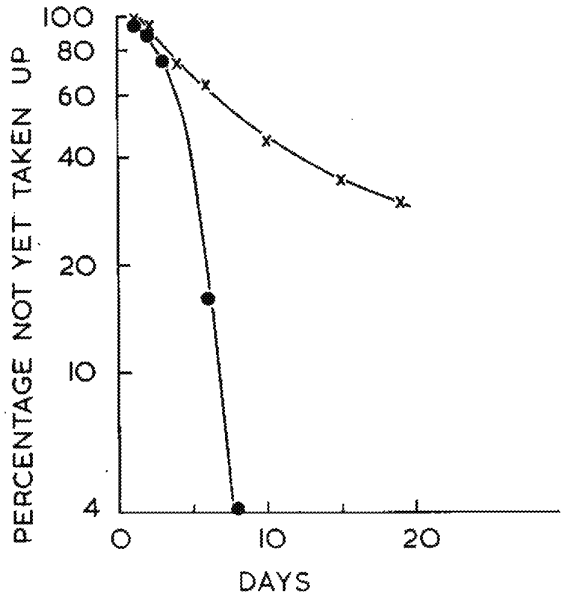

FIGURE 22. Rate of appearance of ^{51}Cr-labelled red cells in the blood stream after injecting the cells intra-peritoneally.

For convenience, the 'percentage not yet taken up' has been plotted. For example, when 20 per cent of the injected red cells have reached the blood stream the point is plotted as 80 per cent 'not yet taken up'.

×—× uptake after injecting 1 ml. of labelled red cells

●—● uptake after injecting 20 ml. of labelled red cells

In a second case, a child (wt. 18 kg.) with thalassaemia major was given an intraperitoneal transfusion of 350 ml. of citrated blood. There was a 'lag' of about 24 hours before any labelled red cells appeared in the circulation, but by the fourth day after transfusion, more than 50 per cent had been 'taken up'. The rate of increase in haemoglobin concentration agreed well with the rate of increase of labelled cells in the circulation (see Fig. 23).

In investigations of this kind allowance has to be made for the fact that chromium is eluting from the red cells. Thus, for example, if 100 per cent of the red cells were taken up from the peritoneal cavity the amount of ^{51}Cr in the circulation at 48 hours would only be 90 per cent of the theoretical 100 per cent value because the normal ^{51}Cr survival at 48 hours is

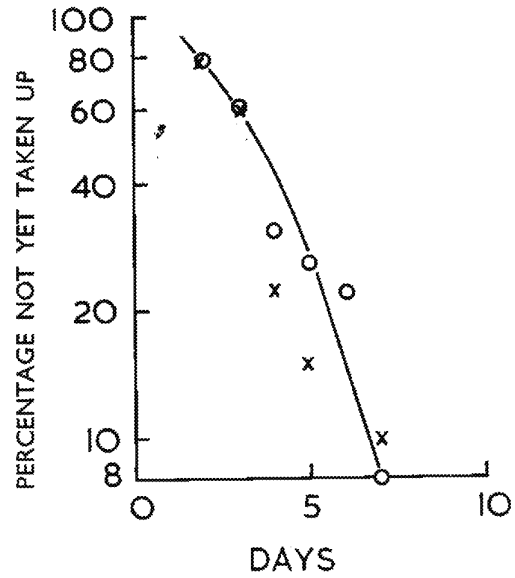

FIGURE 23. Rate of appearance of ^{51}Cr-labelled red cells in the blood stream after an intra-peritoneal transfusion of approximately 370 ml. of citrated blood (containing labelled red cells) given to a child weighing approximately 18 kg., suffering from thalassaemia major.

○—○ uptake of red cells shown as percentage not yet taken up from the intra-peritoneal cavity

×—× haemoglobin changes shown as percentage of 'Haemoglobin rise not yet achieved'; i.e. immediately after injection, 100 per cent and when haemoglobin rise maximal, 0 per cent.

about 90 per cent. In calculating the values shown in Figs. 22 and 23 this was taken into account.

Perhaps a warning should be inserted here about injecting ^{51}Cr-labelled red cells intraperitoneally into children. In girls the dose of radiation given to the gonads must be borne in mind, and as a general rule the injection of radioisotopes into the peritoneal cavity of female children is contra-indicated in any case in which the child has an expectation of reaching adult life.

RCPE0002068_0071

Use of Intraperitoneal Route for Therapeutic Purposes

In children with difficult veins who require regular transfusion, the intraperitoneal route has something to commend it. Several successful cases were reported by Newman (1959). Nevertheless the method is not free from disadvantages. The introduction of a large amount of blood into the peritoneal cavity may interfere with the movement of the diaphragm, and the author knows of two cases in which a child has become dangerously ill during an intraperitoneal transfusion, due, apparently, to respiratory embarrassment.

Re-transfusion of Blood obtained from the Peritoneal Cavity

Before the development of transfusion services, it was a fairly common practice to collect at operation blood shed into the peritoneal cavity, particularly when operating on patients with a ruptured ectopic gestation or ruptured spleen, and to re-inject the blood immediately into the patient's circulation. That this was a reasonably safe procedure is shown by such experiences as those of Tiber (1934), who reported 123 autotransfusions in ruptured ectopic gestation with only one death. There are many reports in the German literature during the years 1920–38 of haemoglobinuria, jaundice and anuria following autotransfusion of blood obtained from the peritoneal cavity and there is some suggestion that these complications may have been connected with the re-transfusion of blood which had been in the peritoneal cavity for some days (e.g. Grossman, 1954).

It seems advisable not to re-infuse blood shed into the peritoneal cavity unless it is reasonably certain that the blood has been there for less than 24 hours.

Intrasternal Transfusion

Tocantins and O'Neill (1941) described the giving of transfusions intrasternally. They achieved rates of up to 25 ml./minute, but on the average of only 3–3½ ml./minute, giving citrated blood by gravity. The method is said to be rather painful and has not become very popular.

Intramuscular and Subcutaneous Injections

Hollingsworth (1954) injected ^{51}Cr-labelled red cells intramuscularly and subcutaneously into rabbits, and concluded that 3 per cent entered the circulation, reaching a maximum concentration at 4–13 days after injection. Stohlman and Schneiderman (1956) carried out similar experiments in dogs and found that following subcutaneous injections only about 1 per cent of the cells entered the circulation; after intramuscular injections they found that 20 per cent of the cells entered the circulation within 5 days.

The author has carried out two experiments in rabbits but has found even less uptake than that observed by Hollingsworth. 5 ml. of a 40 per cent suspension of ^{51}Cr-labelled red cells were injected intramuscularly into three or four different sites; the red cell volume of each of the two animals was determined by the intravenous injection of the animal's own ^{32}P-labelled red cells. Blood samples were taken on each of the following 3 days and then at longer intervals for 16 days. The maximum radioactivity in samples of washed red cells from the animals never exceeded 0.5 per cent of the injected dose in one case and 0.2 per cent in the other.

It may be concluded that intramuscular injection of blood is no substitute for intravenous transfusion, although from the point of view of isoimmunization such an injection is an effective stimulus.

'GRAFTING' OF BONE MARROW

Tissue grafts are referred to as autologous when the tissue comes from the subject himself, isologous when the tissue comes from an identical twin or — in animals — from another member of a closely inbred strain, homologous when the tissue comes from a member of the same species and heterologous when the tissue comes from a member of another species.

Gorer (1960) pointed out that this terminology, which is at present widely used by those engaged in grafting tissue, differs from that which has been standard in immunology for over half a century and he proposed the use of the terms 'isogeneic' instead of 'isologous', 'allogeneic' instead of 'homologous' and 'heterospecific' instead of 'heterologous'. Although the terms proposed by Gorer seem preferable, they may be unfamiliar to many readers and therefore the terms which are more widely used have been retained in the following account.

In normal subjects only autologous or isologous tissue grafts are permanently successful; homologous grafts may 'take' initially but after a short period (days or weeks) the recipient develops an immune response and the graft is destroyed. Homologous grafts may succeed when the recipient's antibody-forming mechanism has been destroyed or damaged or if the recipient has acquired 'immunological tolerance' through exposure to the same homologous tissue in embryonic life. Occasionally even heterologous grafts may succeed when the species are closely related as in

RCPE0002068_0072

the original successful transfusion of rat marrow cells to mice previously exposed to an otherwise-lethal dose of irradiation (Barnes and Loutit, 1956).

The circumstances in which bone-marrow grafts from one human to another have been attempted will be summarized briefly, with a few references to the experimental background.

Autologous Marrow

The chief use for infusions of autologous marrow appears to be to enable a subject to be treated with an otherwise dangerous dose of irradiation; that is, marrow is taken before irradiation and infused back into the circulation immediately after irradiation. It has been shown that in dogs this procedure enables the animal to recover from an otherwise lethal dose of irradiation (Mannick *et al.*, 1960). Apparently successful results in man have been reported by Kurnick *et al.* (1958) and Newton *et al.* (1959).

In view of the fact that marrow cells can probably be stored for indefinite periods at low temperatures (see below), it has been suggested that subjects who are exposed to a special risk of accidental heavy irradiation should have marrow taken and stored, to be ready for use in emergency.

Isologous Marrow

In man, isologous marrow is available only if the subject has an identical twin but in that case the success of a marrow transfusion should be assured.

Homologous Marrow

In man, the infusion of homologous bone marrow has been carried out in subjects accidentally subjected to high doses of irradiation. Evidence was obtained that the injected cells multiplied but the persistence of the graft was only transitory (see review by Mathé, 1960).

Infusions of homologous marrow have also been given to subjects with malignant disease, particularly leukaemia, after giving large doses of irradiation or of cytotoxic drugs. There is a strong suggestion that the desired result may be obtainable only with closely compatible tissue, e.g. from a near relative. In fact, the only case reported up to 1960 in which prolonged success had been achieved was the one reported by Beilby *et al.* (1960).

A patient with lymphadenoma developed marrow aplasia after treatment with X-rays and chlorambucil. Fifteen days after the marrow had become aplastic

40 ml. of marrow was obtained from the patient's sister and mixed with ACD. The mixture was expelled through a fine needle to break up the larger particles and was then spun at 1000 rev./min. for 10 minutes, after which the supernatant fatty material was removed. The remaining suspension, containing a total of 1.1×10^9 nucleated cells, was injected intravenously within 1 hour of taking. There was an almost immediate clinical response and thereafter the patient made steady progress; 16 weeks later her blood count was normal.

The donor was group O MN Rh+ and the recipient O M Rh−. Four months after the marrow transfusion, red cells of the donor's type could be demonstrated with anti-D and with anti-N sera; a proportion of red cells of the donor's type was present in all samples taken subsequently and in some samples exceeded 30 per cent of the total number present.

It appears that even if the problem of performing successful grafts with homologous tissue could be overcome, it is almost certain that it would prove impossible to eradicate the leukaemic process by irradiation without causing lethal damage to other tissues.

Graft against host reaction. Another problem which arises in homologous transplants of marrow is so-called 'secondary disease'. This is due to the development of an immune response by transplanted cells, directed against the tissues of the host. It has been suggested that foetal tissue should be transplanted instead of adult tissue since this is more likely to display tolerance towards foreign antigens (Barnes, Ilbery and Loutit, 1958); and banks of foetal marrow have in fact been established.

Injection of homologous marrow into embryos. If homologous cells are injected into an embryo at a certain stage of development, the cells are not recognized as foreign and they may then form a permanent graft. When the cells concerned are haematopoietic the blood of the animal may then permanently contain two antigenically separate types of cells, so-called chimaerism (see Chapter VII).

It has been suggested that successful marrow transplantation might be life-saving in certain blood diseases. For example, if Rh-negative marrow were injected into an Rh-positive embryo, whose mother's serum contained potent Rh antibody, the infant might escape severe disease by making sufficient Rh-negative red cells. Alternatively an embryo with homozygous sickle-cell disease might develop into a reasonably healthy adult if injected in embryonic life with normal bone marrow. The technical difficulties of injecting marrow into the embryo are considerable and so far few attempts have been made.

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Techniques of Marrow Transfusion

Obtaining Marrow

Adult marrow has been obtained by multiple bone-marrow punctures under general anaethesia (for ref. see Mathé, 1960) or from the marrow of fresh corpses (e.g. Ferrebee, Atkins *et al.*, 1959).

The number of cells obtained by multiple bone marrow aspirations is of the order of $5\text{-}10\times10^9$ (Wilson, 1959). Very much larger numbers ($60\text{-}80\times10^9$) can be obtained from the vertebral bodies of corpses by the technique described by Ray *et al.* (1961) although this method has not yet been used for the attempted transplanting of viable marrow.

Foetal haematopoietic tissue has been obtained simply by chopping up the liver and spleen of embryos obtained at hysterotomy (Ferrebee, Lochte *et al.*, 1959).

The dose of cells required to produce a rapid response in man is not known. In mice optimal results have been observed with the transfer of 0.5×10^6 nucleated cells using isologous tissue (Urso and Congdon, 1957). On a weight for weight basis this would mean administering $2\text{-}3\times10^9$ nucleated cells to a man but the dose needed would probably be greater when homologous rather than isologous tissue was available.

Storage of Bone Marrow at Sub-zero Temperatures

Barnes and Loutit (1955, 1956) reported that haematopoietic cells from mice could be successfully preserved in glycerol at −80° C.; that is, after injection into the circulation of other mice the cells were capable of reproduction. Ferrebee, Lochte *et al.* (1959) obtained blood-forming tissue from the liver and spleen of human foetuses, and after passing it through screens of different sizes down to 86 μ they mixed the cells with 50 per cent AB serum, 15 per cent glycerol and 35 per cent Hanks' solution and froze it at −70° C. After thawing, the glycerol concentration was reduced by adding first 35 per cent glucose, then normal saline. The mixture was then passed through a sieve (43 μ) and injected intravenously into three patients without obvious ill effects. Although no evidence of the viability of the cells was obtained from observations *in vivo*, studies with ^{14}C-formate *in vitro* suggested a fair rate of DNA synthesis.

Bone-marrow cells of dogs have been shown to remain viable after storage in glycerol at −79° C. for long periods. Thus dogs who had received a dose of radiation which would normally have been lethal but who were then given intravenous infusions of dog marrow stored in 15 per cent glycerol at −79° C. and then diluted in glucose so as to lower the glycerol concentration to 2.5 per cent, returned to normal health; the number of cells infused was approximately 3.5×10^9 (Mannick *et al.*, 1960).

As mentioned in Chapter I, dimethyl sulphoxide has been used successfully instead of glycerol for the storage of mouse marrow cells at −79° C. (Ashwood-Smith, 1960). Whether dimethyl sulphoxide will prove to be superior to glycerol for the storage of human marrow at low temperatures is not yet known.

Administration of Marrow

From animal experiments it is known that the intravenous route is far the most effective. This does not exclude the possibility that in man infusion into a marrow cavity may be equally or more effective. The material obtained by marrow biopsy is spun and the cells are then separated from the fat which is discarded. Some authors prefer to pass the marrow through 'screens', that is a metal mesh, before injection but Dr E. A. McCulloch (personal communication) has given many intravenous injections of unfiltered bone marrow tissue without observing any ill effects.

EFFECTS OF TRANSFUSION ON THE CIRCULATION

Some Effects of Rapid Intravenous Injections

In subjects with a previously stable blood volume rapid transfusion produces a transient rise in venous pressure, but venous pressure falls to normal as soon as the transfusion is stopped, even though blood volume may remain above normal for many hours after this. For example, in a normal subject studied by Sharpey-Schafer and Wallace (1942b) the transfusion of 1600 ml. of serum in 14 minutes produced a considerable increase in plasma volume as shown by a fall in haemoglobin concentration of 23.5 per cent. Venous pressure rose from 0 to 10.5 cm. of water during the transfusion; 15 minutes after the end of the transfusion venous pressure was only 1 cm., although the haemoglobin concentration was still 20 per cent below the pre-transfusion level.

After large rapid transfusions of serum or blood, vital capacity is diminished showing that part of the added fluid is accommodated in the blood vessels of the lungs. However, the degree of reduction in vital capacity accounts for only a part of the additional volume and doubtless

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the larger veins and subpapillary venous plexuses also accommodate extra fluid (Loutit *et al.*, 1942; Sharpey-Schafer and Wallace, 1942b).

Rate of Extrusion of Plasma from the Circulation

Madden and Whipple (1940) found that in dogs the plasma protein concentration was raised for only a few hours following plasma transfusions. Florey and Jennings (1941) gave rapid massive transfusions of plasma to cats. In some cats who, in less than 1 hour, received an amount of plasma equivalent to their initial plasma volume, all of the injected fluid left the blood stream within 24 hours. Broadly similar results have been observed in man; for example, Sharpey-Schafer and Wallace (1942a) studied a group of subjects with a stable blood volume who received between 700 and 2100 ml. of normal serum in 7-27 minutes. In most cases the haemoglobin fell by 15 per cent to 20 per cent, but in all except three of these the haemoglobin was back to within 5 per cent of its original value within 1 hour. In the remaining three cases the haemoglobin was still below its original value after 24 hours. A second group of subjects received 300 ml. of four-times-concentrated serum (corresponding to 1200 ml. normal serum) in 15 minutes. The results were similar to those of the group receiving normal serum: in some subjects there was hardly any change in the haemoglobin level; in others the haemoglobin fell and remained low for periods between 2 and 24 hours. Thus after rapid transfusion of serum to subjects whose blood volume is previously stable, in some cases blood volume is readjusted as fast as the serum is transfused, whilst in others readjustment takes more than 24 hours.

Rate of Readjustment of Blood Volume after Transfusion

Relatively few observations have been made concerning the rate of readjustment after transfusions of whole blood or red cell suspensions. In four cases described by the present author transfusions of approximately 500 ml. of concentrated cell suspensions were given in 20-40 minutes and samples were taken 5 minutes after transfusion and at the end of 24 and 48 hours. Haemoglobin concentration, haematocrit and donor cell concentration were all approximately 10 per cent greater in the 24 and 48 hour samples than in the sample taken immediately after transfusion (Mollison, 1947).

In eight further cases an average of 1010 ml. citrated blood was transfused in periods varying from 20 to 85 minutes, and samples were taken in the same way. Again, the estimates at 24 and 48 hours all showed a rise in

values of about 10 per cent compared with the immediate post-transfusion sample. Notice that these figures do not refer to the total change in values produced by transfusion but to the shift of values following the end of transfusion.

The average figures suggest that when 500 ml. of concentrated cell suspension are transfused in about half an hour, blood volume is temporarily increased by an amount approximately equal to the volume transfused. In many cases readjustment of blood volume is complete within 24 hours.

Because some subjects take as long as 24 hours to readjust blood volume, the amount of blood transfused in 24 hours must be severely restricted in patients whose venous pressure is raised; detailed recommendations are given later in this chapter.

Rate of Readjustment of Blood Volume in Normal Infants

In newborn infants there is a good opportunity to study the readjustment of blood volume after birth. The normal infant gains about 100 ml. of blood from its placental circulation during the 5 minutes after birth, unless the cord is tied earlier.

The magnitude of this transfer can be appreciated by considering a few figures. A 7 lb. infant (= 3 kg.) has at the moment of birth about 260 ml. of blood in its body and at least another 100 ml. of blood in the placental circulation. The 260 ml. of blood in the body consists of approximately 115 ml. red cells and 145 ml. plasma. If such an infant receives 100 ml. of blood from the placenta, it will receive approximately 50 ml. red cells and 50 ml. plasma. De Marsh *et al.* (1942) found that the plasma volume of infants recovering their placental blood was no greater than that of those deprived of it. Mollison *et al.* (1950), working with a rather smaller experimental error, found that plasma volume was actually a little lower in infants who recovered their placental blood, the readjustment being virtually complete within 3 hours of birth. Thus, a newborn infant rapidly 'transfused' with an amount of plasma equivalent to 35 per cent of its initial plasma volume can extrude all the extra plasma from its circulation in a matter of 3 hours or less.

It is not surprising that after giving rapid and similarly massive transfusions of blood by scalp vein to infants, equally rapid readjustments are observed. For example, infant R., with haemolytic disease of the newborn, received a scalp vein transfusion of 75 ml. citrated blood at the age of 5 weeks. The transfusion was completed in 20 minutes and the following

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observations were made (all the haematocrit determinations were made on small venous samples taken from other scalp veins):

	Venous haematocrit
Before transfusion	29.5
5 minutes after transfusion	37.5
1 hour later	39.0
Next day	37.0

The donor's citrated blood had a haematocrit of 42 per cent.

Rate of Transfusion in Subjects with a Normal Circulation

In subjects who have no degree of circulatory failure there is no harm in producing some temporary increase in blood volume. It has to be remembered that for many patients transfusion is an ordeal which they wish to get through as quickly as possible. It is perfectly safe in the ordinary way to transfuse 1000 ml. of citrated blood within a period of 2 or 3 hours; the same volume of a packed suspension of red cells (i.e. 1000 ml. of suspension derived from 2000 ml. of blood) may be transfused in 3 or 4 hours.

Most giving sets deliver about 20 drops per millilitre. The time taken for a bottle (540 ml.) to empty itself can be roughly predicted from the following figures:

40 drops a minute	4 hours
60 drops a minute	2¾ hours
200 drops a minute (just countable)	50 minutes

TRANSFUSION IN SEVERE ANAEMIA

In Chapter III the circulatory adjustments to very severe anaemia were described and below an example is given of the ease with which patients in this condition may pass into cardiac failure. Sharpey-Schafer (1945) has suggested that the heart behaves like an overloaded heart-lung preparation. Any considerable increase in venous pressure may precipitate failure and the problem is thus to introduce red cells without producing any material rise in venous pressure.

Restriction of Amount and Rate of Transfusion

Two main proposals have been made: Marriott and Kekwick (1940) suggested that in very severe anaemia (haemoglobin concentration less

than about 4 g./100 ml.) the rate of transfusion should not exceed 0.5 ml. per pound of body weight per hour. For an adult weighing 140 lb. this would mean giving the transfusion at the rate of 70 ml. per hour.

Sharpey-Schafer (1945) suggested that it might be wise to limit the amount of the transfusion to less than 500 ml., to use concentrated cell suspensions and to take various measures to reduce right auricular pressure; for example, to prop the patient up in a sitting position. The jugular venous pressure should be observed and any rise should be an indication for slowing or stopping the transfusion.

Support for the idea that it may be wise in extreme cases to limit the volume of the transfusion to 250 ml. comes from a case reported by Drummond (1943). The patient was a pregnant woman with severe chronic anaemia and oedema of pregnancy. She was transfused with 400 ml. of a concentrated red cell suspension in 6 hours. At the end of that time she complained of a sensation of constriction in the chest and developed a cough. The transfusion was promptly stopped and the patient recovered.

In the following case the transfusion of as little as 200 ml. at slightly too fast a rate had a fatal result:

> A woman of 68 was admitted to hospital with severe anaemia; on admission her haemoglobin concentration was 2.6 g./100 ml. and her packed cell volume 9 per cent. She was transfused with 200 ml. of blood in about 1½ hours. She became very restless and her jugular venous pressure started to rise. Twelve hours later the jugular venous pressure was found to be raised to the level of her ears and crepitations were heard over the lung bases. The patient died 18 hours after the transfusion with massive oedema of both lungs.

It is suggested that if a patient with a very low haemoglobin concentration has to be transfused an initial transfusion not exceeding 250 ml. of a concentrated red cell suspension (i.e. the red cells from a single bottle of stored blood) should be given at a slow drip rate (taking 4 to 6 hours). The transfusion should be stopped immediately if any untoward signs develop. If the transfusion does not provoke such signs, it can be repeated after 24 or 48 hours. Each transfusion of this amount should produce a rise in haemoglobin of 1.0-1.5 g./100 ml. In view of the need to keep a close watch on the patient, the transfusion should not normally be allowed to continue at night unless the patient has special nursing care.

Other Safety Measures

Warming the patient. Warming the body opens up blood vessels in the limbs and decreases the amount of blood in the pulmonary circulation;

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Glaser (1949) found that the vital capacity might increase by 200-500 ml.

There is evidence that part of the increased blood volume produced by a rapid transfusion is normally accommodated in the pulmonary circulation. It is therefore probable that, if, by warming the patient, the amount of blood in the pulmonary circulation is diminished before transfusion, the risk of overloading will also be diminished.

Preliminary or concomitant venesection. In newborn infants with severe anaemia and a raised venous pressure, the infant's condition can sometimes be greatly improved by preliminary venesection, followed by exchange transfusion, using a concentrated red cell suspension (see Chapter XVII). A similar procedure is almost certainly safer than simple transfusion in severely anaemic adults. Walsh (1953) reported a case of a pregnant woman whose haemoglobin concentration was 2.6 g./100 ml. 1200 ml. of blood was withdrawn from one arm while a similar volume of a concentrated red cell suspension was transfused into the other. The procedure was completed in 30 minutes, during which time the patient's condition improved rapidly.

Signs and Symptoms of Overloading

Overloading produces a rise in systemic venous pressure, an increase in the amount of blood in the pulmonary blood vessels and a diminution in vital capacity. This may produce the symptoms of dullness in the head, tightness in the chest and dyspnoea; a dry cough is common. If these warning signs are neglected, pulmonary oedema soon develops and moist râles can be heard at the lung bases. If dyspnoea develops and the jugular venous pressure is found to be raised, the transfusion should be stopped at once and, if the patient is recumbent, he should be propped up. If this does not produce rapid improvement, venesection (500 ml.) should be tried.

It is not unusual for a patient to display some or all of the signs of overloading during transfusion and then apparently recover from them. A few hours later, pulmonary oedema develops insidiously and the patient's death may then appear not to be due directly to the transfusion.

Any signs of circulatory overloading developing during transfusion must be treated as a grave matter, and even if, following the measures outlined above, the patient appears to improve, a very close watch should be kept during the next 12 hours.

An occasional consequence of transfusion in patients with nephritis is the occurrence of attacks of hypertensive encephalopathy.

The Use of Red Cell Suspensions

Castellanos (1937) seems to have been the first to use concentrated suspensions of red cells rather than whole blood for transfusion in cases of anaemia. The use of such suspensions is logical whenever it is important to introduce red cells into a patient's circulation with the least possible disturbance of blood volume. In fact, the widespread use of concentrated red cell suspensions was initially determined by other considerations. In the Second World War plasma was collected on a large scale. It was mainly the hope of salvaging the red cells that were otherwise being wasted that led to clinical trials of the red cell residues (MacQuaide and Mollison, 1940; Cooksey and Horwitz, 1944).

Indications for Use of Red Cell Suspensions

Concentrated suspensions of red cells are preferable to whole blood in transfusing patients with severe anaemia. They are prepared by removing most of the supernatant plasma-citrate from the blood which has been centrifuged, or has simply been allowed to settle. Approximately 80 ml. of plasma-citrate should be left with 180 ml. of red cells; the resulting suspension will have a packed cell volume of 70 per cent but will still flow through the standard 'giving' needle.

Concentrated suspensions of red cells are preferable to whole citrated blood for exchange transfusions in infants, since it is desirable to limit the amount of citrate introduced; it is also desirable that the injected blood should have a packed cell volume of approximately 55 per cent (see Chapter XVII); accordingly, about 180 ml. of plasma-citrate should be discarded from each bottle of citrated blood. The bactericidal power of stored blood is expected to be less than that of fresh blood. Therefore, if there is any possibility that bacterial contamination, however slight, may have occurred during the preparation of the red cell suspension, the suspension must be transfused within the next few hours.

Washed red cells are only rarely indicated. They are needed when transfusing patients with paroxysmal nocturnal haemoglobinuria whose red cells are sensitive to a factor present in normal plasma; in rare cases in which the patient's serum contains an iso-antibody against a plasma protein (see Chapter XV) and perhaps when transfusing group O red cells to a subject of another group, for investigational purposes. In many cases a single wash will suffice.

By 'a single wash' is meant: preliminary centrifugation of the whole citrated blood, removal of as much supernatant as possible, replacement

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by saline, further centrifugation and removal of the supernatant. This should leave not more than 5 per cent of the original plasma. The packed cells may be re-suspended in a small volume of saline for transfusion.

It is hardly ever necessary to wash red cells more thoroughly than this. It was formerly believed that in transfusing patients with paroxysmal nocturnal haemoglobinuria (PNH) it was wise to wash red cells three times before blood transfusion (Dacie, 1948); and Crosby and Stefanini (1952) stated that in one patient studied by them reactions occurred unless the red cells were washed six times before transfusion. When transfusing PNH patients of group A with red cells from a group O donor it is doubtless wise to wash the cells very thoroughly because group A red cells from patients with paroxysmal nocturnal haemoglobinuria are very readily lysed by anti-A sera (Dacie, 1949). Apart from this it is now thought that it is probably the removal of white cells, rather than the removal of plasma, which is important. Washing of the red cell suspension is not an efficient way of removing white cells, and the reader is referred to Chapter XV for more satisfactory methods.

CHAPTER FIVE

METHODS OF ESTIMATING THE SURVIVAL TIME OF RED CELLS *IN VIVO*: RESULTS IN NORMALS

Most of our present knowledge about the survival time of transfused cells was obtained by applying the method of differential agglutination, and this method will be discussed first.

THE METHOD OF DIFFERENTIAL AGGLUTINATION (OR DIFFERENTIAL HAEMOLYSIS)

A direct method for investigating the fate of red cells after transfusion from one animal to another was first described by Todd and White (1911). This consisted in preparing a serum which, *in vitro*, would haemolyse the red cells of one bull (A) but not those of another bull (B). After transfusing blood from bull B to bull A the mixture of cells in a sample from bull A could be analysed by adding anti-A serum; the recipient's (A) cells were haemolysed and the unhaemolysed cells of the donor (B) were then counted.

Ashby (1919) applied this principle to the investigation of red cell survival in man. After transfusing group O blood to group A recipients she took blood samples and incubated them with anti-A serum; the A cells were agglutinated, and the group O cells could be counted.

Landsteiner *et al.* (1928) showed that differences in MN groups between donor and recipient could be used in the same way, and others have successfully used differences in Rh groups (Mollison and Young, 1942; Wiener, 1943).

Differential agglutination can be used in two ways. Either the recipient's red cells can be agglutinated and the donor's red cells recognized by their failure to agglutinate ('indirect' differential agglutination) or the donor's red cells can be agglutinated using a serum which does not react with the recipient's red cells ('direct' differential agglutination; see Dekkers, 1939).

'Indirect' Differential Agglutination

'Indirect' differential agglutination enables the number of surviving red cells to be counted. Provided that highly potent and specific antisera are used and a sufficient number of red cells counted, reliable quantitative estimates can be obtained.

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Although the method is tedious and has for purposes of routine diagnosis been entirely replaced by the far more convenient method of labelling with ^{51}Cr, it remains the final court of appeal in some situations. The value of using an appropriate serological difference as a label is that the label cannot be lost and cannot be used by other cells.

Suitable Combinations of Donor and Recipient

In order to obtain quantitative estimates of the number of donor cells surviving in the recipient's circulation, potent specific antisera are needed. In practice, the only satisfactory sera likely to be available are: anti-A, anti-B, anti-M and, possibly, anti-Rh. These will suffice for almost all cases in which the problem is simply to discover whether normal red cells survive normally in the recipient's circulation. Thus, if the patient is A, B or AB, red cells of group O can be transfused, and differential agglutination carried out with anti-A or anti-B. If the patient is group O there are four chances in five that he will be M-positive, so that, if group N blood is selected, differential agglutination can be carried out with anti-M.

If the recipient is group ON, the survival of transfused red cells can sometimes be estimated satisfactorily provided that the recipient is also Rh-positive. Then, after transfusing Rh-negative blood, differential agglutination can be carried out with an anti-Rh serum. It should be added that only the most potent agglutinating anti-Rh sera are worth using, and even then the results are not likely to be very accurate, although their precision can be greatly improved by transfusing relatively large amounts of Rh-negative blood; for example, 2000 ml. of citrated blood to an adult.

Technique of Differential Agglutination

Dacie and Mollison (1943) modified Ashby's original method by using centrifugation to enhance agglutination. The details of their method are as follows:

From a sample of venous blood take 0.1 ml. and mix it with 5 ml. of saline; this gives a dilution of 1 in 51. If preferred, a rather larger volume of blood (e.g. 1 ml.) can be used and pipetted into saline in a 50 ml. volumetric flask (Young and Rafferty, 1947).

Although it is undoubtedly easier to obtain precision when relatively large volumes are pipetted, with practice even 0.02 ml. amounts of blood can be delivered with a coefficient of variation of less than ± 1 per cent (Mollison and Cutbush, 1949a).

The next step is to add a volume of cell suspension to a volume of agglutinating serum. Since the object is to add equal volumes, and the

absolute amounts added are of no account, it is very convenient to use an automatic pipette (see Fig. 24).

With this device it is a moment's work to transfer one volume of cell suspension to a small test tube and to add to it a volume of agglutinating serum. The pipette should be made to deliver approximately 0.2 ml.

The final dilution of the patient's blood in the mixture will now be 1 in 102. For most purposes it will not be worth while to correct the result finally to take into account the fact that the dilution is 1 in 102 instead of 1 in 100, since usually one is interested in serial changes rather than absolute concentrations. The concentration of approximately 1 in 100 is chosen on the assumption that the count of donor cells in the recipient's blood will be about 1,000,000 per c.mm. after transfusion. With many grouping sera it will be found best not to use more concentrated cell suspensions if maximum agglutination is to be secured, although with very potent sera,

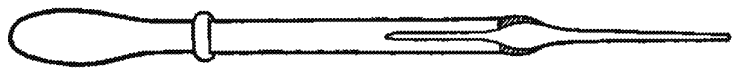

FIGURE 24. 'Automatic' pipette after Wright (1912).

the dilution can safely be reduced to 1 in 50 and this is an advantage if only small amounts of blood are to be transfused.

The mixture of serum and cell suspension must be left for a short period. When using very potent sera, 15 to 30 minutes is quite sufficient, but with less satisfactory sera, e.g. anti-Rh, it is an advantage to wait for 2 hours. The mixture can be left at room temperature except when using anti-Rh, when 37° C. is preferable.

The next step is to centrifuge the mixture. 1000 rev./min. in an ordinary laboratory centrifuge (approx. 150 g.) for 1 minute is sufficient. The tube must then be tapped sharply to break up the clump of red cells. The centrifuging should then be repeated. This time one will know how hard to tap the tube in order to break up the agglutinated cells into a few large fragments. After a third centrifugation, the tube is tapped once more and then held still for a few seconds; during this period the large clumps rapidly fall towards the bottom of the tube. Then with a dry Pasteur pipette a drop of cell suspension is removed from the upper layer of the mixture and immediately transferred to a counting chamber.

At least two separate 'spreads' should be examined. The number of cells

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counted will depend upon the accuracy required; for routine purposes 1000 cells will do, but for more accurate work the number may well have to be increased to 4000.

In making differential counts with anti-Rh, the mixture should be left at 37° C. for 2 hours and must then be handled a little more gently than when using anti-A, anti-B and anti-M. Nevertheless, if the mixture will not stand up to firm tapping it is useless to expect precise results. It is well to try the effect of different amounts of shaking on mixtures containing Rh-positive cells only, to make sure that the number of free cells is not significantly affected by the amount of shaking necessary to ensure an even suspension in the mixtures containing Rh-negative and Rh-positive cells.

Selection and Preparation of Antisera

The commonest serum used is one containing anti-A. Sera produced in rabbits by injecting the artificial A antigen described by Morgan (see below) are by far the most satisfactory, but since most laboratories will have only human anti-A sera available, their testing will be described first.

Sera with a high agglutinin titre usually give the most complete agglutination. Accordingly a few sera of known high titre should be tested by the method described above. Sera from group O donors are likely to contain a slightly more potent anti-A than sera from group B donors and the presence of anti-B does not matter. Sera from group O donors may be sufficiently potent to exhibit a prozone with A_1 cells. That is to say, the most complete agglutination is obtained when the serum is diluted. The results of testing such a serum are shown in Table VII. This serum gave the best results with A_1 cells when it was diluted 1 in 4 or 1 in 8; with A_2 cells it worked best undiluted; with B cells it gave insufficiently complete agglutination.

TABLE VII

OCCURRENCE OF A 'PROZONE' IN A GROUP O SERUM

Dilution of serum 'O 19'	*Number of unagglutinated cells per c.mm.*		
	A_1 cells	*A_2 cells*	*B cells*
1/1	44,000	12,000	93,000
1/2	26,000	29,000	137,000
1/4	10,000	27,000	
1/8	12,000	65,000	
1/16	46,000	—	

Anti-A sera prepared in rabbits by the method of Morgan (1943) are more potent than any human antisera. The author tested the sera originally produced by Morgan and found that with some, all but 0.04 per cent of cells could be agglutinated; that is to say, working with normal adult A_1 blood, all but 2000 cells per c.mm. were agglutinated (Morgan, 1943). Similar results were obtained by Barnes and Loutit (1947) with the same sera.

A great advantage of using Morgan's artificial A antigen rather than human group A red cells as an antigen is that the rabbits are not stimulated to form other unwanted antibodies such as anti-M. Thus the sera do not require much absorption.

Morgan's artificial A antigen is made by combining human A substance with a protein prepared from *Shigella shigae*. Although injections of purified group-specific substances in solution stimulate the production of antibodies in man (see Chapter VIII), in rabbits immune responses have been obtained only when the substances are combined with protein or are adsorbed on to bacteria or on to rabbit's tanned red cells before being injected.

It is essential to select rabbits capable of responding to the A antigen. Thus, some rabbits have A-like substance in their serum and a negligible titre of naturally occurring anti-A, and these fail to respond; others lack A substance and have an appreciable titre of naturally-occurring anti-A and these are suitable for the production of immune anti-A.

Morgan (1943) selected rabbits with a naturally-occurring anti-A agglutinin of 1 in 128 or more, but the present author has had to be content with titres of 1 in 32. Personal experience has shown that three intravenous injections of 1 mg. of Morgan's preparation, given at intervals of 7-10 days, usually stimulates the production of very potent anti-A in such rabbits. The rabbits are bled about 10 days after the last injection. The sera are inactivated and then absorbed once with an equal volume of group O red cells, at 4° C. Serial dilutions of the serum are then tested with suspensions of A_1, A_2 and O cells, following the usual technique for differential agglutination described above. Usually after a single absorption the sera fail altogether to agglutinate O cells; with A_1 cells the most complete agglutination is usually obtained at a dilution of 1 in 10 or more; the count of free cells is then almost always less than 5000 per c.mm. With A_2 cells the sera usually react best at a dilution of 1 in 2 or 1 in 4, and usually give counts of about 10,000 free cells per c.mm.

The sera are stored at —20° C., undiluted. When serum is to be used for a particular case a range of dilutions is prepared and tested with a 1 in 50 suspension of the patient's cells. The dilution which is found to give the

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lowest count of free cells before transfusion is then used for all samples obtained after transfusion in that particular case.

Anti-M and anti-N sera may be produced in rabbits by injecting group OM or ON red cells. The red cells should be washed thoroughly in saline and injected intravenously as a 50 per cent suspension; 0.5 ml. is an appropriate dose. The injections may be given at intervals of 7-10 days. After three injections the rabbits are bled and the serum is inactivated. The serum is then diluted 1 in 10 or 1 in 20 and absorbed with a mixture of AN and ON cells (for anti-M sera) or with a mixture of AM and OM cells (for anti-N). It has been suggested that the heterologous agglutinins for A, B and O human red cells can more conveniently be removed by heating the diluted serum to 70° C. for 60 minutes (Menolasino, Davidsohn and Lynch, 1954).

Use of Haemolytic Antisera

When anti-A is used to distinguish between the cells of recipient and donor, it is possible to haemolyse rather than agglutinate the recipient's cells. Woronow (1936) used this technique, preparing haemolytic sera by injecting incompatible blood (A or B) into several subjects. Hurley and Weisman (1954) found that differential haemolysis gave the same results as differential agglutination, provided that sufficient complement was present in the haemolytic tests. They found that they could ensure this by adding a volume of fresh serum from the recipient to a volume of cell suspension and a volume of immune anti-A serum.

Eadie and Brown (1955) obtained haemolytic anti-A sera by injecting a group B donor with commercial AB substance. As a source of complement, and as a further source of anti-A, they used the serum of a guinea-pig injected with meconium from a group A infant.

The 'Error' of Differential Agglutination

In this case the term 'error' is applied to the extent to which the estimate of the number of foreign cells present in a blood sample deviates from the number actually present. The minimum error of the estimate can easily be assessed: it is that of ordinary red cell counting. In addition one has to assess the effect of variations in the 'blank' count; that is to say, the number of the patient's own cells which fail to agglutinate. If, for example, it were true that the presence of a large number of inagglutinable (e.g. group O) cells interfered with the agglutination of group A cells, the 'blank' count might vary and add to the ordinary error of red cell counting. Thus two

separate kinds of error have to be investigated: firstly, variability round a mean; secondly, deviation of this mean from the truth. Many workers have demonstrated that the variability of replicate samples is no greater than is expected from that of ordinary red cell counting. For instance, Mollison and Young (1940) made a series of thirty-one counts on mixtures prepared from the same suspension, and found that when only 500 cells were counted the coefficient of variation was 5 per cent. This compares closely with errors of ordinary red cell counts on samples of this size. Similarly, Mollison (1951, p. 262) found that the standard deviation on counts of 1000 cells after differential agglutination was 33.6, compared with the theoretical minimum of 31.6 based on the Poisson distribution.

To discover whether there are any systematic errors involved in differential agglutination, estimates can be made on mixtures containing known numbers of group O and A red cells. It might be suspected that in mixtures containing predominantly O cells, the O cells might interfere with the agglutination of A cells and thus lead to an overestimate of the number of O (i.e. unagglutinated) cells present; or that in mixtures containing predominantly A cells some O cells might get caught up in the agglutinates of A cells, leading to an underestimate of the true number of O cells present.

In practice, no evidence has been obtained by the author of systematic overestimates or underestimates in mixtures containing varying proportions of O and A cells (Mollison, 1956, p. 120).

Jones and Kaneb (1960) have published observations which suggest that, in mixtures containing predominantly A cells with only a small proportion of O cells, after the addition of anti-A some O cells become attached to the agglutinated A cells. Nevertheless, from experiments referred to above one must conclude that the phenomenon does not interfere seriously with estimates made by differential agglutination. Jones and Kaneb showed that non-specific clumping of O cells was prevented by the addition of EDTA to the suspensions and it might therefore be a good idea when testing mixtures containing only a very small proportion of O cells, to add EDTA (e.g. in a concentration of 4 mg. per millilitre).

A further estimate of the overall error of the method may be obtained in cases in which a measured number of cells are introduced into the circulation of a subject whose red cell volume is measured by an independent method. In a series of nine cases investigated in this way, Chaplin, Crawford *et al.* (1956) found that the mean count of cells observed at 24 hours was within 1 per cent of the expected figure; the standard deviation

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of the observations was equivalent to a coefficient of variation of 3.7 per cent. In all cases the observed count of cells by the method of differential agglutination was within 7 per cent of that expected from the number of cells transfused and the estimated red cell volume of the recipient.

Sensitivity of the Method

When really good agglutination is obtained so that the blank count is less than 5000 cells per c.mm., the survival of volumes of blood as small as 20 ml. can be detected (Mollison, 1951, p. 268). Before the introduction of convenient methods of labelling red cells with radioactive isotopes it was occasionally valuable to estimate the survival of red cells in 20–50 ml. volumes of blood, as a test of compatibility. Now it is far more convenient to label the red cells with ^{51}Cr; the survival of less than 1 ml. of red cells can then be estimated (see Chapter XIII).

Direct Method of Differential Agglutination

Recognition of the survival of foreign red cells by directly agglutinating them, using a serum which does not react with the recipient's own red cells, is valuable chiefly in the investigation of suspected incompatibility. For example, use may be made of chance differences in MN type between donor and recipient and it may then be possible to say whether or not the circulation of the recipient contains a considerable proportion of transfused cells. This method was used by Wiener and Peters (1940) in investigating the first cases in which anti-Rh was identified as a cause of incompatibility in blood transfusion.

Technique

The technique is the same as that of the indirect method until the point is reached where a drop of the mixture has to be placed on the red cell counting chamber. At this point it is important to obtain a representative drop of free cells and clumps, rather than to try, as in the indirect method, to avoid taking up any clumps.

The representative drop of the mixture is now placed on the counting chamber and examined with the low power of the microscope (see Fig. 25). If moderate or large agglutinates are present, their number and size can be assessed and recorded, e.g. 'scattered clumps of twenty to thirty cells'. If, under the low power, only a few suspicious-looking clumps of three to five cells are seen they should be scrutinized under the one-sixth objective. True agglutinates can usually be distinguished by their brownish

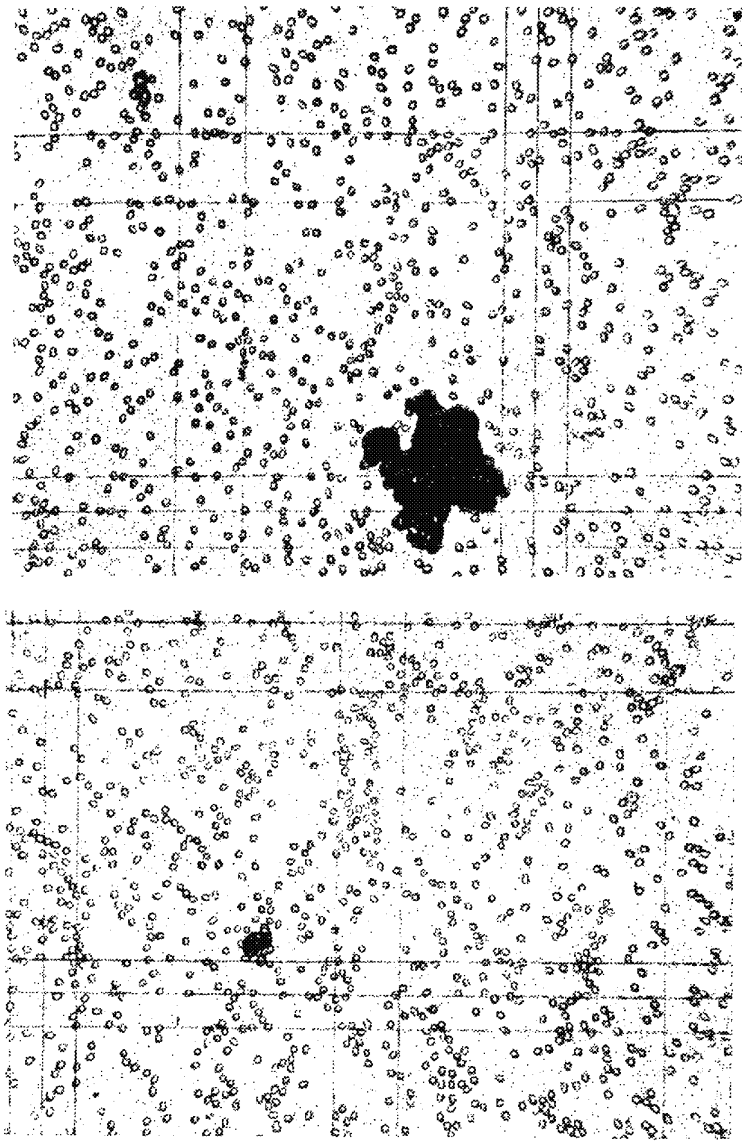

FIGURE 25. 'Direct' differential agglutination of 1 in 100 suspensions of blood using anti-M powdered serum (magnification, × 200).
(*a*) One part of MN with nine parts of N, i.e. 1 in 10. Occasional large agglutinates in a sea of free cells.
(*b*) One part of MN with ninety-nine parts of N, i.e. 1 in 100. The small agglutinate near the centre of the field consists of about twenty cells.

Facing page 142.

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colour; a single clump should never be taken as evidence of the presence of foreign cells.

Sensitivity

The sensitivity of the method can be determined by making mixtures of known amounts of agglutinable and inagglutinable cells (see Fig. 25). One part in fifty can be detected with ease. This corresponds to the survival of less than 10 per cent of the red cells of 400 ml. whole blood given to an adult with moderate anaemia.

In fact smaller proportions of foreign cells can be detected with suitable agglutinating sera. Even one part in 200 produces scattered clumps of approximately eight cells. Under the low power of the microscope these appear as small brown clumps, and under the one-sixth magnification can be seen as small, firm agglutinates.

Detection of very Small Numbers of Foreign Red Cells

The mixed antiglobulin reaction of Coombs, Marks and Bedford (1956) was ingeniously applied by Jones and Silver (1958) to the detection of very small numbers of foreign red cells, the so-called 'minor population'. The principle of this test is to take a suspension of red cells containing a small number of foreign red cells and to add an antibody which will combine only with the 'foreign' cells. Next, antiglobulin serum is added which will also be taken up only by the foreign cells; no agglutinates will form since the agglutinable cells are too scarce. Finally 'detector' red cells are added, that is red cells of the same type as the minor population, sensitized with an appropriate antibody. The cells of the minor population which have taken up antibody, then antiglobulin serum, will combine with the 'detector' cells and form visible agglutinates.

The method used by Jones and Silver (1958) was as follows: approximately 0.25 ml. of a suspension of Rh-negative red cells containing 1 in 10,000 or less Rh-positive red cells was incubated for 30 minutes with anti-D; the cells were then washed four times and incubated for 5 minutes with 0.25 ml. of antiglobulin serum; the cells were washed again four times, then mixed with washed, sensitized red cells and finally examined for the presence of agglutinates. The authors found that they could detect foreign red cells when the mixtures contained only one part in 100,000; they used not only anti-D but also anti-C, anti-c, anti-E, anti-e, anti-K and anti-M.

Use of fluorescent antibodies. Cohen *et al.* (1960) prepared globulin from sera containing anti-A, anti-B, anti-D and anti-c and conjugated the

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product with fluorescein isocyanate. They were able to detect 'foreign' red cells in mixtures containing only one part per 100,000. They applied the technique to studying the survival of Rh-positive red cells in an Rh-negative subject and to detecting Rh-positive red cells in the circulation of Rh-negative women pregnant with an Rh-positive foetus (see Chapter IX).

Survival of Transfused Red Cells in Normal Subjects

Early studies of the survival of transfused red cells by the method of differential agglutination suggested that there was great variation from one subject to another, and that the removal of red cells was an active process, the intensity of which waxed and waned with a definite rhythm (Ashby, 1921). A somewhat similar idea was put forward much later by Denstedt *et al.* (1943) who suggested that cells were sequestered in the spleen and released back into the circulation.

It is now realized that apparent gross irregularities in the slope of elimination of transfused red cells are due mainly to technical errors and that when a really satisfactory technique is used more or less smooth curves are obtained.

Wearn *et al.* (1922) were the first to maintain that the elimination of transfused red cells was a steady process. This was confirmed by Wiener (1934), although he published no details of his findings but simply stated that transfused red cells had a relatively constant survival time of 3 to 4 months.

Mollison and Young (1942) found that in fifteen cases of hypochromic anaemia, transfused with blood stored for 0-4 days, the total survival time of the transfused cells was, on the average, 109 days. This figure was calculated from the percentage of red cells surviving at 60 days, on the assumption that elimination was strictly linear. Callender *et al.* (1945) found that in four normal subjects the life-span of the red cells was 120 days; later (1947) they concluded that although this was the life-span of the majority, some of the red cells were destroyed by extrinsic factors before they reached the age limit. This proportion was greater in women but could not be accounted for solely by the losses of menstruation.

Eadie and Brown (1953) pointed out that the number of carefully studied cases was small and that the normal limits of red cell life-span in man had yet to be determined. The same authors (1955) studied the survival of red cells from two donors, transfused in each case to three different recipients; the 'potential life-span' of the red cells was estimated to be 114 (±8) and 129 (±5) days respectively.

The Slope of Elimination

As mentioned above, after transfusion the number of surviving red cells diminishes steadily over a period of 110-120 days; a typical result is illustrated in Fig. 26.

As Schiødt (1938) pointed out, this suggests that red cells have a constant life-span. Transfused blood is then presumed to contain cells of all ages, in equal numbers; that is to say, approximately one-hundredth of the total number are 1 day old, another hundredth 2 days old, and so on. Thus on

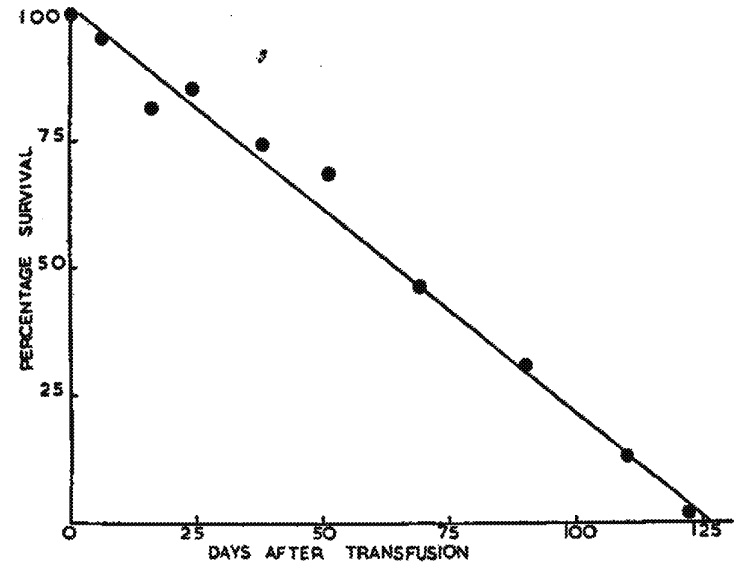

FIGURE 26. Survival of transfused red cells in a healthy male volunteer. 430 ml. of blood were removed by venesection before transfusing 480 ml. of concentrated suspension of red cells. The recipient was Rh negative and the transfused cells were Rh positive but this has not interfered with survival and the slope of elimination is linear (from the data of Mollison, 1944).

each day after transfusion one-hundredth of the number reach the end of their life-span and disappear from the circulation.

The hypothesis that red cells have a constant life span implies that after a certain period in the circulation the red cells either disintegrate spontaneously or become susceptible to some 'physiological' removal mechanism. Of the various differences known to exist between old and young cells, an important one may be their enzyme content. It is known that mature red cells cannot synthesize enzymes and it has been shown that the

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enzyme content of red cells diminishes with age (Pritchard, 1949; Sabine, 1951; Allison and Burn, 1955; Allison, 1960).

The simple hypothesis that all red cells normally live for 110–120 days is subject to modification on three accounts. Firstly, it can safely be assumed that there is some variation around the mean life-span; secondly, there is some evidence from other sources that there may normally be a proportion of red cells with a relatively short life-span; and thirdly, there is evidence that in some normal subjects there may be some degree of random destruction of red cells, irrespective of their age. These three possibilities must now be considered in a little more detail.

Variation in Life-Span

Dornhorst (1951) has pointed out that if there is a variation in red cell life-span, the slope of elimination of transfused red cells will be expected

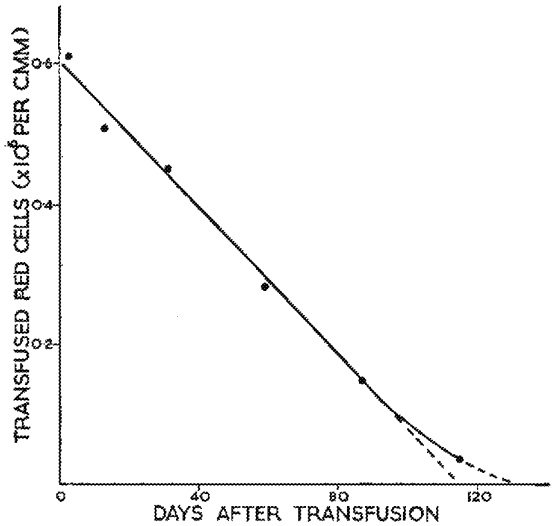

FIGURE 27. Survival of transfused red cells in a male adult. Until elimination of the cells is almost complete the points fall on a straight line. Extrapolation of the line to the time axis gives an estimate of mean cell life (114 days). The persistence of a few transfused cells beyond 114 days is due to variation in red cell life-span.

to show a 'tail', as illustrated in Fig. 27. The mean life-span of the cells is deduced by extrapolating the initial slope to the time-axis, and the coefficient of variation of life-span can be shown to be approximately 2.5 times the percentage survival actually observed at this point. Thus, in an example

given in the first edition of this book, the mean cell life was found to be 120 days and 2 per cent of the transfused cells were still present at that time; the coefficient of variation was thus calculated to be (2.5×2), or 5 per cent; that is to say, 6 days. The conclusion was that 95 per cent of the cells live for 120±(2×6) days, or 108–132 days. It was emphasized that such an estimate was necessarily subject to considerable error, since the method of differential agglutination is least accurate when only a small number of transfused cells are present in the circulation.

Nevertheless, a similar estimate of variation in red cell life-span was made by Brown and Eadie (1953) in studies in dogs with radio-iron. Thus they concluded that 95 per cent of cells died within 12–14 days of the mean life-span.

In a study of the survival of red cells from two human donors, using differential agglutination, Eadie and Brown (1955) found evidence of 'tailing' in one case but not in the other.

Differences in density and osmotic fragility between old and young red cells. Stephens (1940) showed that reticulocytes were less dense than mature red cells and that they tended to collect in the upper layers of a centrifuged sample of red cells. This was confirmed by Chalpin (1956) who also showed that young red cells were more resistant than mature cells to hypotonic haemolysis.

Borun *et al.* (1957) showed very neatly that the density of human erythrocytes increases with age: radio-iron (^{59}Fe) was injected into eight haematologically normal subjects; blood samples were taken at intervals, centrifuged in plastic tubes at a force of 2000 g. for 40 minutes, then frozen and cut into layers. During the first 15 days after labelling, radioactivity was highest in the top layer; then, as time passed, the radioactivity became higher in progressively lower layers. Estimates of red cell life-span were made from the time between the mid-point of appearance in the top layer (4–6 days) and the mid-point of decrease in the bottom layer, which was of course followed by reappearance of radioactivity in other layers as the radio-iron was re-utilized. In six subjects the mean life-span was estimated to be 118 days.

Prankerd (1958) used essentially the same method although he spun the red cells in 30 per cent bovine albumin to get a better separation of red cells by density. He concluded that the lesser density of young cells was due to their higher content of lipids.

Several authors have confirmed that the osmotic fragility of young red cells is less than that of mature red cells. Simon and Topper (1957) and Marks and Johnson (1958) gave ^{59}Fe to normal subjects and then separated the more 'resistant' and more 'fragile' red cells by mixing samples with various concentrations of sodium chloride. Initially most of the radioactivity was found in the most resistant cells although Simon and Topper also found a small amount of radioactivity in the most fragile cells. At 90 to 115 days most activity was in the least resistant cells.

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Short-lived Red Cells

Evidence from experiments with ^{15}N-labelled glycine suggests that there may possibly be a small proportion of very short-lived red cells. Thus Evans (1954) pointed out that after the administration of labelled glycine the amount of labelled stercobilin appearing in the faeces is that which would be expected if one-eighth of the red cells were short lived. If it is arbitrarily assumed that these red cells have an average life-span of only 1 day, and that the remaining seven-eighths of the cells have an average life-span of 115 days, then the number of short-lived cells in the blood stream at any one time would be very small, namely:

$$\left[\frac{(1\times 0.125)}{(1\times 0.125)+(115\times 0.875)}\times 100\right] \text{ per cent;}$$

that is to say, about 0.1 per cent.

This is because the circulation at any given time contains all the long-lived cells produced during the previous 114 days but only those short-lived cells which have been produced during the previous 24 hours, since this is the maximum time for which any of them survive in the circulation. Evidently it is very difficult to detect the presence of a small proportion of short-lived red cells, as first emphasized by Mills (1946).

Mills (1946) also pointed out that the average life-span of red cells in the circulation is given by the initial slope of disappearance of a labelled population. In the example taken above, the presence of short-lived red cells would reduce the average life-span from 115 days to about 100, the average life-span being given by:

$$\left(\frac{12.5}{100}\times 1\right)+\left(\frac{87.5}{100}\times 115\right) = \text{approximately 100 days}$$

The effect on the survival curve would be simply that the percentage survival at 24 hours would be 99.0 instead of 99.13, indicating that the average rate of destruction in the first 24 hours was 1 per cent a day (corresponding to an average life-span of 100 days), rather than 0.87 per cent, corresponding to an average life-span of 115 days. No available method can distinguish between 99.0 and 99.13 per cent survival and therefore in practice the possible existence of a small proportion of very short-lived red cells has to be ignored in determining 'average life-span'.

In estimating the survival of transfused red cells by differential agglutination it is common to take as 100 per cent survival the count of transfused cells found at 1, or even 2, days after transfusion. This is done because the count is usually distinctly greater on the day after transfusion

than it is a few hours after transfusion due to the rather slow readjustment of blood volume. If the counts of transfused red cells are corrected for changes in packed cell volume it is possible to estimate the true percentage survival at 24 hours. In one small series, Hughes Jones and Mollison (1956) estimated the survival at 24 hours to be 99.1 per cent (S.E of mean, 1.7) and thus apparently obtained evidence against the presence of an appreciable number of short-lived cells, but they emphasized that the method of differential agglutination was not accurate enough to settle the question.

Random Destruction of Red Cells

Random destruction of red cells, irrespective of their age, must produce some curvature in the slope of elimination. In normal men the slope of elimination usually appears to be linear but obvious curvature is often seen in women (see Fig. 28), as Callender *et al.* (1947) pointed out. The most obvious explanation for the random loss of red cells in women is men-

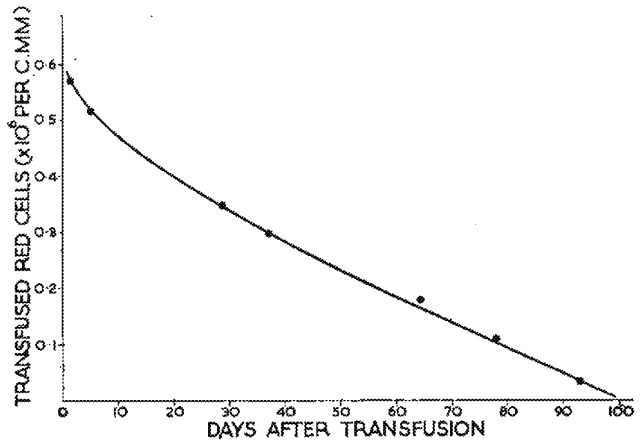

FIGURE 28. Survival of transfused red cells in a woman with hypochromic anaemia; the slope is curvilinear, as it often is in female recipients.

struation and it is true that in non-menstruating females the slope of elimination is sometimes strictly linear (see Mollison, 1951, p. 101, for an example). Nevertheless, Callender *et al.* (1947) deduced from their observations that the loss of red cells from the circulation in females was greater than could be accounted for by menstruation alone. This conclusion seems likely to be upheld, since evidence of random loss of red cells in normal males has been obtained by Eadie and Brown (1955).

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The mechanism of random destruction of red cells in normal subjects is unknown. It is far more prominent in some animals, e.g. cats, than in man (Brown and Eadie, 1953).

The effect of splenectomy on the survival of normal red cells. It is uncertain whether the survival of normal red cells is prolonged in the absence of the spleen. Miescher (1956b) found no significant difference in the curve of disappearance of ^{51}Cr-labelled red cells between thirty-one normal rabbits and ten splenectomized rabbits. On the other hand Belcher and Harriss (1958) found that in splenectomized rats the red cell life-span was greater (65 days) than in normal rats (59 days); the amount of random destruction of red cells was just as great (0.48 per cent a day) in splenectomized as in normal rats. The evidence of Belcher and Harriss seems very strong. The rats' own red cells were labelled by injecting radio-iron and the curve of disappearance of ^{59}Fe from the circulation was plotted. The fact that the ^{59}Fe label was not being transferred from old cells to young was checked by an auto-radiographic technique which showed that the number of 'grains' per red cell remained constant throughout the red cell life-span. The time at which the main fall in ^{59}Fe counts in the blood was occurring corresponded with the time at which excretion of ^{59}Fe in the stools reached a maximum.

As yet there does not seem to be any good evidence to show whether the effect of splenectomy on red cell life-span is the same in man as in the rat.

The Survival Time of Infants' Red Cells

The survival time of the red cells of newborn infants can be measured by obtaining blood from the placenta of one infant at the moment of birth and transfusing it to another infant. In work referred to in the first edition of this book, the results of transfusing placental blood from full-term infants to four anaemic premature infants were described. Each infant also received a transfusion of blood from an adult donor so that the survival of a control population of red cells could be studied. This was a very necessary precaution because the blood volume of infants changes very rapidly as the infant grows and it is also affected by changes in the packed cell volume, and some allowance must be made for this if the true survival curve is to be obtained.

The survival of the placental red cells was first expressed as a percentage of the survival of the adult red cells. That is to say, as long as the ratio of the counts remained the same as it was in the immediate post-transfusion sample, the survival of the placental cells was considered to be 100 per cent of that of the adult cells. When the ratio of the count of placental cells to that of adult cells fell below this initial figure, it was taken to indicate a lesser survival of the placental cells. The assumption was now made that the adult cells were eliminated at the rate of 1 per cent a day and the true

survival of the placental cells was calculated on this assumption (see Fig. 29).

In four cases studied by Vest (1959, p. 67) red cells obtained from the umbilical vein of newborn premature infants were transfused to other newborn infants. Although it is not clear whether any correction was made for blood volume changes, the results strongly suggest that at least in two out of the four cases the average life span of the red cells was shorter than that of adult red cells.

It may tentatively be concluded that the blood of newborn infants does in fact contain a proportion of red cells with a shorter life-span than that of adult red cells. In reaching this conclusion no account has been taken of the

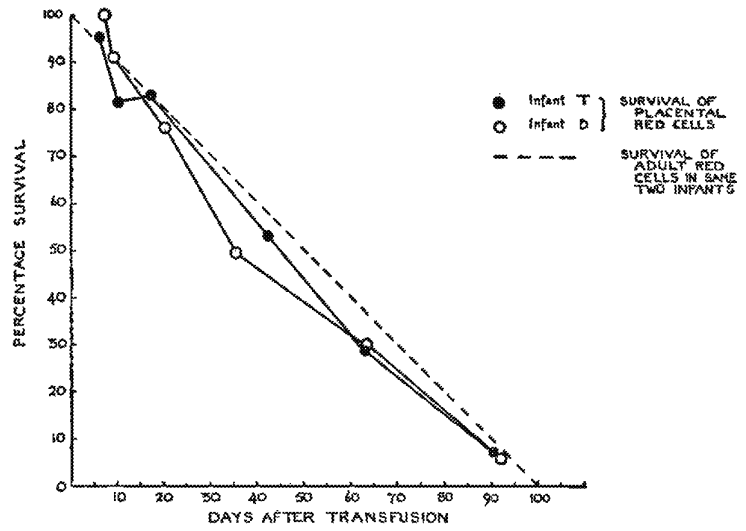

FIGURE 29. Survival of 'placental' red cells in two infants, compared with the survival of adult red cells, transfused simultaneously.

fact that placental blood must contain a disproportionate number of young cells. During the last 100 days of pregnancy the foetus trebles its weight and thus, very approximately, trebles its blood volume. The daily output of red cells must increase greatly during this period. Accordingly, placental blood cannot, like adult blood, contain cells of all ages in equal proportions, but must instead contain an undue proportion of young cells. Therefore if the life-time of the red cells in placental blood were the same as that of adult red cells the proportion surviving at, say, 50 days after transfusion, should be greater. In fact, as Fig. 29 shows, it is less. Thus the

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conclusion that some, at least, of the red cells produced during late foetal life survive for a shorter time than adult red cells is reinforced.

Although the observations reported here suggest that the survival curve of red cells from the placenta falls slightly below that of cells from adult blood, Seelemann (1954) transfused placental blood to four infants and considered that the survival of the cells was no different from that of adult cells in newborn infants.

Results obtained with radioactive chromium apparently support the idea that the red cells of newborn infants have a shorter mean cell life than those of adults (see below).

The Effect of Plethora

The author has previously reported that if the newborn infant is rendered plethoric by transfusion the survival of transfused red cells is not impaired (Mollison, 1943b; 1948). In the first edition of this book an example was given of the data from which this conclusion was drawn, but this has been omitted from the present edition because the matter is no longer controversial.

LABELLING OF RED CELLS WITH ^{51}Cr

The technique of labelling red cells with $Na_2\,^{51}CrO_4$ for the estimation of red cell volume has been described in Chapter II. When the method is to be used for estimation of red cell survival rather larger doses of radioactivity have to be used, but otherwise the method is essentially the same.

Dosage of ^{51}Cr for Estimating Red Cell Survival

The principle is to use the least amount of radioactivity consistent with obtaining a reliable result. It may be necessary to continue observations for about 30 days; at the end of that time about 50 per cent of the injected dose will have left the circulation and the ^{51}Cr which remains will have decayed to half of its original activity, the radioactive half-life of the isotope being 27.5 days. Thus the counting rate will be approximately 25 per cent of that observed immediately after injection.

Suppose it is decided that one needs a count of at least three times 'background' to achieve a sufficiently accurate result without having to count for too long a period. In the ordinary well-type counter this means having a concentration in the blood of approximately 0.2 μc./100 ml. To have this concentration present about 1 month after injection one would have to have about 1 μc./100 ml. in the blood immediately after injection, and

this would be achieved by injecting 50 μc. When survival is to be followed for more than 30 days the dose may be increased to 100 μc.

Hughes Jones (1957) has calculated that even when 100 μc. of ^{51}Cr are injected the ionizing radiation received is only about 0.01 r. per week (averaged over the 13 weeks following injection) and is thus well below the recommended upper limit. This estimate was confirmed by Veall and Vetter (1958) who calculated that a dose of 100 μc. of ^{51}Cr as a red cell label resulted in a dose of radioactivity equivalent to 3 days at the maximum permissible level of radiation for an occupationally exposed worker; that is to say, 3 days' exposure at the level to which a person may be exposed throughout his working lifetime without suffering any appreciable bodily injury.

Although 100 μc. seems to be a very safe amount to use in normal subjects it may be too high when there is a possibility that the dose may be concentrated in the spleen; see Chapter VI.

Correction for Radioactive Decay

If blood samples from a particular subject are counted at different times a correction must be made for radioactive decay. Since counting equipment is not completely stable it is unsatisfactory to correct estimates simply from the known rate of radioactive decay of ^{51}Cr. Instead a standard solution of ^{51}Cr should be used; it is convenient to prepare a solution which gives about 20,000 c.p.m. and then to solidify it by adding agar-agar in a concentration of 3 per cent. The agar is first dissolved by warming; the mixture is then poured into a counting cup and allowed to set and finally covered with vaseline and adhesive tape. This serves as a satisfactory standard for a period of at least 2 months (Hughes Jones and Mollison, 1956). It is desirable to count this standard before and after every sample so that a correction can be made for any instability in the counting equipment as well as for radioactive decay.

It is, of course, possible to avoid a correction for radioactive decay by saving all samples until the end of the investigation and then counting them in a single batch. This may be the most satisfactory for experimental work, but it is unsuitable for clinical studies when the number of samples taken and the time for which the investigation must be continued are determined to some extent by the rate at which the red cells disappear from the circulation.

Volume of Red Cells to be Labelled

The volume of red cells to be labelled should be such that the dose of chromium per millilitre of red cells does not exceed 10 μg. as an absolute

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maximum. As described below, the survival of the red cells is affected if the dose reaches 30 or 40 μg. per millilitre of red cells. When possible, it is doubtless preferable to keep to a level of about 1 μg. per millilitre of cells. If the preparation of $Na_2\ ^{51}CrO_4$ has a specific activity of only 10 mc. per milligramme, a dose of 50 μc. will contain 5 μg. of chromium, so that if this is used to label 5 ml. of red cells the amount of Cr will be only 1 μg. per millilitre. It is now possible to obtain $Na_2\ ^{51}CrO_4$ with a specific activity up to 100 mc. per mg. and this permits the satisfactory labelling of extremely small volumes of red cells.

Use of Unwashed Labelled Red Cells

As explained in Chapter II, when the recipient's red cell volume is to be estimated, the labelled red cells should be washed free from radioactivity in the supernatant before re-injection.

When the object of the test is only to estimate red cell survival, the use of unwashed red cells is less objectionable. No precise estimate of the amount of radioactivity injected is required and therefore it does not matter that there is some radioactivity in the supernatant of the injection suspension. There will be an appreciable amount of radioactivity in the recipient's plasma for a day or two after re-injection of the labelled samples and this will have to be estimated if precise results are required.

When unwashed labelled red cells are injected, it is usual to follow the method of Read (1954) and to add 50 mg. of ascorbic acid to the sample after allowing time for uptake of ^{51}Cr; the ascorbic acid converts any remaining $Na_2\ ^{51}CrO_4$ to the reduced form (Cr_2O_3) and so stops further uptake and also ensures that no hexavalent Cr is injected, some of which would otherwise label the recipient's red cells after injection into the circulation. When a sample of the recipient's own red cells is being labelled *in vitro* and the object of the test is simply to estimate their survival *in vivo*, there seems to be no point in adding ascorbic acid before injecting the labelled cells because it does not matter if some of the circulating red cells are also labelled by any $Na_2\ ^{51}CrO_4$ present in the supernatant of the injection solution. Indeed it may be better not to add ascorbic acid under these circumstances since when hexavalent Cr is injected into the circulation the amount which labels the plasma protein is less (i.e. about 20 per cent of the injected dose) than when trivalent chromium is injected (when about 40 per cent of the injected dose labels plasma protein).

One may now consider exactly how much ^{51}Cr is likely to be found in the plasma when unwashed labelled red cells are injected. Suppose that after 80 per cent of the ^{51}Cr has been taken up by the red cells, ascorbic

acid is added so that 20 per cent of the total amount of radioactivity in the injection suspension is in the supernatant in the trivalent form. Approximately 40 per cent of this trivalent ^{51}Cr will be found in the plasma immediately after injection so that in blood samples taken from the recipient immediately after injection 80 per cent of the ^{51}Cr will be in the red cells and approximately 8 per cent in the plasma. Evidently, the

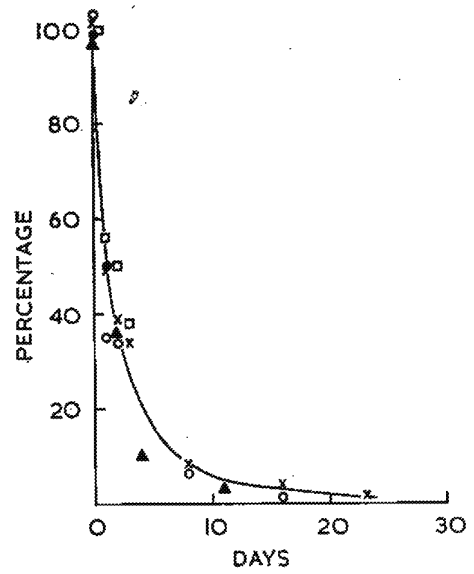

FIGURE 30. Rate of disappearance of ^{51}Cr from the plasma after injecting ^{51}Cr intravenously and taking as 100 per cent the amount of ^{51}Cr in the plasma 10 minutes after injection.

○ × □ Three subjects injected intravenously with 'reduced' (trivalent) ^{51}Cr; amount in the plasma at 10 minutes = 35–50 per cent of the injected dose.

● ▲ Two subjects injected intravenously with $Na_2\ ^{51}CrO_4$; amount in the plasma at 10 minutes = 20 per cent of the injected dose.

assumption that all the radioactivity is in the red cells will lead to a considerable error. On the other hand the amount of ^{51}Cr in the plasma diminishes rapidly, see Fig. 30, so that by the end of 48 hours after injection it can be ignored.

When unwashed labelled red cells are injected, it will as a rule be necessary to estimate the amount of ^{51}Cr in the plasma, as well as in the red cells

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of blood samples taken from the recipient for the first few days after injection. It is really less trouble to wash the labelled red cells and since there is good evidence that this does not cause measurable damage it is recommended that it should be done as a routine. The only circumstance in which it is advantageous not to have to wash the red cells is when chromium labelling has to be carried out in a place where there is no centrifuge, such as in a patient's home.

Labelling with ^{51}Cr in vivo

When $Na_2\,^{51}CrO_4$ is injected intravenously, approximately 10 per cent of the dose labels the red cells (Ebaugh *et al.*, 1953; Hughes Jones and Mollison, 1956); 20 per cent of the dose is found in the plasma immediately after injection, but the amount declines fairly rapidly (see Fig. 30). Because so little of the injected dose labels the cells, the method is unsuitable for routine work, but the results are of theoretical interest and are described below.

Results observed with ^{51}Cr-labelled Red Cells

^{51}Cr Disappearance Curve in Normal Subjects

When the red cells of a normal subject are labelled by adding $Na_2\,^{51}CrO_4$ to blood mixed with ACD or heparin, all owing time for uptake and then washing the cells before re-injecting them, the results shown in Fig. 31 are observed. As the figure shows, about 50 per cent of the Cr leaves the circulation in about 25 days; this estimate agrees closely with that of Read *et al.* (1954) and Donohue *et al.* (1955), but it differs from some other estimates and this is discussed below.

The Cr survival curve is the resultant of at least two processes: loss of red cells by ageing and leakage of Cr from surviving cells — so-called 'elution'. The first process produces a linear slope on arithmetical graph paper, and a curve which is convex on semi-logarithmic paper (see Fig. 32). Cr elution is an exponential process, and is thus linear on a semi-logarithmic plot (see Fig. 32). When the two curves are combined the resulting curve is slightly curvilinear, although, on a semi-logarithmic plot it is approximately linear for the first 40 days (see Fig. 32).

Direct measurement of Cr elution. If a relatively large volume of red cells of a suitable group is labelled with ^{51}Cr, survival of the cells can be estimated by differential agglutination and compared with the Cr survival figures. Estimates of Cr elution obtained in this way give values for the half elution time of 77 days (Ebaugh *et al.*, 1953) or 64 days according to Hughes Jones and Mollison (1956).

If it is assumed that the Cr survival curve is the resultant simply of red cell senescence and Cr elution, then the rate of Cr elution can be estimated by removing the factor of red cell senescence, that is to say, multiplying each estimate by the expression $\frac{115}{(115-t)}$ where t is the number of days following injection.

If such corrections are applied to estimates of Cr survival in normal

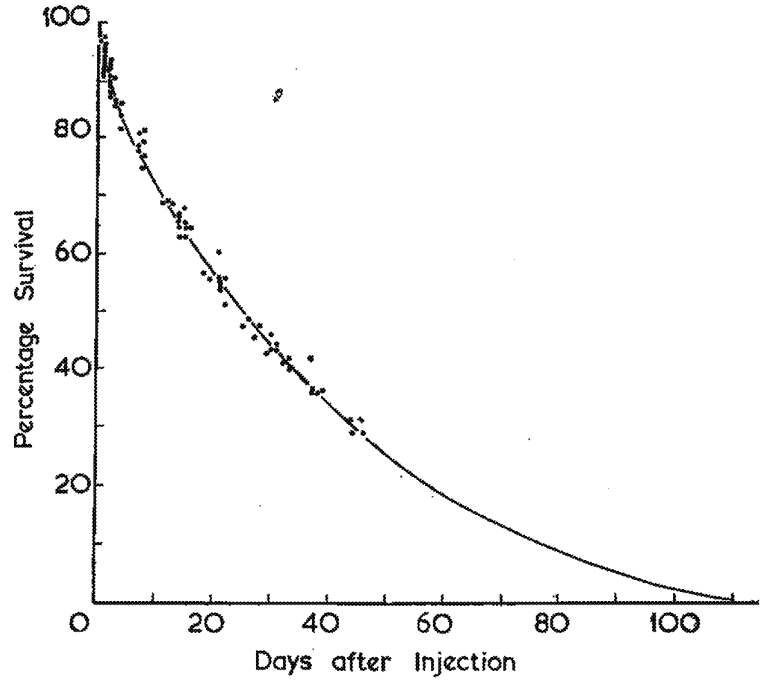

FIGURE 31. Rate of disappearance of ^{51}Cr from the circulation in 11 normal subjects, after injection of the subject's own red cells, labelled *in vitro* by adding ^{51}Cr to whole blood mixed with ACD (from Mollison and Veall, 1955).

subjects and the corrected data, representing only Cr elution, are plotted, a two-component curve results: 5-10 per cent of the Cr elutes rapidly (half-time of the order of 1 day) and the remainder elutes much more slowly (half-time in the case of the data of Mollison and Veall, 1955, about 50 days). The initial more rapid elution of Cr is sometimes referred to as the 'early loss' of Cr and it is a constant feature of Cr survival curves in man.

The early loss of Cr may be overlooked if the Cr survival data are

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plotted on semi-logarithmic paper and an attempt is then made to fit a straight line to the data. This is simply because the data are in fact fitted by a curvilinear slope. If the best straight line is fitted to the data it is found that points beyond 30 or 40 days all fall below the line as do the initial points, the intermediate points falling above the line. This is illustrated by Fig. 32 which shows a theoretical curve without early loss; from the regression line it would be calculated that the percentage Cr at zero time

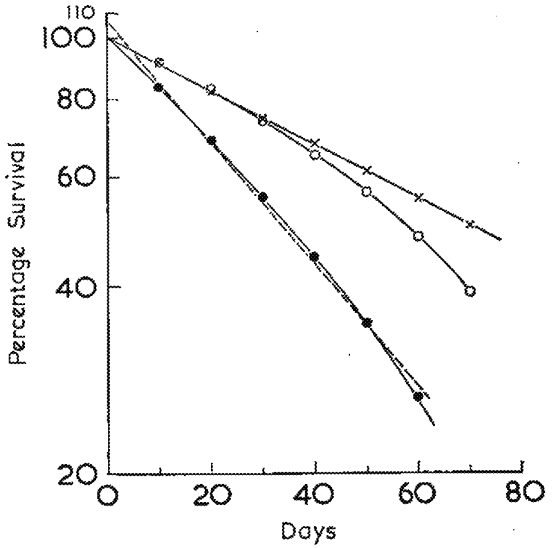

FIGURE 32. Hypothetical figure to show ^{51}Cr survival curve expected when true red cell survival curve is linear with a mean life-span of 115 days and Cr elution is uniform with a half-time of 70 days.

○—○ Red cell survival

×—× Rate of Cr. elution

●—● Above two curves combined .The resulting slope is curvilinear. If a regression line is fitted to the points and extrapolated, it cuts the time axis at about 107 per cent.

was about 106 per cent. Fig. 33 shows an actual example of a Cr survival curve with normal early loss; here the regression line suggests that the Cr survival at zero time was 100 per cent.

It seems almost certain that the 'early loss' is due to the leakage of loosely-bound chromium from the intact red cells. Perhaps the best evidence is derived from observations after the intravenous injection of $Na_2\,^{51}CrO_4$. Since 'early loss' is observed under these conditions although the PCV remains constant, it seems clear that the loss cannot be of red cells

and must be of the label alone (Hughes Jones and Mollison, 1956). Indirect support for this view comes from observations in sheep. Thus, when sheep red cells are labelled with ^{51}Cr either *in vitro* or *in vivo*, about 40 per cent of the label is lost in 24 hours although there is no evidence of red cell destruction (Drury and Tucker, 1958).

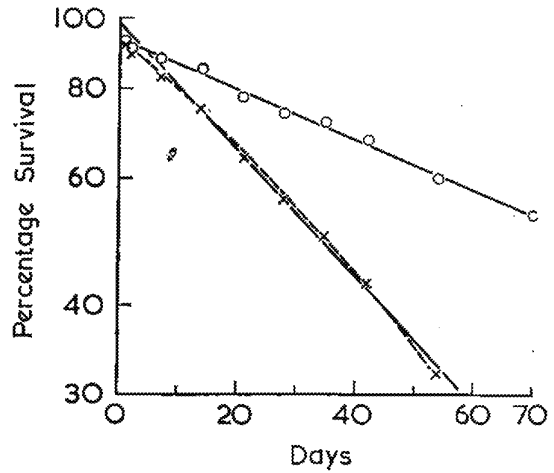

FIGURE 33. To show the effect of fitting a linear curve to normal Cr survival data.

× Observed Cr survival in a normal subject; (the red cells were labelled with ^{51}Cr after a preliminary wash in citrate, and $T\frac{1}{2}Cr$ was about 34 days; see text).

○ The same estimates multiplied by $\frac{115}{(115-t)}$ when t is the time in days, to correct for loss of red cells by ageing.

The dotted line has been drawn by eye to fit approximately the curve of disappearance of Cr. The continuous lines are regression lines (by the method of least squares) fitted to the observed Cr survival curve and corrected Cr survival respectively. Note that the calculated regression line fitted to the Cr survival data cuts the time axis at 100 per cent, falsely suggesting that there was no early loss.

Calculated data for Cr elution are well fitted by a straight line.

The variable rate of the slow component of Cr elution is troublesome, although not very important in practice; the implications are considered in detail in the following chapter.

As mentioned above, not all workers have found that 50 per cent of the Cr disappears in about 25 days. Some authors have in fact reported that the time taken for 50 per cent of the Cr to disappear is as long as 32 days (Jandl *et al.*, 1956). Possible causes for this discrepancy must now be considered.

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Effect of Ignoring Changes in PCV

In a normal subject, provided the PCV remained constant, it would make no difference whether ^{51}Cr counts were expressed in terms of unit volume of red cells or of whole blood; but in practice PCV does fluctuate, being affected by posture and by venous stasis; see Fig. 10 on page 61.

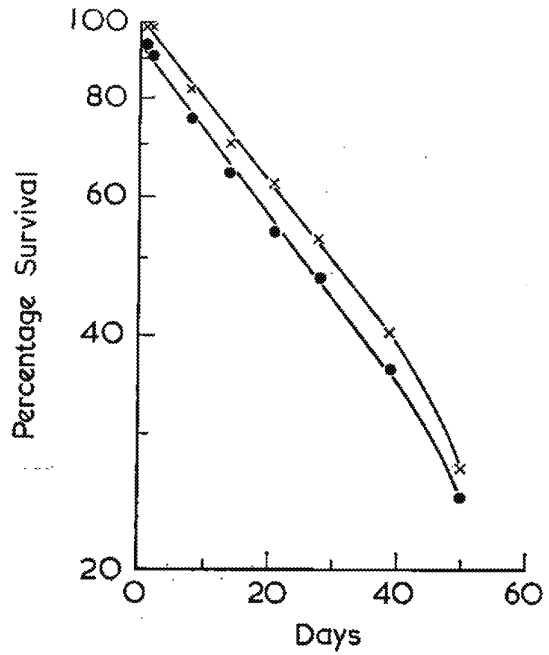

FIGURE 34. Effect on the Cr survival curve of ignoring PCV changes.
●—● PCV taken into account. Cr survival expressed as counts per unit volume of red cells; T½Cr 25 days.
×—× PCV not taken into account. Cr survival expressed as counts per unit volume of whole blood. T½Cr 30 days.
The difference between the curves is accounted for by the fact that when the first sample was taken from the subject 10 to 20 minutes after injecting the labelled cells, PCV was relatively low, that is 42 per cent, and most subsequent samples had a PCV of approximately 44 per cent.

If, following the injection of ^{51}Cr-labelled red cells, changes in PCV are ignored, and ^{51}Cr survival is expressed in terms of counts per unit volume of blood, survival may be overestimated or underestimated. Fig. 34 shows an example; in this instance the red cells of a normal subject were labelled with ^{51}Cr and re-injected with the subject recumbent. A

sample taken 10 minutes after injection therefore had a relatively low packed cell volume (41.1 per cent). In taking blood samples subsequently the subject was seated for only a minute or two before the sample was withdrawn, and PCV therefore tended to be high (about 44 per cent). As the figure shows, if ^{51}Cr counts were expressed in terms of whole blood, survival was overestimated (T½Cr 30 days instead of 25 days).

Effect of Ignoring the First 24 or 48 Hours

Some authors take as the point of 100 per cent survival the ^{51}Cr count in a 24- or even 48-hour sample. If this had been done with the data shown in Fig. 31, results would have been as follows: taking the 24-hour survival as 100 per cent, 50 per cent is reached in 28 days, that is, 27 days later, or, taking the 48-hour sample as 100 per cent, 50 per cent is reached at about 29.5 days, that is, 27.5 days later. This method of expressing the results thus gives a slightly longer 'T½Cr' than the method of taking as 100 per cent the count in the sample obtained within an hour of injection.

Effect of Method of Labelling on Cr Survival Curve

If red cells are washed in 10–20 volumes of citrate solution at a pH between 6.4 and 6.9 before being labelled with ^{51}Cr, the Cr survival curve is found to be different from that shown in Fig. 31. Thus, the point of 50 per cent survival is reached at about 32 days instead of 25 days (Mollison, 1961). If the results are corrected for loss of cells by ageing, as described above, a two-component curve results. About 5 per cent of the Cr is lost rapidly ('early loss') and thereafter Cr elutes with a half-time of about 77 days (see Fig. 33).

It now seems that differences in the 'T½Cr', that is the time taken for 50 per cent of the chromium to leave the circulation after injecting ^{51}Cr-labelled red cells, as reported by various authors are due to a large extent to differences in the rate of elution of Cr, depending upon the medium in which the red cells are suspended at the time of labelling and whether they have been washed before being labelled (Mollison, 1961).

Recommended Method of ^{51}Cr Labelling

It is evidently desirable to label red cells in such a way that the subsequent elution of Cr is as slow as possible and preliminary washing in citrate before labelling is therefore recommended: sufficient heparinized blood to provide about 3 ml. red cells is centrifuged and the plasma

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discarded. Approximately 15 ml. of one of the following two solutions is now added:

(*a*) 9 volumes 3.0 per cent trisodium citrate
1 volume 2.5 per cent disodium citrate, or

(*b*)

Trisodium citrate	3.0 g.
$NaH_2PO_4.2H_2O$	0.015 g.
Glucose	0.2 g.

Water to 100 ml. (After autoclaving at 20 lb./sq. in. for 30 minutes, the pH of the solution should be 6.9.)

TABLE VIII

PERCENTAGE SURVIVAL OF Cr EXPECTED ON VARIOUS DAYS AFTER THE INJECTION OF LABELLED RED CELLS

(*a*) When $T\frac{1}{2}Cr$ is 25.5 days, (*b*) when $T\frac{1}{2}Cr$ is 32.5 days; and the correction factors which will convert these percentage Cr survivals to the true percentage red cell survival, assuming that the true red cell survival curve is linear and the mean red cell life-span is 115 days.

	Expected Cr survival (*a*)	(*b*)	*Correction factors* (*a*)	(*b*)
Days				
1	94	95	1.05	1.04
2	90.5	92	1.08	1.07
3	87	90	1.12	1.08
4	84.5	88	1.14	1.10
5	82	86.5	1.17	1.11
6	80	85	1.19	1.12
7	78	83.5	1.21	1.13
8	76	81.5	1.23	1.14
9	74	80	1.25	1.15
10	72.5	78.5	1.26	1.16
12	69	75.5	1.30	1.18
14	66	73	1.33	1.20
16	63	70	1.37	1.23
18	60	67.5	1.41	1.25
20	57	65	1.45	1.28
22	54.5	62.5	1.49	1.30
24	52	60	1.53	1.33
26	49	57.5	1.58	1.35
28	47	55	1.61	1.37
30	44.5	53	1.66	1.40
35	39	47.5	1.78	1.46
40	34	43	1.91	1.52
45	29.5	38	2.06	1.60
50	26	34	2.18	1.66
60	19	26	2.49	1.81

The mixture is spun and almost all the supernatant discarded, just enough being left to enable the red cells to be easily re-suspended; radioactive sodium chromate is now added and the mixture is incubated at 37° C. for

10 minutes. The red cells are then washed twice in saline and re-suspended in saline for re-injection into the subject. Table VIII shows the average Cr survival on various days after the re-injection of labelled cells in normal subjects (*a*) when $T\frac{1}{2}Cr$ is 25.5 days (as observed when whole heparinized blood or blood mixed with one-quarter of its volume of ACD is labelled), and (*b*) when $T\frac{1}{2}Cr$ is 32.5 days (as observed when red cells washed in

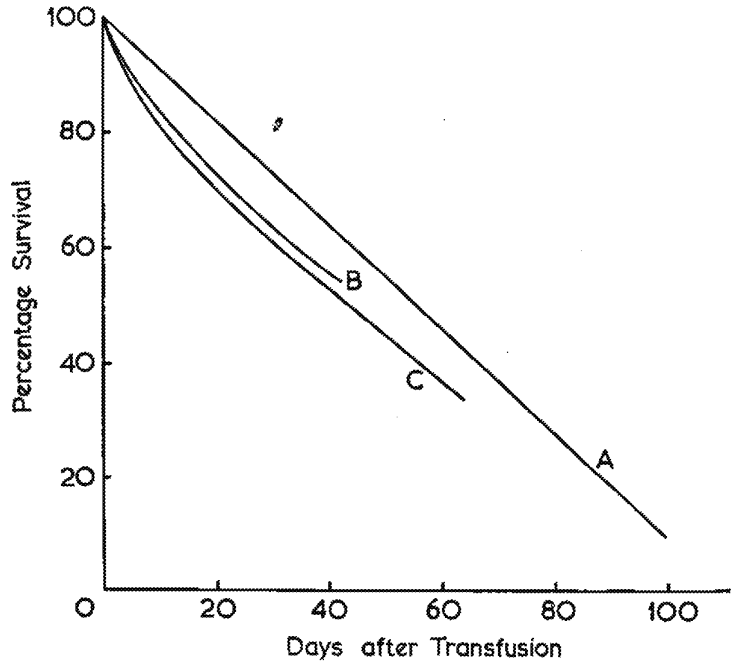

FIGURE 35. Comparison of ^{51}Cr survival curves corrected for chromium elution (half-period 64 days), with the usually accepted normal curve of the survival of transfused red cells (A). Curve B is derived from data obtained by labelling red cells *in vivo* by the intravenous injection of radio-active sodium chromate; Curve C is derived from the curve shown in Fig. 31 (from Hughes Jones and Mollison, 1956).

citrate (pH 6.4-6.9) are labelled). The table also shows correction factors which convert observed Cr survival on any particular day to true red cell survival when the mean life-span is 115 days and the true survival curve is linear.

If the existence of the rapidly eluting component of Cr is ignored and corrections for uniform elution of Cr are made to the normal Cr survival curve, the resulting corrected curve is curvilinear; see Fig. 35. As described

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in the following chapter it seems that the same 'early loss' of Cr is obtained in haemolytic anaemias as in normal subjects and that a correction for uniform elution is therefore never appropriate.

Results Observed After the Intravenous Injection of $Na_2{}^{51}CrO_4$

When red cells are labelled with ^{51}Cr *in vivo* by injecting radioactive sodium chromate, the survival of the labelled red cells can be followed if samples are washed free from labelled plasma. It is found that the ^{51}Cr survival curve is very similar to that obtained when red cells are labelled *in vitro*. Hughes Jones and Mollison (1956) made estimates in seven subjects with the particular object of discovering whether there was 'early loss' under these conditions. Their data showed clearly that there was early loss; thus in all seven subjects the percentage of Cr in the circulation 24 hours after injection was between 95 and 96 per cent of the amount found 30 minutes after injection. Nevertheless, the early loss was rather less than that observed when red cells were labelled *in vitro*. It was estimated that 50 per cent of the Cr was left after 26.5 days, but this figure was based on rather scanty observations. In a series of cases investigated by Sutherland and McCall (1955), 50 per cent of the Cr was left at 30 days; (the figure given was 32.5 days but was based on the assumption that a sample taken 24 hours after injection contained '100 per cent').

Effects of Washing on the Survival of Labelled Red Cells

Donohue *et al.* (1955) stated that the loss of ^{51}Cr from labelled red cells during the first 24 hours after injection was 10 per cent when the cells were washed and only 4 per cent when the cells were not washed, but Mollison and Veall (1955) could detect no difference between washing the cells once and washing them three times, and noted that their results with three-times-washed cells were almost identical with those of Read (1954), who did not wash the cells at all. Cells washed ten to fifteen times apparently survive as well as cells washed two or three times (Hughes Jones and Mollison, 1956).

Similarly, O'Brien (1958) observed a normal ^{51}Cr survival curve with twenty-five-times-washed red cells from one human subject and with fifty-times-washed red cells in rabbits.

Toxic Effects on Red Cells of Labelling with Sodium Chromate

Donohue *et al.* (1955) found that the ^{51}Cr survival curve was apparently not affected by varying the dose of Cr between 0.6 µg. and 20 µg. per millilitre of red cells, but at a dose of 50 µg. of Cr per millilitre of red cells

a shorter survival time was observed (21–24 days instead of 26 days), and at a dosage of 100 µg. per millilitre half the Cr disappeared from the circulation in 16 days.

A slightly different effect was reported by Hughes Jones and Mollison (1956), who found that in two subjects whose red cells were labelled with 30–40 µg. of Cr per millilitre the survival curve followed the normal path

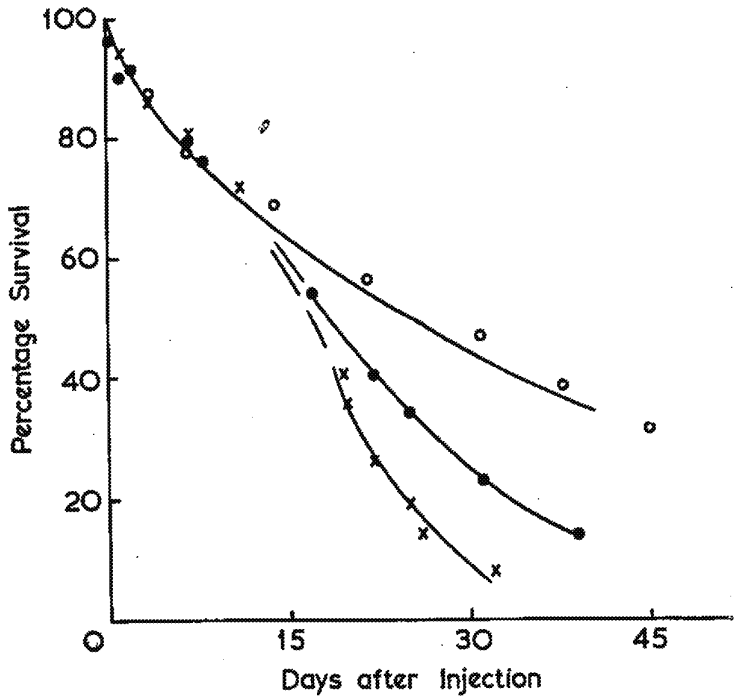

FIGURE 36. Effect of labelling the red cells of three volunteers with relatively large amounts of sodium chromate as $Na_2{}^{51}CrO_4$; dosage in terms of chromium per ml. of red cells was 18 µg. (○), 35 µg. (×) and 40 µg. (●). The upper continuous line is the curve shown in Fig. 31 for red cells labelled with 0.2–2.0 µg. chromium per ml. of red cells.

for about 15 days but then fell much more rapidly than normal (see Fig. 36).

Although most authors refer to chromium toxicity, it is possible that chromate is the toxic agent. When red cells are labelled with chromium by incubation with sodium chromate, in a dose corresponding to about 35 µg. chromium per millilitre of red cells, there is considerable methaemo-

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globin formation due to the oxidizing activity of chromate (Hughes Jones and Mollison, 1956). According to Jandl *et al.* (1956), when the molar ratio of chromate to Hb exceeds 0.1, i.e. about 22 μg. Cr, or 70 μg. $Na_2{}^{51}CrO_4$ per millilitre of red cells, detectable amounts of methaemoglobin appear. It is unlikely that methaemoglobin itself causes any interference with red cell survival, since red cell survival is normal in congenital methaemoglobinaemia (Hurley and Weisman, 1954), and it must be presumed that other substances, possibly enzymes, are damaged by the oxidizing effect of chromate. According to Jandl *et al.*, when chromate is added to red cells even in the proportion of 10 μg. per millilitre of red cells, glycolysis is significantly depressed.

Evidence that ^{51}Cr is not Re-utilized by Red Cells

One great advantage of the ^{51}Cr method is that when labelled red cells are destroyed, the ^{51}Cr does not label other red cells.

Ebaugh *et al.* (1953) labelled red cells with ^{51}Cr *in vitro* and then haemolysed them by freezing and thawing; fresh red cells incubated with this haemolysate did not take up ^{51}Cr. When the haemolysate was injected intravenously no labelling of the recipient's red cells could be detected.

Mollison and Cutbush (1955) found that in one case, on the day following the injection of labelled incompatible red cells, no radioactivity could be detected in a sample of red cells taken from the recipient; the plasma contained only about 1 per cent of the injected radioactivity.

When radioactive sodium chromate is injected intravenously, about 10 per cent is taken up by the red cells and 20 per cent or more of the radioactivity is found in the plasma immediately after injection. If a sample of this plasma is incubated with fresh unlabelled red cells *in vitro*, the red cells do not take up any radioactivity (Sutherland and McCall, 1955; Hughes Jones and Mollison, 1956), suggesting that the Cr is in the trivalent form and bound to plasma protein.

Cr Survival Curve in Children

Remenchik *et al.* (1958) estimated the survival of autologous red cells in fifteen children, of ages between 2½ and 17 years. The average Cr survival at 24 hours was 96.5 per cent, and 50 per cent was reached at 24-40 days. The authors concluded that the survival curve was the same in children as in adults.

Cr Survival Curve in Normal Newborn Infants

If red cells from newborn infants are labelled with ^{51}Cr and injected into normal adults, the Cr survival time is found to be distinctly shorter

than that observed when adult red cells are labelled. Thus, Hollingsworth (1955) found that about 50 per cent of the Cr disappeared in 20 days; and Foconi and Sjölin (1959) found that 50 per cent of the Cr disappeared in 22.8 days when the red cells of full-term infants were labelled and injected into adults, compared with 27.5 days when adult cells were labelled; and

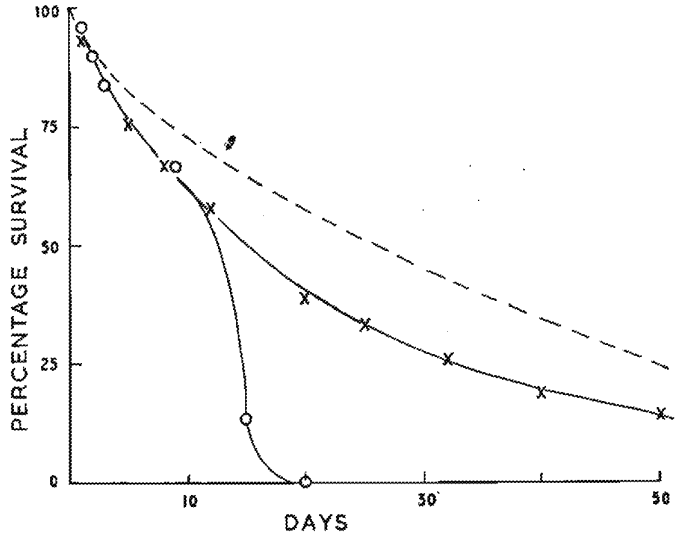

FIGURE 37. Survival of ^{51}Cr-labelled red cells from two newborn infants injected into a normal adult. Small samples of blood were taken from two infants, the first aged 35 hours and the second aged 50 hours. The samples were mixed with ACD and then labelled with ^{51}Cr in the usual way.

O—O Cr survival of red cells from the first infant; 2 ml. of red cells were labelled.

×—× Cr survival of red cells from the second infant; 8 ml. of red cells were labelled.

The interval between the two injections was 34 days.

The dotted line shows the normal Cr survival curve observed when red cells from normal adults are labelled with ^{51}Cr in the same way.

(Unpublished observations made in collaboration with Dr Eliose R. Giblett.)

in six cases in which red cells from premature infants were injected into adults 50 per cent of the Cr disappeared in 15.8 days.

Similarly Dr Eloise Giblett (unpublished observations, made while working in the author's laboratory in 1955) found that ^{51}Cr-labelled red cells from normal mature infants when injected into normal adults had a $T\frac{1}{2}Cr$ of the order of 20 days; an example is shown in Fig. 37.

Gilardi and Miescher (1957) studied the survival of ^{51}Cr-labelled

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autologous red cells in seven premature infants. The mean $T\frac{1}{2}Cr$ was 16 days (range 15–19 days) compared with 24 days (range 21–26 days) in five full-term infants, and with an average of 31 days in older children and of 30 days in adults.

Foconi and Sjölin (1959) found that in eleven out of twenty-seven cases the survival curve was normal for the first 5 to 15 days but then 'collapsed'. This phenomenon was also observed in ten out of forty-one cases studied by Kaplan and Hsu (1961) and in three out of ten cases studied by Dr Giblett. The same phenomenon is observed, with about the same frequency, when cross-transfusion experiments with small volumes of ^{51}Cr-labelled red cells are carried out in adults. It is believed to be due to blood-group incompatibility, although as a rule no blood group antibodies can be found; see Chapter XIII.

In view of the fact that when the survival of red cells from cord blood is estimated by differential agglutination it is found to be only slightly less than that of adult red cells, it seems possible that the Cr elutes more rapidly from the red cells of cord blood than from those of adults. Evidence in support of this suggestion has been published by three groups of workers:

Suderman *et al.* (1957) labelled red cells from cord blood and adult blood with ^{51}Cr, then lysed the cells and dialysed the haemoglobin against saline. The rate of diffusion of Cr from the cord blood haemoglobin was twice as rapid as that from adult haemoglobin. The results of Erlandson *et al.* (1958) were similar although these authors were unable to discover any increased rate of elution *in vitro* from intact red cells. Foconi and Sjölin (1959) found that when ^{51}Cr-labelled red cells were stored in their own plasma at 4° C., the rate of loss from cord red cells was very slightly greater than that from adult red cells (2 per cent instead of 1 per cent after 12 days' storage).

Further support for the idea that Cr may be less firmly bound by foetal haemoglobin than by adult haemoglobin is provided by the observations of Pearson and Vertrees (1961). These authors found that in a patient whose red cells contained a mixture of haemoglobins H and A, the haemoglobin H took up twice as much ^{51}Cr as haemoglobin A. Since haemoglobin H has 4 beta polypeptide chains (β_4) and haemoglobin A has only two beta chains ($\alpha_2\ \beta_2$), it appeared that Cr was bound predominantly by beta chains and this was confirmed by recombination experiments.

Foetal haemoglobin has the formula $\alpha_2\ \gamma_2$, that is, it has gamma chains instead of the adult beta chains and thus might well bind Cr differently from adult haemoglobin.

Other Methods of Labelling Red Cells

The methods of differential agglutination and of using ^{51}Cr both label a sample of the entire red cell population of the donor. As explained in the following chapter, there are circumstances in which more useful information would be obtained if it were possible to label the red cells produced over a restricted period of time. This can, in fact, be done, using labels such as ^{59}Fe or ^{15}N-glycine, which are incorporated in newly formed red cells, but in practice both labels have severe limitations.

Use of Radioactive Iron

Two isotopes are available, ^{59}Fe, with a half-life of 46 days, and ^{55}Fe, with a half-life of 2.94 years. ^{59}Fe is much to be preferred for investigations in human subjects.

When radioactive iron is injected intravenously the iron is incorporated into newly formed haemoglobin; in healthy men 70–90 per cent of the injected dose is used for haemoglobin synthesis within the first 6 to 9 days (Dubach *et al.*, 1946).

Two main types of experiment have been performed with red cells labelled with radio-iron. In the first radioactive iron is injected into a prospective donor. Initially, red cells formed during a limited period of time are labelled; when these cells break down at the end of their life-span the radio-iron is liberated and re-utilized and appears in newly formed cells. In time, cells of all ages are labelled. If blood is now taken from the subject and used for transfusion, the fate of a uniformly labelled population of cells can be studied. This method has been used for studying the effect of storage on red cell survival (Ross *et al.*, 1947; Gibson, Evans *et al.*, 1947; Gibson, Peacock *et al.*, 1947; Gibson, Sack *et al.*, 1947).

The use of radio-iron for studying the survival of transfused cells has one considerable disadvantage: when labelled red cells break down in the circulation, the radioactive iron which is liberated labels cells newly formed by the recipient and appears in the circulation again after about 24 hours. Thus if any large proportion of the donor's red cells break down during the immediate post-transfusion period the survival of the transfused red cells can be followed only for some 24 hours after transfusion; after that time there is no method of telling how much of the circulating radioactivity is in the donor's cells and how much in the recipient's.

In the second type of experiment with radio-iron, the isotope is injected directly into the circulation of the subject, the object being to study the survival of red cells throughout their life-span. From what has been said it will be apparent that this method can be used only when re-utilization of

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liberated radio-iron is negligible. This will be the case in subjects with greatly increased iron stores and in subjects suffering from aplastic anaemia (Finch *et al.*, 1949). Experiments in such subjects give results which agree well with those of differential agglutination; e.g. mean cell life 110 days in one case (Finch *et al.*, 1949).

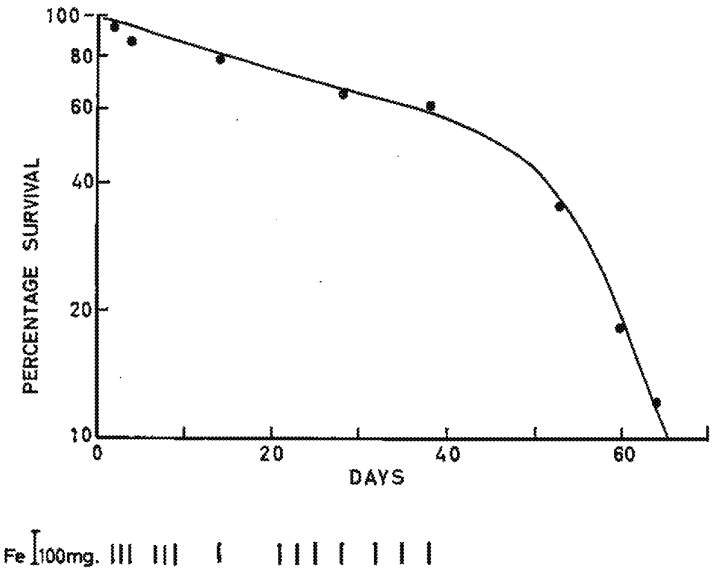

FIGURE 38. An experiment in a rabbit to show the survival *in vivo* of reticulocytes labelled *in vitro* with ^{59}Fe. The rabbit was bled of about 270 ml. during a 3-week period in order to stimulate a brisk reticulocyte response. A sample of blood was then obtained containing 25 per cent reticulocytes and this was labelled by incubating it *in vitro* at 37° C. for 1 hour with ^{59}Fe. The red cells were then washed and reinjected. During the following month approximately 700 mg. of imferon were injected intramuscularly to suppress reutilization of ^{59}Fe released from destroyed red cells.

In healthy animals, the re-utilization of radio-iron can be prevented by administering large doses of non-radioactive iron. Thus Brown and Eadie (1953) injected ^{55}Fe as dilute ferric chloride intravenously in dogs. When the uptake into newly formed red cells was near its 'plateau', large doses of saccharated iron oxide were given intravenously; the amount of radioactivity in the red cells remained constant for about 100 days and then fell precipitously; it was concluded that 95 per cent of the cells lived for 107±14 days. Eadie and Brown (1953) have discussed elsewhere the

advantages of this type of experiment, in which red cells formed over a limited period of time are labelled, in providing evidence of the true life-span of red cells.

Labelling of reticulocytes in vitro *with radioactive iron.* Normal red cells do not take up any appreciable amount of iron *in vitro*, but when red cells from a patient with pernicious anaemia, who is responding to treatment, are incubated with iron, uptake is found to parallel the reticulocyte count (Walsh *et al.*, 1949).

The method of labelling reticulocytes *in vitro* with radio-iron has theoretical advantages over labelling *in vivo* because a more limited population is labelled. Labelling of normal red cells *in vitro* with radio-iron can be carried out successfully by first bleeding the subject repeatedly so as to obtain a good reticulocyte response. Although this can be done well enough in rabbits (see Fig. 38), it is not suitable for studies in man, particularly when it is remembered that large doses of inactive iron will have to be given to prevent re-utilization of the label when the red cells are destroyed.

Labelling of reticulocytes with radio-iron to study the mechanism of destruction of red cells by antibodies is described in Chapter XIII.

Use of Radioactive Di-Isopropylfluorophosphonate ($DF^{32}P$)

When $DF^{32}P$ is injected, a part becomes irreversibly bound to the cholinesterase in red cells and in other cells, e.g. platelets; a part becomes bound to plasma cholinesterase, and the remainder is rapidly excreted or broken down in the body. Thus by measuring the amount of radioactivity in the red cells, their survival can be measured; estimates in two normal subjects gave values of 116 and 129 days for the mean life-span (Cohen and Warringa, 1954).

Bove and Ebaugh (1958) studied red cell survival in eight normal males after injecting $DF^{32}P$ intramuscularly and concluded that the average life-span was 124.6±11 days. In four further subjects a special study was made of the early part of the curve, and it was found that in the first 10 days the rate of fall was twice that of the subsequent part of the curve.

From an extensive study, Eernisse and van Rood (1961) concluded that the elution of $DF^{32}P$ from red cells, sometimes observed in the first few days after labelling, is related to the period for which the $DF^{32}P$ is stored before use; they considered that it ought to be possible to avoid this early elution and that in any case it was usually confined to the first 24 hours after injection. Eernisse and van Rood estimated red cell survival by label-

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ling with $DF^{32}P$ and (subsequently) with ^{51}Cr in a variety of cases of haemolytic anaemia and found that the ^{51}Cr results, after correction for Cr elution, agreed closely with the uncorrected DFP results.

Hjort *et al.* (1960) took a more serious view of the 'early loss' of DFP from red cells. They found that even after labelling human red cells with a very small dose (32 μg. $DF^{32}P$ injected intravenously), there was a loss of 20 per cent from the red cells in the first 24 hours, taking as 100 per cent the amount bound to red cells 5 minutes after injection, which was in fact about 85 per cent of the injected dose. They considered that $DF^{32}P$ should not be used as a red cell label when accurate information was wanted about red cell survival during the first 3 days after labelling.

Although, under some circumstances, $DF^{32}P$ seems to be a very satisfactory label for red cells and platelets, it seems unlikely to come into routine use for the following reasons: (1) Red cells must be washed free of plasma before being counted and platelets must first be separated from red cells and plasma; (2) The dose of radioactivity is greater than that involved when ^{51}Cr is used; (3) DFP is a 'nerve gas' and although the toxic dose is of the order of 5 mg., which is very much greater than the amount needed for a labelling experiment, care is needed in handling the compound; (4) $DF^{32}P$ may be less suitable than ^{51}Cr when information about the first 24 hours is needed, as when studying the survival of stored red cells, because more is known about the magnitude of the early loss of Cr than of $DF^{32}P$.

Bove and Ebaugh (1958) reported the results of labelling red cells *in vitro* with $DF^{32}P$: in two cases 80 per cent of the label was lost during the first 24 hours after re-injection of cells into the circulation.

Use of ^{15}N*-labelled Glycine*

Shemin and Rittenberg (1946) tagged a subject's own red cells by feeding ^{15}N-labelled glycine. The glycine was rapidly incorporated in newly-synthesized haemoglobin, although the concentration of labelled nitrogen per unit mass of red cells did not reach its peak for about 25 days after the beginning of the experiment. At about the eightieth day the concentration of labelled nitrogen began to decline and then fell steeply. The time separating the mid-point of the rise and the declining portion of the graph was found to be 127 days, and this was taken to be the average life-time of the cells. The concentration of labelled nitrogen did not fall to zero after declining but remained at a concentration equivalent to about 10 per cent of the maximum that had been reached; there was little change even at the end of 200 days. Presumably this nitrogen was incorporated

in haem manufactured long after the start of the experiment. Very similar findings were reported in two further normal subjects (London *et al.*, 1949). In one male the mean cell life was found to be 120 days and in one female 109 days.

Although these experiments provide valuable information about haemoglobin metabolism they do not add anything important to our knowledge of the life-span of human red cells. In particular, they do not provide accurate information about the variation in life-span of red cells because the labelled population does not contain cells of uniform age. Thus in some experiments, the amount of labelled haem continued to increase for at least 25 days after the start of the experiments and some labelling of haem continued for much longer periods because, as mentioned above, labelled haem was still present in the blood even after 200 days.

Shemin and Rittenberg (1946) stated that labelled haem, once incorporated in circulating red cells, did not leave them until the cell died. In some of their normal subjects studied subsequently, the amount of labelled haem in the circulation apparently started to diminish about 60 days after labelling and they therefore concluded that there was a large variation in the life-span of human red cells. Thus they suggested that one quarter of the labelled population died between 60 and 106 days, that half the cells died between 106 and 141 days and the rest subsequently (London *et al.*, 1949). In view of the fact, mentioned above, that appreciable amounts of labelled haem were present as long as 200 days after the start of the experiment, it does not seem possible to draw any precise conclusions about the number of cells living for more than the average life-span. In view of the evidence from other sources about the comparatively small standard deviation of red cell life-span, it must be concluded either that the apparent decrease in labelled haem after 60 days was due to errors of measurement or that there is some loss of ^{15}N from intact red cells.

It would be expected that valuable information would be obtained from an experiment in which red cells were taken from a donor comparatively soon after feeding ^{15}N glycine and were transfused to another subject, because in such an experiment a much more limited population of red cells could be studied. In an experiment of this kind carried out by James *et al.* (1953), ^{15}N-glycine was fed to a normal group O subject; 'after a maximum constant tag was observed' 500 ml. of red cells were taken and, after washing, transfused to a normal group A recipient. ^{15}N-labelled stercobilin started to appear in the stools 70 days after transfusion, reached a peak on the one hundred and twenty-second day and then decreased. Since some of the red cells may already have been 20 or 30 days

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old at the time of transfusion, this experiment does not provide any precise evidence about the standard deviation of life-span.

^{14}C-Labelled Compounds

Berlin *et al.* (1954) gave intravenous injections of glycine-2-^{14}C to patients with chronic leukaemia to measure red cell life-span; the specific activity of CO_2 derived from combustion of extracted haemoglobin was measured in an ionization chamber. Their curves showed evidence of prolonged uptake and considerable re-utilization of ^{14}C, and the method thus appears to be open to the same criticisms as the ^{15}N method. It seems doubtful if any precise conclusions about mean cell life-span can be drawn from the curves illustrated. For further data, including comparisons with ^{59}Fe and differential agglutination, see Berlin *et al.* (1957).

The same objection appears to apply to the use of ^{14}C-labelled amino acids prepared from ^{14}C-algal protein, although in experiments in rabbits reported by Piha (1954, 1956) the isotope content of the red cells increased rapidly after administration of the 'label', and a plateau was reached after 5 days. In these rabbit experiments the plateau of maximum radioactivity was maintained until 30 days after labelling. Since when ^{59}Fe is used to label red cells in rabbits no plateau is found (Brown and Eadie, 1953; see also Fig. 38), it must be suspected that the 'plateau' observed by Piha may have been a balance between loss of red cells from the circulation and the appearance in the circulation of newly labelled cells.

Unsatisfactory Labels

Sulphhaemoglobin as a label. Jope (1946) made observations on a group of subjects who had been exposed to TNT and had developed sulphhaemoglobincythaemia. After exposure to TNT had ceased the sulphhaemoglobin disappeared only very slowly from the blood stream, since red cells cannot re-convert the pigment to haemoglobin. In six subjects the average time taken for sulphhaemoglobin to disappear from the blood stream was 116 days, and since the curve of disappearance was approximately linear it was concluded that this provided independent confirmation of the normal red cell survival curve. Jope's interpretation of his results was criticized by Silver *et al.* (1956) who produced evidence that sulphhaemoglobinaemia, produced experimentally, is associated with diminished red cell survival. They suggested that the slow disappearance of sulphhaemoglobin from the blood stream observed by Jope could have been due partly to the continued formation of small amounts of the compound.

There is in fact much evidence that sulphhaemoglobin formation is associated with a decreased red cell survival. McKerns and Denstedt (1949) produced sulphhaemoglobinaemia in rabbits by the administration of acetophenetide and found that the pigment persisted in the blood stream for about 20 days; if the blood was transfused to another rabbit the pigment persisted for only 10 days. Since the life-span of rabbit red cells is of the order of 60 days, the observation suggested that the production of sulphhaemoglobin in red cells considerably shortened their survival.

Silver *et al.* (1956) produced sulphhaemoglobinaemia in dogs and in rabbits by the administration of sulphur together with various drugs, including TNT. All these drugs produced haemolysis at a rate apparently unrelated to sulphhaemoglobin formation. Red cells containing sulphhaemoglobin were labelled with ^{55}Fe and shown to have a diminished survival.

Israels *et al.* (1957) induced sulphhaemoglobin formation *in vitro* by exposing cells to isotonic phosphate buffer through which H_2S had been bubbled. The red cells were labelled with ^{51}Cr and found to be rapidly destroyed *in vivo* ($T\frac{1}{2}Cr$ 2 days). Sulphhaemoglobinaemia was induced *in vivo* by the administration of para-amino-propriophenone and calcium sulphide. This caused a fall in the animal's haemoglobin concentration and a greatly reduced Cr survival time.

Miss Margaret Robinson in the author's laboratory tried to label red cells with very small amounts of ^{35}S, as $Na_2{}^{35}S$, but severe red cell destruction was always produced. When the amount of sulphhaemoglobin formation in the red cells was as low as 3 per cent, red cell survival (judged by ^{51}Cr labelling) was still greatly shortened.

Despite this evidence, which shows that substances which produce sulphhaemoglobinaemia also produce red cell destruction, it still seems likely that the presence of sulphhaemoglobin in red cells is not necessarily associated with a haemolytic process. Miller (1957) described an infant whose blood regularly contained 1 g. per 100 ml. of sulphhaemoglobin and who otherwise had a normal blood picture.

P-Radio-Iodo-Phenylhydroxylamine. If this substance is injected intravenously into an animal, about 50 per cent is taken up by the red cells; there is severe transient methaemoglobinaemia, but this is rapidly reversed. When the red cells break down, the label is promptly excreted from the body (Crick and Jackson, 1953). Tests with this substance have been conducted in man because of its potential advantage as a red cell label. Thus, if it did not damage red cells it would appear to provide a way of studying red cell survival by means of external body counting: that is to say, if it could be assumed that all the labelled compound in the body was present in circulating red cells. However, samples produced so far have

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proved unsatisfactory for the labelling of human red cells (personal communication from Dr H. Jackson and Dr N. Veall).

Elliptocytes were used as 'labelled' red cells by Hedenstedt (1947), but subsequent work suggested that the characteristic elliptical shape was progressively lost after transfusion to a normal subject, and that the data obtained were of very limited value (Berlin and Hedenstedt, 1952).

CHAPTER SIX

DIMINISHED SURVIVAL OF RED CELLS IN DISEASE

Haemolytic syndromes can be divided into two main groups:

(*a*) those in which 'faulty' red cells, incapable of a normal life-span are produced, and,
(*b*) those in which normal cells are produced whose life-span is diminished by extrinsic factors.

The evidence for this division was originally derived from transfusion experiments; and until the antiglobulin test was introduced, estimates of the survival of transfused red cells remained indispensable as a means of distinguishing between the two types.

Until ^{51}Cr was introduced as a red cell label the method of differential agglutination also provided the only practicable means of obtaining a quantitative estimate of red cell survival.

Although transfusions are now very seldom given for the purpose of distinguishing between one type of haemolytic anaemia and another or of estimating red cell life-span, knowledge of the survival of transfused red cells and of the patient's own red cells in various types of haemolytic anaemia is very helpful in predicting how valuable transfusion will be in a particular case.

ANAEMIAS ASSOCIATED WITH THE PRODUCTION OF 'FAULTY' RED CELLS

In the conditions described in this section transfused normal red cells usually survive normally, whereas the patient's own red cells have a diminished life-span. By transfusing the patient's 'faulty' red cells to another subject, or by labelling a sample with ^{51}Cr and re-injecting them, their survival can be estimated and their mean life-span deduced.

Estimation of the Mean Cell Life of 'Faulty' Red Cells

As already mentioned, the mean life-span of a population of red cells is obtained by plotting the curve of their disappearance from the blood stream and extrapolating the initial slope to the time axis (Mills, 1946;

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Dornhorst, 1951). This may be illustrated by taking a hypothetical example:

Suppose a patient is producing two types of red cell: 90 per cent of the red cells produced each day live for 10 days, but the remaining 10 per cent of each day's production live for 90 days. Now although nine times as many short-lived cells are being produced, it can be shown that at any time the blood contains equal numbers of short-lived and long-lived cells: thus suppose the total daily output of cells is 100 (90 short-lived cells and 10 long-lived cells), a blood sample will contain only those short-lived cells produced during the previous 10 days (that is to say, 10×90, or 900) but will contain all the long-lived cells produced during the previous 90 days (that is to say, 90×10, or, again 900). Thus although, each day, nine short-lived cells are produced for each long-lived cell, the blood at any moment contains equal numbers of the two types of cell. Now suppose a volume of the blood is labelled which contains the (900+900) cells described above, the number surviving in the circulation at intervals after re-injection of the cells will be made up as shown in Table IX.

TABLE IX

HYPOTHETICAL EXAMPLE OF THE SURVIVAL OF A MIXED POPULATION OF SHORT-LIVED AND LONG-LIVED RED CELLS

Time after transfusion	*Short-lived cells*	*Long-lived cells*	*Total*
Immediately after	900	900	1800
1 day	810	890	1700
2 days	720	880	1600
3 days	630	870	1500
10 days	nil	800	800
12 days	nil	780	780
14 days	nil	760	760
90 days	nil	nil	nil

If the total numbers of cells surviving at various intervals are plotted graphically, the results shown in Fig. 39 are obtained. Application of the simple rules described above (extrapolation of the initial slope to the time axis) shows that the mean cell life is 18 days. This agrees with the estimates from the original data:

$$\text{Mean cell life} = \left(\frac{90}{100} \times 10\right) + \left(\frac{10}{100} \times 90\right) = 18 \text{ days}$$

It should be noted that the validity of this estimate depends upon two assumptions: (1) that the patient's red cell production has been constant

before transfusion for a time not less than the life-span of the longer-lived red cells; (2) that if survival is estimated after transfusing the 'population' of red cells from one subject to another, the red cell environment is not changed in any relevant way.

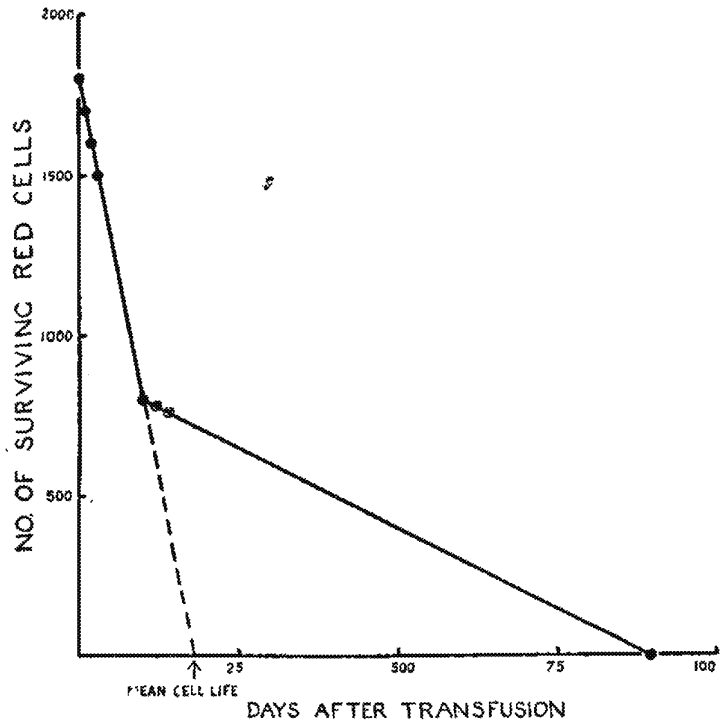

FIGURE 39. Hypothetical example of the survival in a normal recipient of red cells derived from a donor producing short-lived (10-day life) and long-lived (90-day life) cells in the proportion of 9 to 1. Although relatively few long-lived cells are produced, they are as numerous in the donor's blood as are the short-lived cells and this accounts for the large 'tail' of the survival curve. The mean cell life can be estimated by extrapolating the initial slope to the time axis. See text for further details.

Examples of the different survival of 'faulty' red cells in the circulation of the recipient and of the donor himself are given later in this chapter.

Even when the above two assumptions are justified an accurate estimate of the mean cell life depends on the presence in a survival curve of a well-defined initial linear slope; examples of such cases are shown in Figs. 44 and 45.

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Importance of the Initial Slope of the Red Cell Survival Curve

Since mean cell life is estimated by extrapolating the initial slope of the curve to the time axis a serious error will be introduced if the initial slope is steep and changes abruptly within 24 hours. This has been discussed in Chapter V and is illustrated by the theoretical example shown in Fig. 40. Nevertheless, it will be appreciated that it has been necessary to take an extreme and improbable case in order to make the point.

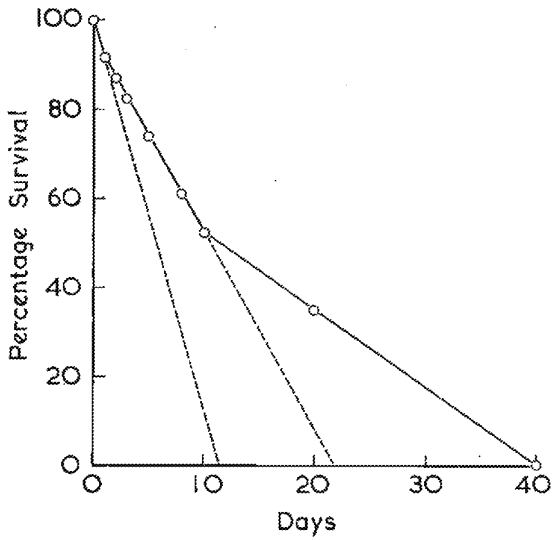

FIGURE 40. Hypothetical example to show the effect on estimates of mean cell life of ignoring the existence of very short-lived red cells. In this example 50 per cent of red cells produced each day have an average life-span of only 1 day, 30 per cent have a life-span of 10 days, and 20 per cent of the daily production have a life span of 40 days. The short-lived red cells make up only 4 per cent of the total number present in the circulation at any time. If estimates of survival are begun at 24 hours when all the very short-lived red cells have been eliminated, and the next part of the survival curve is extrapolated, mean life-span is estimated to be 22 days instead of the true figure of 12 days.

Analysis of curves into components. When the curve of elimination changes abruptly, as in the theoretical examples shown in Figs. 39-41, and the latter part of the curve is linear, the magnitude of the slow component of the curve can be estimated reliably. For example, in Fig. 39 the slow component is seen to consist of 50 per cent of the cells and to correspond to a mean cell life of 90 days.

It might seem equally safe to plot the differences between the initial slope and the extrapolated slow component and thus to define the rapid component. In practice it is very difficult to distinguish between two main components and a continuous distribution of life-span as the example in Figs. 41 and 42 shows.

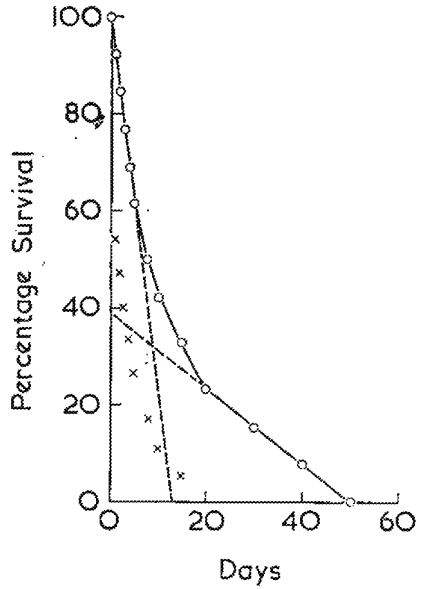

FIGURE 41. Hypothetical example of the survival curve of a population resulting when each day's production consists of 50 per cent of cells with a mean life of 5 days, 25 per cent with a mean life of 10 days, 15 per cent with a mean life of 20 days and 10 per cent with a mean life of 50 days. The survival of the whole population is shown by the continuous line. The slowest component can be extrapolated to zero time and the differences between this slope and the rest of the survival curve can then be replotted (×). On a semi-logarithmic plot all the points (×) are well-fitted by a straight line (see Fig. 42), falsely suggesting that the original curve consisted of only two components.

Curves indicating a very wide distribution of life-span have been described in the following conditions: paroxysmal nocturnal haemoglobinuria (Dacie and Mollison, 1949; Hughes Jones and Mollison, 1956); sickle-cell disease (Callender *et al.*, 1949; Lewis, Szur and Dacie, 1960); Hb C disease (Singer *et al.*, 1954); and thalassaemia (Vullo and Tunioli, 1958).

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Effect on Estimates of Life-span of the Presence of a Proportion of Normal Transfused Cells

It may happen that an estimate of red cell life-span is required in a patient who has been transfused within the previous 4 months. It must then be remembered that if the number of surviving red cells is appreciable the survival curve will be affected. In such cases it is best to find out exactly how much blood was transfused and when, and to relate the probable number of surviving cells to the recipient's total red cell volume.

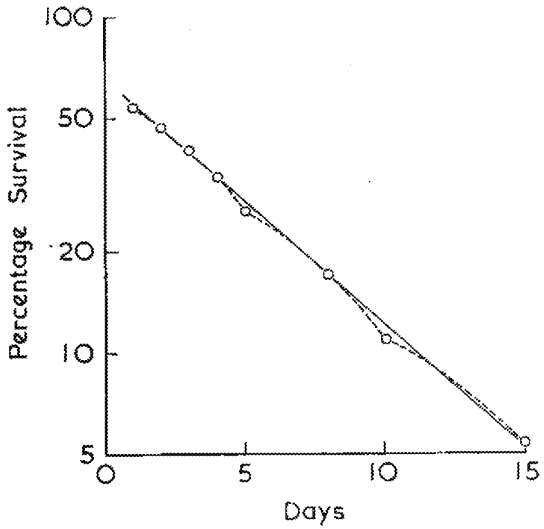

FIGURE 42. To show that a single straight line may give a reasonable fit to a 3-component curve. The points are those marked as crosses in Fig. 41 but in this figure they are plotted on a semi-logarithmic scale. Each of the three curves is fitted by a curvilinear slope but a straight line appears to fit the points fairly well.

For example, suppose an adult weighing 60 kg. with a PCV of 22 was transfused 60 days previously with 1000 ml. of fresh whole blood, the calculation would be: volume of red cells transfused — say, 450 ml.; volume of transfused red cells still in circulation $= \frac{60}{120} \times 450 = 225$ ml. Recipient's red cell volume, assuming normal to be 30 ml./kg. and normal PCV to be 44, $= 30 \times 60 \times \frac{22}{44}$; that is, 900 ml. Proportion of normal cells in blood $\frac{225}{900} = 25$ per cent; that is to say, clearly enough to upset any estimate.

Fig. 43 shows a hypothetical case in which one-third of the cells in the circulation are surviving normal transfused cells, to show the effect on an estimate of mean cell life-span.

Effect of falsely assuming that destruction is random. As described later in this chapter, when red cell destruction is due to an extrinsic mechanism

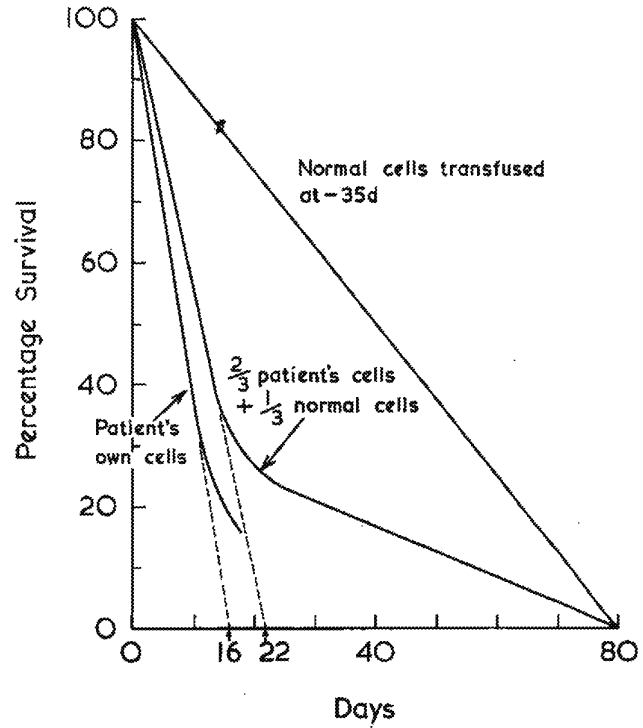

FIGURE 43. To show the effect on the red cell survival curve of the presence of normal transfused red cells. In the example, two-thirds of the red cells in the circulation are the patient's own red cells and these have a mean life-span of 16 days. One-third of the cells are from normal blood transfused 35 days previously and these have an average life-span of 80 days (115 − 35). The middle curve shows the survival curve which would be found if this mixed population were labelled; the average life-span deduced by extrapolating the initial slope to the time axis would be 22 days.

destroying red cells irrespective of their age, the survival curve approximates to a simple exponential and mean cell life may be deduced as the time taken for the number of surviving red cells to fall to 37 per cent of

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the number present immediately after injection. In many cases in which red cell survival is diminished due to 'intrinsic' red cell defects, the curves do not depart greatly from simple exponentials and then it makes little difference whether red cell life-span is deduced by extrapolating the

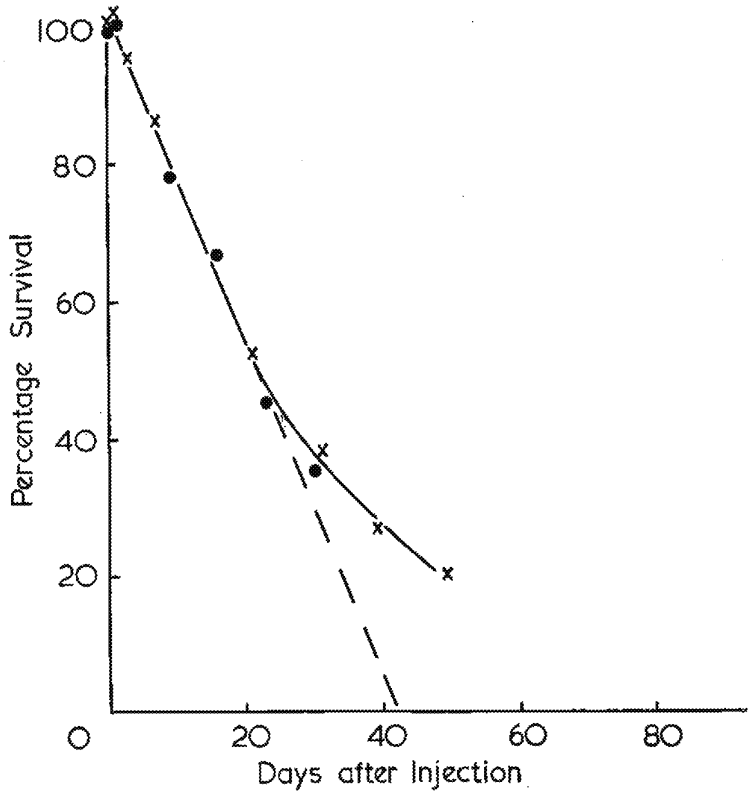

FIGURE 44. Survival of red cells in a patient with an intrinsic red cell defect; the patient's own red cells were labelled with ^{51}Cr on two occasions at an interval of one year (● and ×); the estimates were corrected for chromium elution using the correction factors '(a)', shown in Table VIII. Mean cell life, deduced by extrapolating the initial slope to the time axis, is just over 40 days.

initial slope of the curve to the time axis or estimating the time taken for survival to fall to 37 per cent. Fig. 44 illustrates a case in which extrapolation of the initial slope (the correct method) gave an estimate of 42 days whereas estimation of the point of 37 per cent survival (the incorrect method) gave an estimate of about 30 days.

Effect of applying different corrections for chromium elution. When the mean life-span of the red cells is of the order of 10 days or less, it makes very little difference which set of corrections for Cr elution (see Chapter V) is applied. When the mean life-span is of the order of 30 to 40 days the difference becomes appreciable although it is still of little clinical significance. In the example shown in Fig. 44 mean cell life is estimated to be about 40 days if the 'empirical' corrections are applied, assuming the normal $T\frac{1}{2}Cr$ to be 26 days (see previous chapter); if instead it is assumed that Cr elution is a uniform process with a half-time of 64 days, mean cell life is estimated to be 30 days.

Very little information is available about the rate of Cr elution from abnormal red cells. As mentioned in the previous chapter, if red cells from particular donors with thalassaemia major, with more than 50 per cent of foetal haemoglobin, are labelled with ^{51}Cr and then haemolysed, and the haemoglobin is placed in a dialysis sac, Cr is found to diffuse from the haemoglobin more rapidly than from haemoglobin obtained from normal adult donors (Erlandson *et al.*, 1958).

Difference between Survival of Abnormal Red Cells in their own Circulation and in the Circulation of a Normal Recipient

In clinical research it may sometimes be desirable to study the survival of a patient's red cells in the circulation of a normal recipient in order to demonstrate that the red cells have an 'intrinsic' defect. Although in most cases such a study will give the information that is needed, it must be remembered that the survival of the cells is unlikely to be exactly the same in the recipient's circulation as in the donor's.

For example, the same PNH red cells may survive better in the circulation of one recipient than in that of another; the differences are related to the variable amount of lysis which occurs when PNH red cells are suspended in different samples of serum *in vitro* (Dacie and Mollison, 1949).

The red cells of a patient with hereditary spherocytosis may survive considerably less well in the circulation of a normal recipient than in the circulation of the donor (Jandl *et al.*, 1956; Hughes Jones and Szur, 1957). In this case it has been suggested that the 'sequestering sites' in the patient's spleen are fully occupied even though in subjects with hereditary spherocytosis the sequestering capacity for Rh-sensitized cells is unimpaired (Jandl *et al.*, 1956). Red cells from a patient with hereditary spherocytosis survive normally in the circulation of a splenectomized normal subject (Schrumpf, 1950).

In sickle-cell anaemia without splenomegaly ^{51}Cr-labelled red cells survive better in the patient's own circulation than in the circulation of normal subjects,

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indicating the benefit of 'autosplenectomy' (Jandl *et al.*, 1956; Sprague and Paterson, 1958).

Survival of Transfused Normal Red Cells in Patients with an 'Intrinsic' Red Cell Defect

Normal transfused red cells have been shown to survive normally in hereditary spherocytosis (Dacie and Mollison, 1943), paroxysmal nocturnal haemoglobinuria (Dacie and Firth, 1943; Mollison, 1947; Dacie, 1948); sickle-cell anaemia (Callender *et al.*, 1949) and thalassaemia (Evans and Duane, 1949; Dameshek and Neber, 1950; Hamilton *et al.*, 1950) although in thalassaemia there are exceptions.

Lichtman *et al.* (1953), from observations in twenty children with thalassaemia major who received regular transfusions, concluded that many of them required transfusion unduly frequently. In seven selected cases they measured the survival of transfused cells and found that in six, 50 per cent of the transfused cells were eliminated in 5 to 9 days. No blood group antibodies could be detected. After splenectomy the transfusion requirements of these patients were reduced to between one-fifth and one-third of the pre-splenectomy requirements. Lichtman *et al.* reviewed the literature on this subject and pointed out that although transfused cells had sometimes been found to survive normally in patients with thalassaemia major, the patients concerned had usually had comparatively few transfusions. By contrast in their own series the patients had been transfused regularly for some years.

Examples of Estimates of Mean Life-span of Red Cells with an Intrinsic Defect

The shortest mean life-spans – 4 or 5 days – have been reported in PNH (Dacie and Mollison, 1949; Hughes Jones and Mollison, 1956, see Fig. 45), and in patients with sickle-cell disease and splenomegaly (Sprague and Paterson, 1958).

A mean life-span of 10 to 30 days has been found in patients with hereditary spherocytosis (see data of Loutit and Mollison, 1946; also Crosby and Akeroyd, 1952) and in patients with sickle-cell disease without splenomegaly (see data of Callendar *et al.*, 1949).

In elliptocytosis red cell survival, as judged by the survival of transfused elliptocytes in normal subjects, is usually normal (Berlin and Hedenstedt, 1952), although shortened survival (mean life-span 45 days) was found in one out of three cases investigated by Motulsky, Singer *et al.* (1954). In a single case studied by McBryde *et al.* (1956) the survival time of the patient's red cells in his own circulation was reduced ($T\frac{1}{2}Cr$ 18.5 days).

The relationship of mean red cell life-span to the maximum haemoglobin concentration which the patient can maintain is considered in a later section.

Pernicious anaemia. Several studies have shown that red cells taken from a patient with pernicious anaemia and transfused to a normal subject survive far less well than normal red cells (Loutit, 1946; Singer, King and Robin, 1948; Hamilton *et al.*, 1954). This is not surprising, but it is surprising that normal red cells may survive poorly in the circulation of a patient with pernicious anaemia. This was first reported by Hamilton *et al.* (1953) who found that the amount of random destruction of transfused cells depended upon the time that elapsed between administering vitamin B_{12} and giving the transfusion. When vitamin B_{12} was injected 9 to 12 days before transfusion there was no random destruction, whereas when it was injected only 1-3 days before transfusion random destruction was observed.

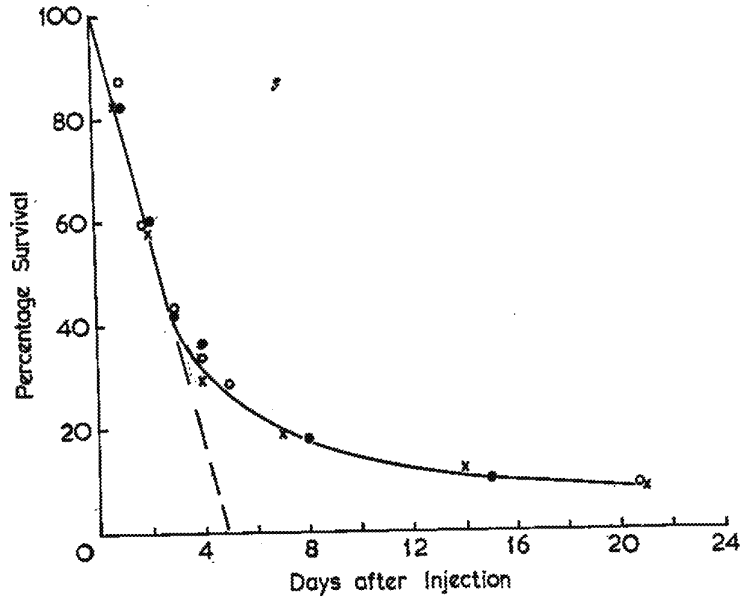

FIGURE 45. Survival of patient's own red cells in a case of paroxysmal nocturnal haemoglobinuria; three sets of measurements on different occasions using varying doses of chromium per ml. of red cells; thus, 7.0 μg. (●), 4.0 μg. (×) and 0.7 μg. (○). Estimates corrected for uniform chromium elution (half-period 64 days). Mean cell life (4½ days) deduced by extrapolating initial slope to the time axis (from Hughes Jones and Mollison, 1956).

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When B_{12} therapy was discontinued in a patient in whom normal red cells were surviving normally, there was a sharp increase in red cell destruction after a lag of 20 days.

In further studies Hamilton *et al.* (1958) confirmed that in untreated pernicious anaemia there was a random loss of red cells which in a small series of cases ranged from 0.6 to 3.0 per cent per day.

Iron-deficiency. There have been relatively few studies of the survival of red cells in patients with iron-deficiency anaemia. Rash *et al.* (1958) studied fourteen iron-deficient infants aged 7-22 months and five haematologically normal infants aged 7-30 months. In every case the subject's own red cells were labelled with ^{51}Cr. In the anaemic subjects $T\frac{1}{2}Cr$ ranged from 13 to 22 days (average 17 days); in the healthy subjects $T\frac{1}{2}Cr$ was between 28 and 35 days (average 32 days). The anaemic infants had haemoglobin concentrations ranging from 1.8 to 7.3 g. per 100 ml. with an MCV of 50-80 μ^3 and an MCHC of 19-28 per cent. They all had positive tests for occult blood in the stools but the estimated loss was only 0.25 to 3.7 ml. a day; that is, too little to account for the reduced red cell survival times.

Verloop *et al.* (1960) studied two women with iron-deficiency anaemia and found $T\frac{1}{2}Cr$ to be 14 days and 13 days (two estimates) in the first and 18 days and $5\frac{1}{2}$ days in the second, this last estimate having been obtained during a haemolytic crisis immediately after a course of parenteral iron therapy. In the first of the two patients, the haemoglobin fell very rapidly after the transfusion of normal red cells, suggesting an accelerated destruction of the transfused cells. The authors concluded that in these two cases there was 'pathological haemolysis' complicating iron-deficiency anaemia. In five other women with hypochromic anaemia and iron deficiency $T\frac{1}{2}Cr$ was found to be 18 to 22.5 days (normal given as 28.3 ± 2.3 days).

Red Cell Abnormalities which need not Disqualify a Donor

In some populations a considerable number of donors have an inherited red cell abnormality and it has to be considered whether their blood can safely be used. Abnormalities of this kind are sickle-cell trait, thalassaemia minor and a deficiency of the enzyme, glucose-6-phosphate dehydrogenase.

It is known that *sickle-cell trait* red cells survive normally in normal subjects (Singer, Robin, *et al.*, 1948; Callender *et al.*, 1949) although in patients with various types of anoxia they survive poorly, being trapped in the spleen (Krevans, 1959). In normal recipients, stored sickle-cell trait red cells survive as well as stored normal red cells (Ray *et al.*, 1959).

The problem of describing the survival of red cells from patients with *thalassaemia minor* is that there appear to be all degrees of severity. The red cells of patients with a minimal degree of the disorder survive normally (Hamilton *et al.*, 1950; Kaplan and Zuelzer, 1950) whereas red cells from more severely affected subjects do not (e.g. Strumia, Taylor *et al.*, 1955).

The red cells of patients with a *deficiency of glucose-6-phosphate dehydrogenase* apparently have a slightly shortened mean life-span, that is about 80 days as determined by labelling with $DF^{32}P$ (Alving *et al.*, 1960), and a $T\frac{1}{2}Cr$ of 18-26 days compared with a normal $T\frac{1}{2}Cr$ of 25.5 to 31 days (Brewer *et al.*, 1961; Kellermeyer *et al.*, 1961) even when no drugs have been administered.

The life-span may be very much shorter than this if the subject ingests one of a large series of substances, for example, primaquine, phenacetin, sulpha drugs, vitamin K or fava beans. It has been shown that if red cells with the enzyme defect are injected into the circulation of a subject with normal red cells and primaquine is now given to the normal subject, about half of the injected red cells (in fact the older half) are rapidly destroyed (Dern, Weinstein *et al.*, 1954; Dern, Beutler and Alving, 1954). Ideally, subjects with this enzyme defect should not be used as blood donors although the transfusion of their blood would be dangerous only under exceptional circumstances.

Red cells taken from patients with *polycythaemia vera* survive normally in the circulation of normal recipients (Merskey, 1949) and in the subject's own circulation (London *et al.*, 1949).

HAEMOLYTIC ANAEMIAS IN WHICH RED CELL LIFE-SPAN IS DIMINISHED BY EXTRINSIC FACTORS

This group of conditions includes all those in which the subject produces normal red cells whose life is shortened by any process other than haemorrhage.

Estimation of Mean Cell Life

As Schiødt (1938) postulated, and Brown *et al.* (1944) demonstrated, the operation of a mechanism which destroys red cells irrespective of their age produces curvilinear slopes of elimination. Mean cell life can as usual be estimated by extrapolating the initial slope to the time axis but an easier method when the curve is a simple exponential (as it usually is), is to plot the points on semi-logarithmic paper and read off mean cell life as the point of 37 per cent survival (Dornhorst, 1951; see Fig. 46).

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Effect of applying different corrections for Cr elution. When the curve of red cell survival is a simple exponential, indicating a uniform rate of destruction, mean red cell life-span can in principle be estimated from any part of the curve. This means that the problem of 'early loss' (the initial more rapid elution of chromium, see Chapter V) can be ignored by taking no account of estimates made within the first few days following the injection

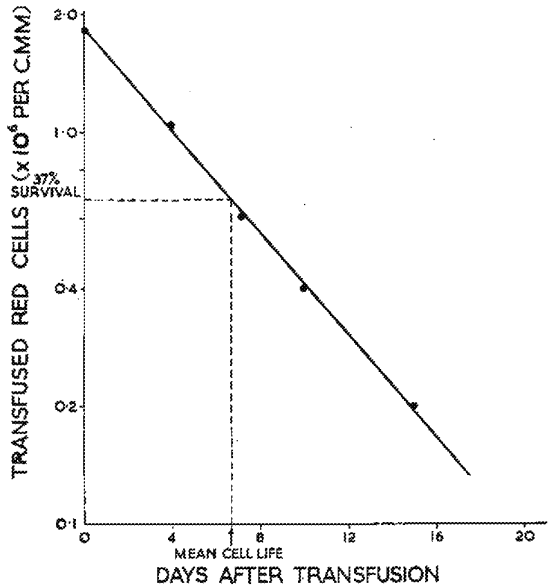

FIGURE 46. Survival of transfused red cells, plotted on a logarithmic scale, in a patient with moderately severe acquired haemolytic anaemia.

of labelled cells. It can then be shown that the particular correction applied for Cr elution has a relatively minor effect on the estimated mean life-span. For example, suppose that, taking as 100 per cent the concentration of Cr in the circulation 3 days after the injection of labelled cells, the concentration falls to 37 per cent after a further 12 days (i.e. by the end of 15 days after injection). Then if Cr elution is taken to have a half-time of 50 days (i.e. the slow component of Cr elution when the normal $T_{\frac{1}{2}}$Cr is 25.5 days, see previous chapter) then the mean life-span of the red cells is calculated to be 14.5 days. If Cr elution is taken to have a half-time of 77 days (i.e. the slow component of Cr elution when the normal $T_{\frac{1}{2}}$Cr is 32.5 days), the mean life-span of the red cells is calculated to be 13.5 days.

In fact it appears that there is the same 'two-component loss' of chromium when the red cells of patients with haemolytic anaemia are labelled as when normal cells are labelled. Thus if the Cr estimates are corrected for uniform elution of Cr and the corrected points are plotted on semi-logarithmic paper and a line is then fitted to the points, ignoring the first 48 hours, this line when extrapolated to the time axis passes

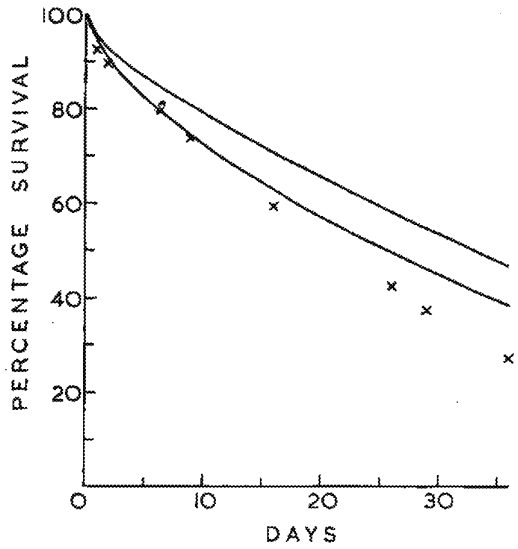

FIGURE 47. To show the effect of comparing abnormal Cr survival results with two different normal Cr survival curves. The crosses record observed Cr survival in a patient with a mild haemolytic anaemia. The continuous lines show normal Cr survival curves when $T_{\frac{1}{2}}$Cr is 25.5 days (lower curve) and when $T_{\frac{1}{2}}$Cr is 32.5 days (upper curve). If the crosses are compared with the lower normal Cr survival curve it appears that survival is completely normal for the first 10 days and departs significantly from normal only after about 20 days. On the other hand if the observations are compared with the upper normal curve they are clearly abnormal throughout (from the data of Mollison, 1961).

through the point of approximately 90 per cent survival rather than 100 per cent (see Fig. 48).

The reason why the present author (Mollison, 1959a, 1960) had previously concluded that 'early loss' is not observed in studying red cell survival in haemolytic anaemia must now be described:

Fig. 47 shows some estimates of Cr survival following the injection of the subjects own ^{51}Cr labelled red cells in a case of haemolytic anaemia. The red cells

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were labelled by adding $Na_2{}^{51}CrO_4$ to a sample of whole blood mixed with ACD. If their survival curve is compared with the curve for the survival of normal red cells labelled in this way, they seem to have a normal survival initially but after 15 days their survival falls increasingly below the normal curve. If their survival is compared with the curve for red cells labelled after being washed in citrate, it is seen to fall below the normal curve from the outset.

Fig. 48 shows the same estimates corrected for Cr elution using three different sets of corrections. These corrections have been described in the previous chapter.

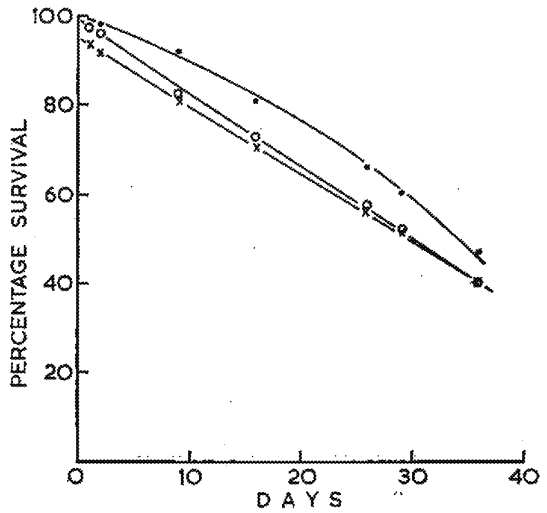

FIGURE 48. Effect of applying three different corrections for Cr elution to the estimates of Cr survival plotted in Fig. 47.
●—● Empirical corrections appropriate when normal $T\frac{1}{2}Cr$ is 25.5 days (see Table VIII).
○—○ Empirical corrections appropriate when normal $T\frac{1}{2}Cr$ is 32.5 days (see Table VIII).
×—× Appropriate corrections when Cr elution is uniform throughout with a half-time of 64 days.
From Mollison (1961).

They are: (1) empirical; namely those correction factors which convert the normal Cr survival curve with a '$T\frac{1}{2}Cr$' of 25.5 days to the true normal survival curve; (2) as (1) but taking the $T\frac{1}{2}Cr$ to be 32.5 days; (3) a correction for uniform elution of Cr with a half period of 64 days.

As Fig. 48 shows, application of correction (1) results in a convex curve suggesting that an over-correction has been applied; corrections (2) and (3) give linear slopes, but correction (3) gives a slope which when extrapolated passes through the point of approximately 90 per cent rather than 100 per cent. Previously (Mollison, 1959a; 1960) the author had been impressed by the fact that

correction (1) gave a convex slope and curve (3), which takes no account of 'early loss', gave a linear slope. The possibility that corrections appropriate to a normal Cr survival curve of 32.5 days might give the best fit was not entertained. On re-examining other curves obtained from patients with haemolytic anaemia it is now apparent that 'early loss' is usually detectable if destruction is not too rapid to mask it (Mollison, 1961). It is not known why corrections appropriate to a normal $T\frac{1}{2}Cr$ of 32.5 days should be applicable when red cells have been labelled by a method which normally gives a $T\frac{1}{2}Cr$ of 25.5 days.

Recommended Procedure

It is suggested that the labelling procedure described in the previous chapter should be followed; that is the red cells are washed in citrate (pH 6.8 to 6.9) before being incubated with ^{51}Cr. The expected per cent Cr survival on various days after re-injecting the labelled cells may be found in Table VIII on p. 162.

When the observed Cr survival deviates only slightly from the expected normal figures, reference to Table X which shows the lower limits of

TABLE X

THE LOWER LIMIT OF NORMAL Cr SURVIVAL (MEAN − 2 S.D.)
(*a*) When $T\frac{1}{2}Cr$ is 25.5 days and (*b*) when $T\frac{1}{2}Cr$ is 32.5 days. The Cr concentration in a sample taken 10 to 20 minutes after injection is taken as 100 per cent

	Cr survival (per cent)	
Day	(*a*)	(*b*)
5	74	76.5
10	66	69
15	59	63
20	52	57.5
25	46	52
30	40.5	47
35	36	42

normal survival (mean—2 S.D.) on certain days after re-injection, may be found helpful.

The coefficient of variation is ±4.5 per cent for method (*a*) and ±6 per cent for method (*b*) (Mollison, 1961). For example at day 30 the mean normal Cr survival by method (*a*) is 44.5 per cent; the coefficient of variation is ±4.5 per cent and the standard deviation therefore ±2 per cent. 95 per cent of normal values should therefore fall within the range 40.5–48.5 per cent.

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Correction for changes in PCV. In determining Cr survival, what is wanted is an estimate of the percentage of Cr remaining in the circulation at any given time as a percentage of the total present immediately after injection. If blood volume remains constant and the ratio: body haematocrit/venous haematocrit (see Chapter II) remains constant, then percentage Cr survival is given directly by the counts in samples of venous blood. Alternatively, if total circulating red cell volume remains constant, then even if plasma volume varies, true Cr survival is given by relating Cr counts to the packed cell volume of each sample; similarly, if PCV varies due to the amount of stasis employed in obtaining blood samples, true survival will still be obtained if Cr survival is expressed as counts per millilitre of red cells.

In subjects who are in a stable state with regard to red cell production and destruction, and in whom it may therefore be assumed that total circulating red cell volume is constant, the PCV of all blood samples should be determined and Cr survival expressed as counts per millilitre of red cells.

When it cannot be assumed that total circulating red cell volume is constant, as for example in a subject who is receiving transfusions, it can usually be assumed that total blood volume remains approximately constant, apart from the 24 hours or so immediately following transfusion. In such subjects, in estimating Cr survival, counts must be expressed in terms of unit volume of whole blood.

In subjects in whom total circulating red cell volume and total blood volume are both changing, as for example in some patients with nephritis, it may be impossible to obtain a reliable estimate of Cr survival without re-estimating total circulating red cell volume. Turnbull *et al.* (1957) made two estimates of RCV (using ^{32}P), one immediately after injecting ^{51}Cr-labelled cells and the other, 2 or 3 weeks later. In twenty-four out of twenty-eight cases there was no change in blood volume; in the remaining four, percentage Cr survival was altered by about 10 per cent by taking the change in blood volume into account. It may be concluded that in practice, when Cr survival is expressed in terms of counts per unit volume whole blood and the assumption is made that total blood volume is constant, misleading results will very seldom be obtained.

Survival in the Patient's Circulation of Normal Transfused Red Cells compared with that of the Patient's Own Cells

There are two reasons why the survival of normal transfused red cells in the patient's circulation might differ from that of the patient's own red cells.

1. *Effect of different age structure of red cell population.* There will be a much greater proportion of older red cells in a population of normal cells than in the patient's own cell population. In a patient with a random haemolytic process, few of the patient's own cells will live to their full potential life-span and except when the haemolytic process is very mild

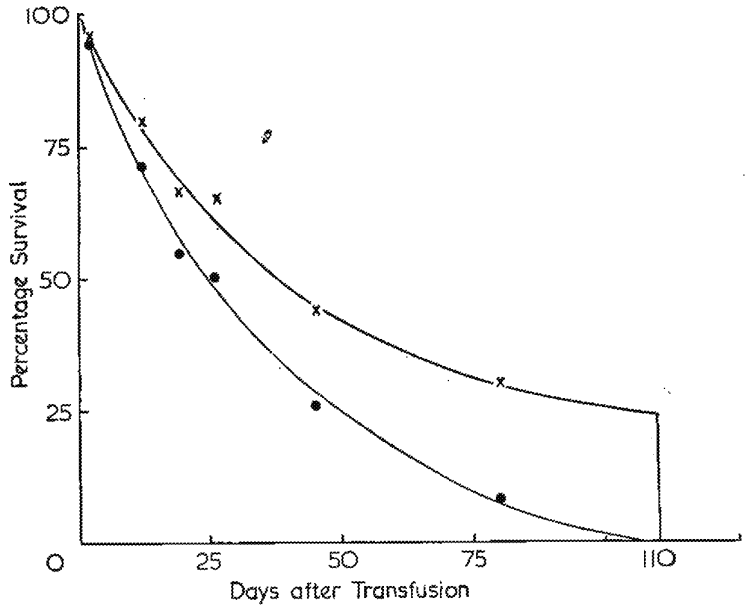

FIGURE 49. Survival of transfused red cells estimated by the method of differential agglutination in a patient with mild acquired haemolytic anaemia. The actual observations are shown as solid dots (lower line). The observations have been corrected for loss of cells by ageing by multiplying each observation by $\frac{110}{110-t}$, where t is the number of days since transfusion; the resulting points are shown as crosses (upper line). The area under the upper curve between 0 and 110 days is divided by the initial height of the curve to give an estimate of the mean cell life of the patient's own cells; in the present case this is found to be 51 days (based on Dornhorst, 1951).

the fraction of red cells dying of old age will be negligible. By contrast, when normal red cells are introduced into the circulation an appreciable proportion will die of senescence. It is true that unless the haemolytic process is very mild this number will be small in relation to the number destroyed by the random haemolytic process. Dornhorst (1951) suggested that the number dying of senescence could be ignored when virtually all

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the transfused red cells were eliminated within 30 days, but that in other cases a correction should be applied if the estimates of the survival of transfused red cells were to be made to yield an estimate of the mean life-span of the patient's own cells.

The correction consists in treating the estimates of survival of the transfused cells so as to eliminate the factor of death by ageing. Assuming the normal mean life-span of red cells to be 110 days, the observed percentage

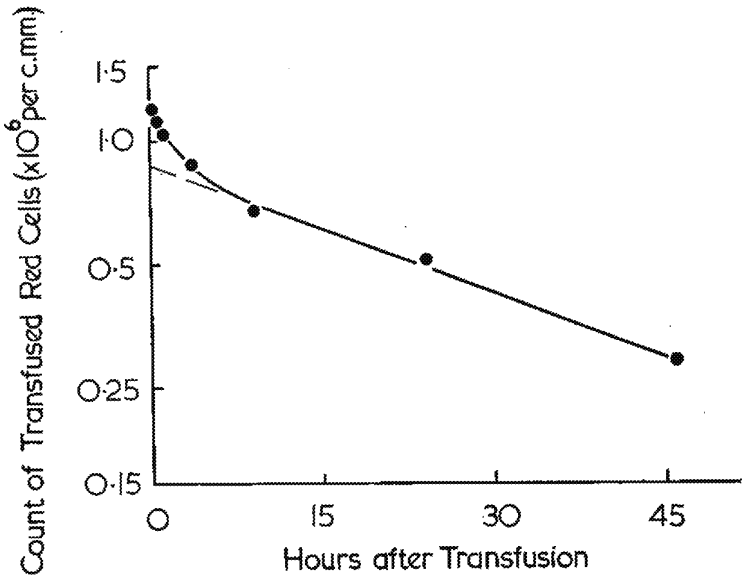

FIGURE 50. Counts of transfused red cells, estimated by the method of differential agglutination, in a very severe case of acquired haemolytic anaemia. The transfusion of 1000 ml. of a packed cell suspension took 1½ hours. Five minutes later the count was 1,200,000 transfused cells per c.mm; mean cell life, calculated as the time taken for the count to fall to 37 per cent of this figure, is 28 hours. If the observations in the first nine hours are ignored, the slope extrapolates back to 900,000 per c.mm. at zero time; 37 per cent of this figure is reached at 39 hours (from Mollison, 1947).

survival of transfused cells after t days is multiplied by $\frac{110}{110-t}$. The corrected estimates are now plotted on squared graph paper and the area under the curve between 0 and 110 days is measured and divided by the initial height to give the mean cell life (Dornhorst, 1951; see also Fig. 49).

It must be emphasized that this method of treating the data is never applicable when the survival of red cells in their own circulation is being studied. It is applicable only when the survival of red cells from a normal donor is being studied in the circulation of a patient with random red cell destruction.

2. *Effect of varying sensitivity of red cells to destruction.* It is sometimes found that the survival of a population of the patient's own red cells is distinctly better than that of a population of transfused normal red cells; that is to say, the difference is greater than can be accounted for by allowing for additional death by ageing in the transfused population. One possible explanation for this difference may be that red cells vary con-

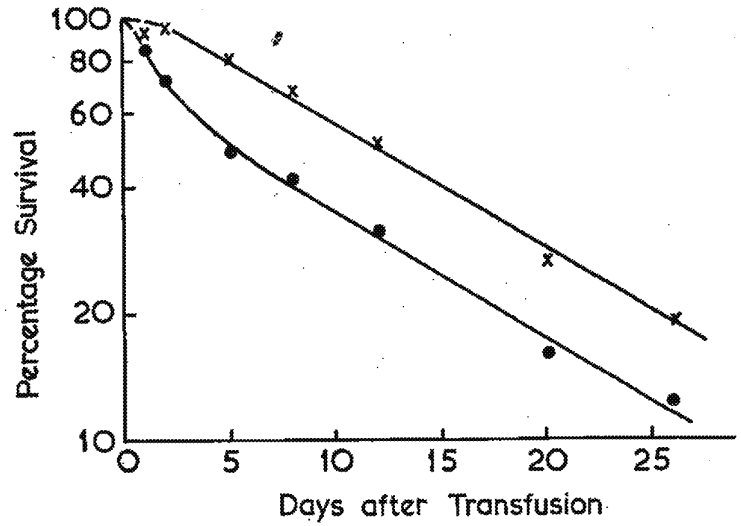

FIGURE 51. Survival of patient's own cells (×) and of transfused cells from a normal donor (●), in patient with acquired haemolytic anaemia. Survival of patient's own cells was estimated by labelling with ^{51}Cr and correcting estimates for a uniform rate of Cr elution. The survival of the transfused cells was estimated by the method of differential agglutination (from Hughes Jones and Mollison, 1956).

siderably in their susceptibility to destruction; this seems reasonably likely since a variation in susceptibility can sometimes be demonstrated when the destruction of red cells by blood-group antibodies is investigated (see Chapter XIII). If some red cells are more resistant to destruction the patient's circulation must contain an undue proportion of resistant cells and these will clearly survive better than an unselected population from a normal donor.

Whatever the explanation, it is a fact that if the survival of a population

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of normal transfused red cells is estimated in a patient with acquired haemolytic anaemia there may be an initial more rapid phase of elimination (see Figs. 50 and 51) whereas the survival curve of the patient's own red cells is substantially linear (see Fig. 51). Similar observations were reported by Hagen *et al.* (1954) in three patients.

Red Cell Survival in Auto-Immune Haemolytic Anaemia

Warm-antibody Type of Acquired Haemolytic Anaemia

As described in Chapter XI in the warm antibody type of acquired haemolytic anaemia the antibody bound to the red cells may exhibit some degree of blood-group specificity. Often this amounts to no more than a tendency to react more strongly with red cells containing a particular antigen; for example it is common for the antibody to react more strongly with e-positive cells than with e-negative cells (Dacie and Cutbush, 1954).

In such a case, in which the antibody reacts with red cells of all groups and merely reacts more strongly with e-positive than with e-negative cells, there may be no difference between the survival of the two types of red cell (see Mollison, 1956, p. 164).

When the antibody exhibits a more definite specificity the group of the donor may strongly influence the survival. Thus in a case reported by Holländer (1954) a patient whose cells were D-positive had anti-D bound to his red cells. When D-positive cells were transfused, 50 per cent were eliminated in one day but when D-negative cells were transfused 50 per cent were still left after 11 days.

In comparing the survival of two types of red cells if only one 'label' is available (e.g. ^{51}Cr) the tests must be made consecutively. If the 'incompatible' cells are injected first and the 'compatible' cells later, the better survival of the 'compatible' cells may be due to the institution of treatment with steroids or to a spontaneous remission. For this reason many other reported cases cannot be regarded as absolutely conclusive (e.g. Crowley and Bouroncle, 1956; Wiener, Gordon and Russow, 1957; Mollison, 1959d).

The investigation can be made really decisive by using a second label so that the survival of two populations can be followed simultaneously. Thus a sample of the patient's own red cells may be labelled with ^{51}Cr and at the same time as these are injected the patient can be given a transfusion of 'compatible' cells whose survival can be followed by differential agglutination. Fig. 52 shows a case in which this was done and there can be no doubt that e— cells survived far better than the patient's own (e+ cells).

In selecting donors for patients with a.h.a. it may be worth preparing an eluate from the patient's red cells and using this to investigate the specificity of the antibody. Thus in Holländer's case reported above in

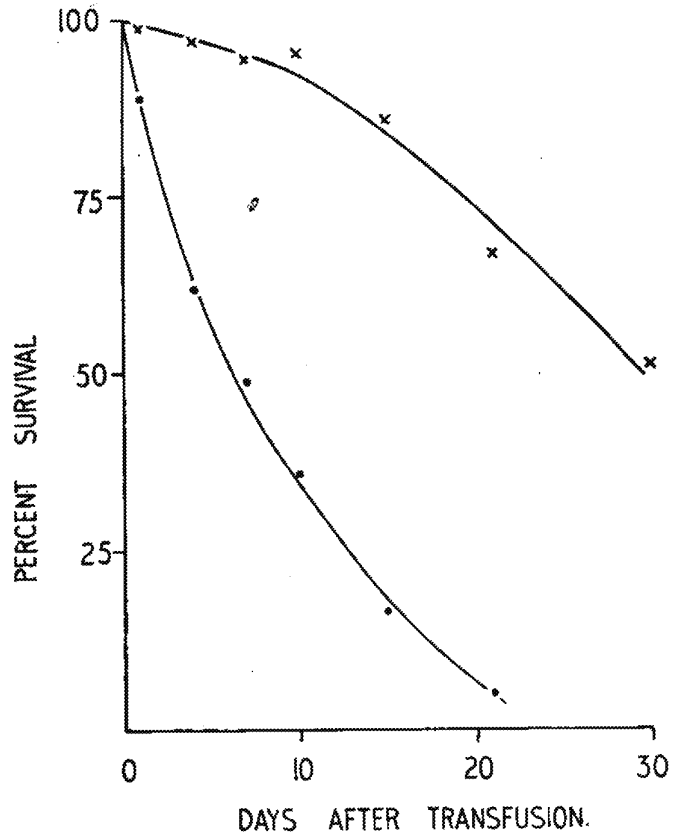

FIGURE 52. Survival of patient's own (e+) red cells, labelled with ^{51}Cr (●—●) and of transfused, e—, red cells, estimated by the method of differential agglutination (×—×) in a patient with auto-immune haemolytic anaemia. The patient's serum contained an auto-antibody with the specificity anti-e (from Mollison, 1959a).

which D— red cells survived well and D+ cells survived badly anti-D was found in an eluate from the patient's red cells but could not be demonstrated free in the patient's serum.

There is some evidence that the amount of antibody on the red cells in

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the warm antibody type of a.h.a. is related to the intensity of the haemolytic process. Thus Fudenberg *et al.* (1958) prepared eluates from the red cells of various cases and estimated the amount of antibody by a test involving inhibition of an antiglobulin serum; they found a good relationship between the amount of antibody eluted and the severity of the haemolytic process, estimated by red cell survival *in vivo*.

The Cold Antibody Type of a.h.a.

It is now realized that the potent cold antibody observed in these patients does display some blood group specificity (usually anti-I). Many patients with cold auto-antibodies do not have a severe haemolytic process and transfusions are not often called for. As yet no studies have been reported on the survival of I-negative (i) red cells but it would certainly be expected that they would undergo little or no destruction.

It has sometimes been stated that blood should be warmed before being transfused to a patient with potent cold agglutinins, but there is good reason to believe that this will not affect the issue. Certainly when patients have potent specific cold agglutinins like anti-P_1 or anti-M in their serum and incompatible red cells are injected, the destruction produced is the same whether the red cells are injected as a cold suspension or a warm suspension (see Chapter XIII). In the previous edition of this book (Mollison, 1956, p. 166) the author gave details of a single experiment in which labelled red cells from a patient whose serum contained a potent cold auto-agglutinin, were injected back into the patient's circulation as a warm suspension and a cold suspension (two different labels); in neither case could red cell destruction be detected during the hour following injection.

Lewis, Dacie and Szur (1960) showed that if the red cells of a patient whose serum contained a potent auto-antibody, were left at 4° C. for 15 minutes with 5 volumes of their own heat-inactivated serum and then rewarmed, to reverse the intense auto-agglutination which had occurred, the cells survived normally. Even when the red cells were left at 4° C. with 5 volumes of their own fresh unheated serum, before being warmed and re-injected, there was no impairment of survival, although there was some trapping of red cells in the liver for an hour or so after injection.

Survival of 'a.h.a.' Red Cells in Normal Recipients

Red cells 'coated' with antibody are not necessarily destroyed at an increased rate. Thus Loutit and Mollison (1946) found that red cells from patients with acquired haemolytic anaemia survived normally when

transfused to normal recipients (see Fig. 53); all four patients who acted as donors were either in remission or not very severely affected. Similarly, if Rh-positive red cells are incubated with moderate amounts of Rh antibody and then transfused to Rh-positive subjects, survival is found to be approximately normal, even though the red cells give a positive direct antiglobulin test for many weeks after transfusion (Mollison and Paterson, 1949).

As described in Chapter XIII studies made subsequently have shown that the rate of destruction of sensitized red cells depends upon the degree

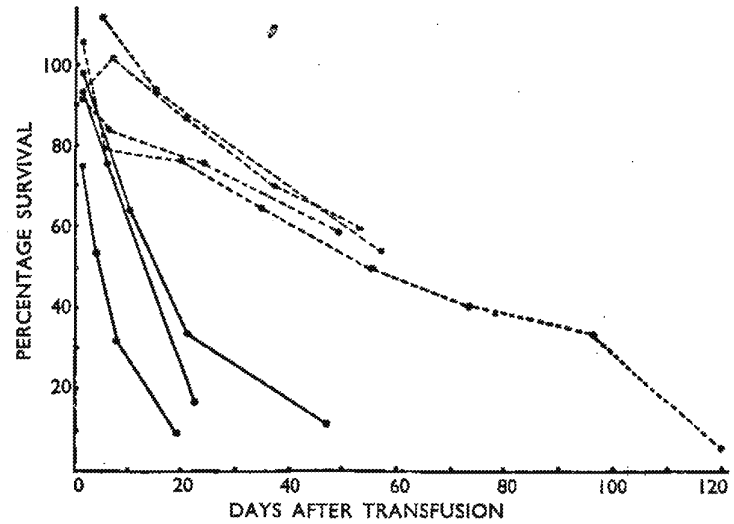

FIGURE 53. Survival in normal recipients of transfused red cells from four patients with mild acquired haemolytic anaemia (broken lines) and from three patients with hereditary spherocytosis (continuous lines) (from Loutit and Mollison, 1946).

of sensitization, that is upon the amount of antibody adsorbed on to the red cells. Moreover when sensitized red cells are injected into the circulation of a normal subject, after a few hours there is a considerable slowing in the rate of destruction of surviving cells due to the fact that the antibody, which was originally bound only to the injected cells, becomes redistributed among all the cells in the circulation so that the load of antibody on the injected cells is greatly reduced. As expected, if red cells are taken from a patient with severe haemolytic anaemia and transfused to subjects without a haemolytic process, some of the red cells are rapidly destroyed.

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Thus Owren (1949) and Selwyn and Hackett (1949) carried out such an experiment and found that 50 per cent of the cells were destroyed in 3 to 4 days but that the rest survived normally; similarly Strumia, Taylor *et al.* (1955) found 35 per cent destruction in the first 24 hours and very little further destruction in the next 5 days. Seelemann (1953) took blood from one infant with only moderately severe haemolytic disease of the newborn and injected it into another anaemic infant (without a haemolytic process). Seventy per cent of the cells were destroyed in 24 hours but the remainder were only slowly eliminated over a period of 27 days.

Further information about the pattern of destruction of red cells sensitized *in vitro* with antibody will be found in Chapter XIII.

Haemolytic Anaemias Due to the Operation of Extrinsic Factors other than Auto-Antibodies

Hypersplenism

It appears that when the spleen is considerably enlarged there is usually some increased destruction of red cells; certainly when splenomegaly is induced in animals, for example by implanting percorten (Miescher, 1956a) red cell survival is found to be diminished.

In considering the benefits of splenectomy it is worth remembering that a large proportion of the circulating red cells may be present in an enlarged spleen. Accordingly after splenectomy the patient may be able to maintain a higher PCV without increasing the production of red cells.

In a case investigated by the author, the patient before splenectomy had a circulating red cell volume of 1270 ml. or, in terms of body weight, 19.4 ml./kg. and a PCV of 20.7. Six weeks after the removal of the spleen, which weighed almost 1.5 kg., total circulating red cell volume was 1050 ml. but the patient had lost some weight so that in terms of body weight it was still 19.4 ml./kg. PCV had now risen to 26.3.

McFadzean *et al.* (1958) described the syndrome of 'cryptogenetic splenomegaly'; their findings were as follows: (1) Transfused normal red cells were rapidly destroyed but after splenectomy survived normally; (2) Before splenectomy the patient's own red cells had a very short survival when transfused to a normal recipient, perhaps due to the damaging effect on the red cells of the pathological spleen; (3) After splenectomy the patient's own red cells survived normally when transfused to a splenectomized normal recipient but had a slightly diminished survival in a normal (unsplenectomized) recipient, possibly due to the slight damaging effect of a pathological liver.

A point to remember in carrying out red cell survival tests in patients with gross splenomegaly is that the mixing of injected red cells occurs very slowly; an example of this has been given in Chapter II.

The first sample of blood for estimates of survival should be taken not less than 30 minutes after the injection of the labelled cells.

Leukaemia, Lymphadenoma and Other Reticuloses

Patients with leukaemia, particularly with chronic lymphatic leukaemia, may develop severe haemolytic anaemia (for references, see Dacie, 1954b, p. 332). Some diminution in red cell survival is in fact observed in many cases of chronic leukemia, both of the myeloid and lymphatic types (Berlin, 1951; Hagen and Watson, 1950; Brown *et al.*, 1951; Berlin *et al.*, 1954).

Patients with lymphadenoma also occasionally develop severe haemolytic anaemia but more often show a moderate reduction in red cell life-span. In five patients studied by Giannopoulos and Bergsagel (1959) the $T\frac{1}{2}Cr$ varied from 9.5 to 22.0 days with a mean of 16.7 days (corresponding approximately to an average mean cell life of 30 days).

Carcinomatosis

Transfused red cells usually survive normally in patients with localized carcinoma (Mollison, 1947). In patients with leucoerythroblastic anaemia associated with malignant disease, transfused red cells also usually survive normally and the patient's cells (labelled with ^{51}Cr) are found to have a normal life-span (Johnston, 1956), although in patients with widely disseminated malignant disease, increased haemolysis can quite frequently be demonstrated (Hyman, 1954).

Increased red cell destruction following irradiation was reported by Sheets *et al.* (1954) who considered that increased red cell destruction began 7–10 days after X-ray treatment for carcinoma of the cervix. Nevertheless, Davis *et al.* (1950) concluded that there was no conclusive evidence that irradiation caused red cell destruction, certainly any effects observed could hardly be ascribed to direct damage to red cells as Dr M. Ebert (personal communication, 1956) has shown that doses up to 100,000 r. do not impair red cell glycolysis.

Rheumatic Fever, Rheumatoid Arthritis, Disseminated Lupus Erythematosus

Reinhold (1954) found that the rate of disappearance of transfused red cells was increased during exacerbations of rheumatic fever. No studies of the survival of the patient's own cells in his own circulation have been reported.

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In rheumatoid arthritis the patient's own red cells apparently have a normal survival (Ebaugh, 1958). In forty-six out of fifty cases studied by Lewis and Porter (1960) the survival of the patient's own red cells labelled with ^{51}Cr was within normal limits. In these patients the daily blood loss in the stools was measured and found to be usually less than 4 ml. a day; in one or two patients it was as much as 15 ml. a day but even then there was little effect on the red cell survival curve.

It should perhaps be pointed out that the ^{51}Cr method is scarcely capable of detecting a small random loss of red cells from the circulation, mainly because the rate of elution of Cr from red cells varies from one subject to another and is influenced by the conditions under which the red cells are labelled (see Chapter V) and, for all we know at present, may be affected by minor changes in the red cells or their environment. It is certainly possible to conclude that there is no gross shortening of the survival of the patient's own red cells in rheumatoid arthritis but it does not seem possible to exclude a minor degree of shortening on the evidence at present available.

By contrast with this finding many observers have found that the survival of transfused normal red cells is reduced in patients with rheumatoid arthritis. Mollison and Paterson (1949) studied one patient in whom mean cell life was of the order of 25 days. McCrea (1957) found increased destruction of transfused red cells in ten out of fifteen acute cases of rheumatoid arthritis; in only two of the patients was the reticulocyte count above 2 per cent. In all patients the direct antiglobulin test was negative. When clinical improvement was sustained or disease activity was of moderate degree only, red cell life-span was little, if at all, diminished. Alexander *et al.* (1956) found that transfused normal red cells had a diminished survival in eighteen out of eighteen patients who were in an active phase of the disease, with a raised sedimentation rate and anaemia.

Freireich *et al.* (1957) also found an increased rate of destruction of transfused cells, although the increase, that is the random loss superimposed on the normal loss of cells through ageing, amounted only to 0.14 to 1.44 per cent per day.

Alexander *et al.* (1956) found that red cells from 'rheumatoid' donors survived better than red cells from healthy donors, when transfused to other patients with rheumatoid arthritis — an observation which has not yet been explained.

Stoeber and Kölle (1960) found that the survival of normal transfused red cells was diminished in seventeen out of twenty-five children with rheumatoid arthritis and estimated that red cell life-span in these cases was

18 to 34 days. In patients receiving steroids, a normal life-span of transfused red cells was observed but only when steroids were given in much higher doses than was necessary for the treatment of arthritis.

In conclusion, it seems to be established that in patients with rheumatoid arthritis, 'foreign' red cells have a diminished survival, a phenomenon which may prove to be linked to that of 'incompatibility without demonstrable antibodies', see Chapter XIII.

In disseminated lupus erythematosus it is not uncommon to find a weakly positive direct antiglobulin test and an increased rate of red cell destruction. Thus in three cases investigated by the author, mean red cell life estimated from the survival of normal transfused red cells varied between 40 and 70 days; these three patients were moderately anaemic (packed cell volume 26-33 per cent) and their reticulocyte counts were between 2 and 4 per cent.

Nephritis; Pre-Eclamptic Toxaemia

In patients with chronic nephritis in whom the disease is not obviously progressing, and in whom there are no retinal changes, transfused red cells have been observed to survive normally (Chaplin and Mollison, 1953). In such patients during phases in which the degree of anaemia is constant, red cell production can be deduced from the expression:

$$\frac{\text{Observed packed cell volume}}{\text{Normal packed cell volume}}$$

In three patients studied by Chaplin and Mollison, red cell production was estimated to be between 50 and 65 per cent of normal.

In patients with rapidly progressing nephritis, with severe anaemia and with hypertensive retinopathy, transfused red cells are destroyed at an increased rate (Emerson and Burrows, 1949; Loge *et al.*, 1950; Chaplin and Mollison, 1953). Nevertheless, as the above authors stressed, the degree of destruction is usually mild — two to four times normal — and the prominent mechanism in the anaemia of these patients is a depression of erythropoiesis.

In occasional patients the haemolytic process may be much more severe and may then be associated with morphological changes in the red cells; thus Dacie (1954b, p. 365) has described the occurrence of contracted forms of red cells including small triangular cells in films of peripheral blood. In one such case showing 'fragmented' red cells, Shumway and Miller (1957) found that almost all transfused cells were removed within 2 weeks.

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In eclampsia there may be quite severe haemolytic episodes with haemoglobinaemia and haemoglobinuria (Pritchard *et al.*, 1954); in pre-eclamptic toxaemia an increased rate of destruction of transfused red cells has been demonstrated (Chaplin and Mollison, 1953).

Hepatic Cirrhosis

Acute haemolytic episodes have been described in this condition (see Hyman and Southworth, 1951). In patients with advanced liver disease but with no definite evidence of a haemolytic process, as judged by a routine blood examination, red cell survival was found to be reduced to 25-55 days as judged by the survival of transfused red cells (Chaplin and Mollison, 1953).

In various series in which the patient's own red cells were labelled with ^{51}Cr a moderate reduction of life-span was found (Allen *et al.* 1957; Sheehy and Berman, 1960; Cawein *et al.*, 1960).

Infections with Protozoa, Bacteria and Viruses

Malaria may cause mild chronic red cell destruction and episodes of severe intravascular destruction (blackwater fever); in these latter episodes normal transfused red cells, as well as the patient's own cells, are destroyed (Foy, Kondi and Moumjidis, 1941; Mollison, 1947).

It is well recognized that severe red cell destruction occurs in *Clostridium welchii* septicaemia; rapid destruction of transfused cells has been observed in other severe bacterial infections (Brown *et al.*, 1944; Mollison, 1947).

Haemolytic anaemia may complicate various virus infections, particularly virus pneumonia and infectious mononucleosis. In virus pneumonia the patient's serum commonly contains potent cold auto-agglutinins, although haemolytic anaemia only rarely develops. In infectious mononucleosis, auto-agglutinins are rarely demonstrable.

The possible role that infections may play in causing red cell destruction is illustrated by the experiments of Ceppellini and de Gregorio (1953) who sensitized rabbit red cells *in vitro* with Vi antigen. On injection into rabbits previously immunized against the *Salmonella* organism the cells were very rapidly destroyed, whereas in non-immunized rabbits the treated cells survived normally.

Scurvy

In scurvy, transfused red cells are rapidly removed from the circulation, apparently not by haemorrhage (Merskey, 1953). In two cases investigated by Dr A. Goldberg (personal communication) the subject's own red cells

were labelled with ^{51}Cr while the subjects were on a scorbutic diet; $T\frac{1}{2}$Cr was considerably reduced and it was shown that this was not due to loss of Cr (i.e. labelled red cells) in the urine or stools. Red cells taken from one of these subjects were transfused to a normal recipient and shown to survive normally. After the administration of ascorbic acid to these two patients, $T\frac{1}{2}$Cr was re-estimated and found to be normal.

Hyperthyroidism

In two patients with marked hyperthyroidism McClellan *et al.* (1958) found the $T\frac{1}{2}$Cr of the patient's own red cells to be 18 days, compared with 39 to 48 days in normal subjects.

Overdosage with Phenacetin

In patients taking large amounts of phenacetin, red cell survival time, as judged by labelling the patient's own red cells with ^{51}Cr, is reduced (Miescher and Pletscher, 1958). The reduction is much more striking in patients who also have renal insufficiency (Friis *et al.*, 1960a); in these, transfused red cells are destroyed as rapidly as the patient's own red cells; the administration of prednisolone prolongs red cell survival (Friis *et al.*, 1960b).

MISCELLANEOUS TOPICS

Random Loss of Red Cells due to Chronic Haemorrhage

Loss of blood in the stools. In patients with a chronic blood disease with a low platelet count, poor survival of transfused red cells may be due not to haemolysis but to chronic bleeding into the gastro-intestinal tract. If ^{51}Cr labelled red cells are injected into the circulation, the amount of blood lost in the stools can be measured by estimating their ^{51}Cr content (Roche *et al.*, 1957; Hughes Jones, 1958a). Correction for blood loss can then be applied so as to discover whether the survival curve, corrected in this way, is normal. Incidentally, if it is suspected that the amount of blood lost in the stools is only small, before embarking upon the injection of ^{51}Cr labelled red cells it is worth making a quantitative estimate of the amount of blood in the stools by the modified benzidine test described by Ebaugh and Beekin (1959). According to Hughes Jones (1958a) the normal daily loss of blood in the stools is about 0.5 ml. (or 0.2 ml. of red cells); this figure is a little lower than that of Ebaugh and Beekin, who estimated the daily loss as approximately 2 ml. of whole blood.

Loss of blood by venous sampling. Corrections are also needed if substantial amounts of blood are withdrawn during the course of an estimate of red

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cell survival time. It can be shown that when the amount of blood lost from the circulation is x per cent of the blood volume, the corrected survival is given by:

$$(1)\ \text{Observed survival} \times \frac{100}{(100-x)}$$

This is the appropriate correction whatever the percentage of surviving cells at the time the sample is taken. Thus, assuming a normal life-span of 100 days, consider the effect of withdrawing a tenth of the blood volume firstly on the tenth day after transfusion and secondly on the thirtieth day.

In the first case, after the restoration of blood volume, percentage survival will fall from 90 to 81 and by the fiftieth day will be $\frac{50}{90} \times 81 = 45$.

In the second case (withdrawal of blood on the thirtieth day), percentage survival will fall from 70 to 63 after blood volume is restored and by the fiftieth day will be $\frac{50}{70} \times 73 = 45$.

Thus for each substantial withdrawal of blood during the course of a survival experiment the correction (1) should be applied.

Example: Suppose ^{51}Cr labelled cells are injected into a subject whose blood volume is 4500 ml. By the twentieth day after injection, ten samples each of 15 ml. (i.e. total 150 ml. or 3.33 per cent of the blood volume) have been withdrawn. The observed ^{51}Cr survival is 55 per cent; corrected survival is:

$$55 \times \left[\frac{100}{(100-3.33)}\right] = 57$$

Similarly, to correct for loss of blood in the stools, the first step is to calculate the percentage of the blood volume which is lost in each stool. Suppose the subject's red cells have been labelled with 50 μc. of ^{51}Cr and on the tenth day, when the percentage survival in the blood is 72, a melaena stool is passed containing 3 μc. of ^{51}Cr. Since the total amount of ^{51}Cr in the blood at the time is $\frac{72}{100} \times 50$, or 36 μc. (after correction for radioactive decay), the loss in the stool represents $\frac{1}{12}$ or 8.5 per cent of the blood volume. Subsequent estimates of ^{51}Cr survival in the blood must therefore be corrected by $\frac{100}{(100-8.5)}$.

Surface Counting in Haemolytic Anaemias

This subject may seem rather far removed from blood transfusion but in fact there are instances when it becomes highly relevant. The spleen is evidently highly specialized for removing certain kinds of altered red cells

from the blood stream and in some haemolytic anaemias it plays a predominant role in red cell destruction (Korst *et al.*, 1955; Jandl *et al.*, 1956; Hughes Jones and Szur, 1957; Schloesser *et al.*, 1957). Following splenectomy the transfusion needs of a patient may be drastically reduced, at least for a time. In patients with chronic leukaemia who require frequent transfusions it is worth carrying out surface counting after the injection of ^{51}Cr-labelled cells to see whether the spleen is playing a predominant part in red cell destruction.

In a case investigated by the author, a patient with chronic lymphatic leukaemia remained moderately anaemic despite the transfusion of three bottles of blood every 2 weeks. A ^{51}Cr survival test showed that the mean life-span of the red cells was about 10 days and that excess counts were accumulating over the spleen. Thus, taking as 1000 counts per minute the counting rate over the heart after the injection of labelled cells, the initial count over the spleen was 1350 counts per minute (indicating splenic enlargement). Nine days later the heart count had fallen to 370 and the spleen count was 1280, indicating considerable excess destruction in the spleen (see Hughes Jones and Szur, 1957). After splenectomy, the patient needed no further transfusions for 10 weeks and for the following 18 months was maintained with monthly transfusions. Thereafter his condition deteriorated.

In patients with splenomegaly, the rate of mixing of red cells in the circulation may be a useful guide to the amount of splenic 'sequestration' which is taking place. Harris *et al.* (1958) found that in normal subjects the mixing of injected cells in the splenic circulation, as determined by surface counting, could be described by a single exponential. In some subjects with an enlarged spleen when there was no evidence of splenic sequestration the mixing could still be described by the single exponential, although the rate was slower. In some subjects splenic mixing could be described by a two-component curve, the slow component having $T\frac{1}{2}$ of 30-70 minutes; in some of these subjects splenic sequestration was demonstrated subsequently.

^{51}Cr 'dose' in surface-counting measurements. Although it is convenient to use a dose of 100 μc. of ^{51}Cr to label red cells when surface counting is to be carried out, this dose may result in excessive radiation to the spleen if the majority of the red cells are concentrated there. Jandl *et al.* (1956) calculated that if 100 μc. of ^{51}Cr were accumulated entirely by a 200 g. spleen the exposure would amount to approximately 16 rep. which is above the 'maximum permissible level'. They recommended that in subjects with haemolytic anaemia in which there was a possibility that a large proportion of the labelled red cells would be destroyed in the spleen the amount of ^{51}Cr used should be restricted to 50 μc.

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Estimates of Red Cell Production from Measurements of the Mean Life-span of Red Cells

When the patient's red cell volume is stable red cell production must be equal to red cell destruction. For example in a subject who maintains a normal haemoglobin concentration (and red cell volume) despite having a mean red cell life-span of only 30 days, production must be four times normal (assuming the normal red cell life-span to be 120 days).

A subject, for example one with chronic nephritis, who maintains a PCV of only 50 per cent of normal (PCV 22 per cent) despite having a normal red cell life-span must have a red cell production which is only 50 per cent of normal.

Thus in a stable state red cell production is given by:

$$\frac{\text{Normal red cell life-span}}{\text{Observed red cell life-span}} \times \frac{\text{Observed PCV}}{\text{Normal PCV}}$$

Crosby and Akeroyd (1952) concluded that in chronic haemolytic disease anaemia usually develops when the mean life-span of the red cells is less than 15-20 days. From their own data and from published observations, they concluded that in chronic haemolytic disease, red cell production is usually 6-8 times normal.

The present author has pointed out that output may reach 10-12 times normal (Mollison, 1956, p. 149). Thus, in a patient with PNH (see Fig. 45) the average red cell life-span determined on three different occasions was 4.5 days. Without transfusion the patient maintained a PCV of 21 per cent. Thus, taking the normal life-span to be 115 days and the normal PCV to be 42, production was $\frac{115}{4.5} \times \frac{21}{42}$ or about 12 times normal, which may represent the maximum rate of erythropoiesis.

CHAPTER SEVEN

BLOOD GROUPS: GENERAL CONSIDERATIONS

The Term: Blood Groups

The term 'blood group' has several possible meanings. It may be used specifically for a person's ABO phenotype, as in the expression 'blood group A'; or it may be used for any of the other systems of red cell antigens whose inheritance is independent; thus one speaks of the Rh blood group system or of blood group incompatibility due to anti-K, etc. Some people would like to see the term 'blood groups' extended to include not only red cell factors but inherited serum factors like the Gm groups. A further step would be to include inherited differences in the blood of individuals of a species which are not identifiable serologically, for example the high and low potassium content of the red cells of different sheep.

Although all inherited differences in the blood may fall within the widest meaning of the term blood groups, in this book the term is usually used simply for red cell antigens. When the inheritance of a system of antigens is independent of another system the antigens are said to belong to a particular blood group system.

Discovery of Blood Groups

Antigenic differences between different species were recognized before differences within a species. Thus Landois (1875, p. 185) discovered that when the red cells of an animal, for example, a lamb, were mixed with the serum of another animal (a dog) and incubated at 37° C. they might be lysed within 2 minutes. Serological differences between the blood of different members of the same species were discovered a quarter of a century later. Thus Ehrlich and Morgenroth (1900) found that by injecting red cells from one goat into another they could provoke the formation of haemolytic antibodies, 'iso-haemolysins'; and Landsteiner (1900; 1901) found that in humans there were naturally-occurring antibodies — 'iso-agglutinins' — which would react with the red cells of certain other human subjects.

The way in which the various human blood group systems have been discovered is summarized in Table XI. After the discovery of the ABO system in 1901 no new blood group systems were found for 25 years.

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Landsteiner and Witt (1926) examined human sera for antibodies other than anti-A and anti-B but could find only weak agglutinins active at low temperatures. It occurred to Landsteiner and Levine that they might be able to reveal other antigens in human red cells by making immune antibodies against them in animals, and accordingly they carried out experiments in which several different samples of human red cells were injected into rabbits. Antibodies identifying three new human antigens were obtained (Landsteiner and Levine, 1928), and to the first of these the letter M was given to indicate that the antigen had been identified with immune serum ('I' was avoided because of confusion with the numeral 1, Levine, 1944).

Landsteiner and Wiener (1940; 1941) adopted a slightly different approach when they injected the red cells of rhesus monkeys into rabbits

TABLE XI

SUBJECTS IN WHICH THE ANTIBODIES OF THE ELEVEN MAIN BLOOD GROUP SYSTEMS WERE FIRST FOUND

*Blood group systems**	*Antibodies first found in:*
ABO, Le	Normal subjects
MN, P	Rabbits injected with human red cells
Rh	Rabbits injected with rhesus monkey red cells
Rh, K, Jk, Di	Mothers of infants with haemolytic disease
Lu, Fy, Js	Transfused patients

* The terminology used in describing the various blood group systems is discussed later in this chapter.

and tested the resulting serum against human red cells. They hoped that in this way they might reveal the existence of an antigen present both in *rhesus* cells and in human cells, and they were successful. At about the same time Levine and his colleagues were making their highly important discoveries relating to iso-immunization in pregnancy. Levine and Stetson (1939) had found an antibody (which subsequently proved to be anti-Rh) in the serum of a recently delivered woman whose foetus had died *in utero*. They discussed the possibility that the mother had been immunized by the cells of her foetus which had inherited from the father an antigen which was foreign to the mother. This was the beginning of the fascinating work which led to the discovery that iso-immunization in pregnancy is the cause of haemolytic disease of the newborn (Levine *et al.*, 1941).

Of the other blood group systems most have been discovered by finding immune antibodies either in the mothers of infants with haemolytic disease or in patients who have received multiple transfusions.

Inheritance of Blood Group Antigens

The presence of antigens in the red cells is determined by genes, which are carried on the chromosomes. In all the cells in the body except the sex cells chromosomes are present in pairs, and the genes controlling the inheritance of any particular series of blood group antigens are conceived as lying opposite one another, so that the effects are the result of the action of two sets of genes lying on the two members of a pair of chromosomes. When the sex cells are formed, each cell receives only a single set of chromosomes, that is to say, half the number of genes possessed by the body cells.

TABLE XII

PHENOTYPES AND GENOTYPES, ILLUSTRATED BY THE ABO SYSTEM (IGNORING SUBGROUPS)

Phenotypes	*Genotypes**
AB	*AB*
A	*AA* or *AO*
B	*BB* or *BO*
O	*OO*

* It is customary to denote genes by italic letters.

In most cases no distinction need be made between genes and antigens, so that if a person inherits certain genes the antigen can be detected in his red cells. The main exceptions to this rule are provided by cases in which the product of one gene is suppressed or modified by a gene carried on a different chromosome. Examples of both of these exceptions are described in the Chapter on the ABO and Lewis groups.

Some genes do not produce any recognizable effects, and are termed amorphs. For example, ignoring the question of sub-groups, the inheritance of the ABO groups is thought to depend upon the genes *A*, *B* and *O*. *A* and *B* give rise to corresponding antigens, but *O* is probably an amorph, for no product can be recognized. Thus, although there are six possible genotypes, only four phenotypes can be recognized (see Table XII). (The term genotype is used for the sum of inherited genes whereas phenotype refers only to recognizable characteristics.)

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Gene Frequencies

By gene frequency is meant simply the frequency of a particular gene in the population as a whole. For example, in calculating the frequency of the *O* gene one would have to count not only people of group O with a double dose of the gene but also those people of group A or B whose genotype is *AO* or *BO*. A knowledge of the gene frequencies in a blood group system enables one to calculate certain facts about the system very simply: for example, in Britain the frequencies of the three genes in the ABO system are as follows (Dobson and Ikin, 1946);

O	0.6827
A	0.2573
B	0.0600

The frequency of the combination *OO* must be simply 0.6827×0.6827, or approximately 0.47 — that is to say, 47 per cent, which is in fact the frequency of group O in the British population. Similarly one can calculate the frequency of the genotype *AB* as follows: $((0.2573 \times 0.0600) \times 2)$. The '$\times 2$' is accounted for by the fact that *A* can combine with *B*, and *B* with *A*. The above sum comes to approximately 0.03, or 3 per cent, which is the proportion of group AB people in the British population.

In this book, unless otherwise stated, gene frequencies and phenotype frequencies refer to the British Isles. Frequencies for all parts of the world are given by Mourant (1954).

Independent Inheritance of Different Blood Group Systems

The most direct way of showing that one system of antigens is inherited separately from another is to study their inheritance in a suitable family.

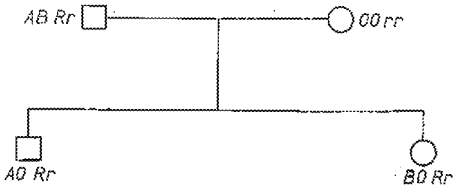

FIG. 54. Separate inheritance of the ABO and Rh genes. In this example, the father passes on *R* to both children but he passes on *A* to one child and *B* to the other.

Thus, Fig. 54 illustrates the separate inheritance of the ABO and Rh antigens.

The group O, Rh negative mother must have the genotype *OOrr*, and must pass on an *O* gene and an *r* gene to all of her offspring. On the other hand the father can pass on *A* or *B* and *R* or *r*. If these genes were all part of one system they would (with very rare exceptions) be passed on in sets, e.g. *A* would always go with *R* and *B* with *r*. The fact that in this particular family *R* has gone with *A* in one case and with *B* in another illustrates the independent inheritance of the ABO and Rh systems of antigens.

Linkage between Blood Group Genes and Other Genes

Genes are said to be linked when they are situated very close to one another on the same chromosome so that they are usually inherited together, that is to say that the two genes stay on the same piece of chromosome when the chromosome material divides.

Blood group genes are potentially very useful gene markers. For instance, if a particular blood group gene were linked with the locus on the X chromosome which determines haemophilia, it would often be possible in a family to distinguish carriers of haemophilia from normal women. Although no really 'useful' linkages of this kind are yet known, a beginning has been made by recognizing the following linkages:

(i) *Lu* and *Se* (Mohr, 1951, although originally thought by Mohr to be a linkage between *Lu* and *Le*).
(ii) *Rh* and one locus for elliptocytosis (Chalmers and Lawler, 1953).
(iii) *ABO* and the locus for the nail-patella syndrome (Renwick and Lawler, 1955).

Influence of ABO Group on Disease of the Gastro-Intestinal Tract

The first well-established claim for a relationship was that between group A and carcinoma of the stomach (Aird *et al.*, 1953). If the risk that a subject of group O or group B will get carcinoma of the stomach is taken as 1, the risk for a group A subject is 1.2 (see review by Fraser Roberts, 1957). Pernicious anaemia, too, occurs more frequently in group A subjects than in group O (Collective Series, 1956).

An even more definite association is that between group O and duodenal ulceration so that if the chance that a person of group A, B or AB will get a duodenal ulceration is taken as 1, the chance for a group O person is approximately 1.4 (Fraser Roberts, 1959). There is an even closer association between 'non-secretors' (i.e. those of the genotype *sese*) and duodenal ulceration (Clarke *et al.*, 1959).

The association of ABO blood groups with diseases of the upper gastro-intestinal tract suggests the possibility of some direct effect of the blood group mucoids since they are present not only in the saliva but also in the

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gastric and duodenal mucosa. The studies of Glynn *et al.* (1957b) have shown that the effect of the secretor status appears to be confined to the specificity of the blood group antigens in the superficial epithelium of both stomach and duodenum. Thus in secretors, A and B substances were abundant in the superficial mucosa, but in non-secretors (presumably of the genotype LL or Ll) were replaced by Le^a substance. In the deeper layers A and B substances were present in non-secretors as well as secretors.

Clarke *et al.* (1959) measured the amount of blood group mucoids in the saliva of secretors and non-secretors and found that the amounts were about the same. They therefore concluded that the relation between ABO group and liability to gastro-intestinal disease was most likely to have an immunological basis.

Influence of Disease on the ABO Groups

In some subjects of group A with leukaemia, red cells may be produced which behave as if they belonged to a very weak subgroup of A or even as if they were a mixture of A and O cells (see following Chapter).

Terminology of Blood Groups

Blood group terminology is chaotic. In some systems, e.g. ABO, antigens are given capital letters (e.g. B) or capital letters with subscripts (e.g. A_2). Genes are denoted simply by italics (e.g. A_2); although the genes in the ABO system are all thought to be alleles, that is to say, alternative forms at a single locus, this is not indicated by the nomenclature; (for example, one allele of A_2 is O). In other systems (e.g. P, Kell) the main alleles are denoted respectively by large and small letters, thus: *P* and *p*; *K* and *k*; here the terminology indicates that the alleles are alternative forms. The MNSs system follows a blend of these two systems of nomenclature, the first alleles to be described being called M and N, and the next pair S and s. The Lutheran, Lewis, Duffy, Kidd, Diego and Sutter systems follow a uniform pattern. The system is denoted by a capital letter and a small letter, thus: Lu, Le, Fy, Di and Js; genes are denoted by superscripts, e.g. Lu^a; phenotypes are written Lu(a+b−), and antibodies thus: anti-Lu^a. This leaves the Rh system, for which there are two entirely different systems of nomenclature, as described in Chapter IX.

BLOOD GROUP ANTIBODIES

Antibodies are often divided into 'naturally-occurring', that is, occurring in the serum of subjects who have not received any recognized antigenic

stimulus and 'immune', that is, arising after a known antigenic stimulus. Most sera containing naturally-occurring antibodies are capable of agglutinating red cells suspended in saline and they usually react more strongly at low temperatures (4° C. or 20° C.) than at 37° C. Sera containing immune blood group antibodies are sometimes capable of agglutinating red cells suspended in saline but if so they usually react as strongly at 37° C. as at lower temperatures (Levine *et al.*, 1940).

Immune blood group antibodies are often incapable of agglutinating red cells suspended in saline and they are then termed 'incomplete'. Sera containing incomplete antibodies may agglutinate enzyme-treated red cells suspended in saline or may agglutinate untreated red cells suspended in a suitable colloid medium; almost all incomplete antibodies will sensitize red cells to agglutination by an antiglobulin serum. The principles of these tests are discussed later in this chapter and the tests themselves are described in Chapter XII.

It is generally believed that all antibodies are gamma globulins but some confusion has been caused by different definitions of this term. Gamma globulins were originally defined as the group of proteins migrating most slowly on electrophoresis at pH 8.6. With the introduction of the technique of immuno-electrophoresis, it was realized that 'gamma' globulins are electrophoretically more heterogenous than had previously been suspected.

In the technique of immuno-electrophoresis, serum is first submitted to electrophoresis in a gel; in the next stage, proteins, which are now spread out along the gel according to their electrophoretic mobility, diffuse towards an antiserum which is placed in a trough in the gel. Where the protein and its corresponding antibody meet a line of precipitation forms.

On immuno-electrophoresis the main component (90–95 per cent) of gamma globulin forms a line extending from the extreme gamma end of the 'spectrum' of proteins forwards as far as the region of the alpha globulin. This main component of gamma globulin has a molecular weight of the order of 160,000 and a sedimentation constant of 7*S*, and is often referred to as 7*S* gamma globulin.

High molecular weight gamma globulin (molecular weight about 1,000,000; sedimentation constant 19*S*) migrates on electrophoresis with a mobility intermediate between that of the gamma and beta globulins and was for this reason termed $gamma_1$ globulin by Deutsch *et al.* (1947). On immuno-electrophoresis this protein forms a separate line of precipitation (Scheiddegger, 1955) now termed $beta_{2M}$ (see Burtin, 1960). The term $gamma_1$ globulin is particularly likely to lead to confusion

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since it includes not only 19*S* gamma globulin but also some 7*S* gamma globulin.

Several other proteins (e.g. beta$_{2A}$) can be recognized by immuno-electrophoresis in the gamma globulin region and these, with the foregoing, are sometimes referred to collectively as immuno-globulins (see, for example, Heremans, 1959).

Classification of Blood Group Antibodies as 19S or 7S Gamma Globulins

So far as blood group antibodies are concerned, the most useful distinction to make seems to be on a basis of molecular size. This is because, as described below, naturally-occurring antibodies are usually composed of 19*S* globulin and immune antibodies usually of 7*S* globulin (although they too are sometimes composed of 19*S* globulin). A second important point from a clinical aspect is that the placenta readily transfers 7*S* gamma globulin but not 19*S* gamma globulin (see Chapter XVII).

The fact that 19*S* gamma globulin is antigenically distinct from 7*S* gamma globulin was demonstrated by Franklin and Kunkel (1957) who prepared antibodies against the two proteins in rabbits. Specific anti-19*S* and anti-7*S* sera were used in the antiglobulin test by Polley *et al.* (1961) to determine which incomplete blood-group antibodies were composed of 19*S* gamma globulin and which of 7*S* (see Table XV on p. 242).

Preparations containing predominantly 7*S* or 19*S* gamma globulin can be obtained by ultracentrifugation or by the use of an ion-exchange cellulose column (Sober and Peterson, 1958). These preparations can then be examined for blood-group antibodies. Another approach is to treat whole serum with a sulphydryl compound which breaks the disulphide linkage between the constitutent parts of 19*S* gamma globulin and destroys the serological activity of any antibodies composed of this protein (Deutsch and Morton, 1957; Grubb and Swahn, 1958).

Grubb and Swahn (1958) mixed one volume of a 1 in 5 dilution of serum with an equal volume of 0.2 M-2-mercapto-ethanol and left the mixture at room temperature for 48 hours. As a diluent for the serum they used a phosphate buffer pH 7.4. After 48 hours at room temperature the mixtures were dialysed against phosphate buffer for a further 24 hours before being tested. In the author's laboratory it has been found that treatment of undiluted serum with an equal volume of 0.2 M-2-mercapto-ethanol at 37° C. for 2 hours followed by dialysis overnight against buffered saline, pH 7.3, inactivates all but the most potent 19*S* gamma globulin antibodies.

The fact that there might be a correlation between the serological characteristics of antibodies and their molecular size was demonstrated by

Campbell *et al.* (1955) who examined a serum containing both agglutinating and incomplete anti-Rh and showed that the agglutinating activity was associated with the 'heavy' component, separated in the ultracentrifuge.

It appears that most antibodies which are capable of agglutinating red cells suspended in saline are composed of 19*S* globulin; for example, anti-M, anti-N and anti-P_1, 'saline agglutinating' anti-Rh and some examples of anti-A and anti-B (Fudenberg *et al.*, 1959); examples of naturally-occurring anti-Le^a and anti-P+-P_1 have also been shown to be composed of 19*S* globulin (Kekwick and Mollison, 1961).

High titre cold agglutinins found in the sera of certain patients with acquired haemolytic anaemia are also composed of 19*S* globulin migrating on electrophoresis as 'fast' gamma globulin (Christenson and Dacie, 1957; Fudenberg and Kunkel, 1957). Similarly Grubb and Swahn (1958) showed that the serological activity of anti-A_1, anti-B and anti-Rh saline agglutinins as well as 'non-specific' cold agglutinins was destroyed by treatment with 2-mercapto-ethanol.

Anti-A and anti-B (and some anti-M, see p. 599) seem to be the only 'saline agglutinins' which may be 7*S* globulin. Fudenberg *et al.* (1959) found that some anti-A and anti-B were 19*S*, some were 7*S* and some were mixtures. Fahey and Morrison (1960) examined two sera from group O subjects who were not known to have been immunized with A or B substances and found most of the anti-A and anti-B activity in the 19*S* (18*S*) globulin with only a small amount in the 7*S* (6.6*S*) globulin. Kekwick and Mollison (1961) examined one immune anti-A serum from a group O donor and found that most of the agglutinating activity was associated with 7*S* globulin.

Antibodies composed of 19*S* globulin do not necessarily behave only as saline agglutinins. Thus naturally occurring anti-Le^a, which is a 19*S* antibody, behaves as a saline agglutinin at room temperature but as an incomplete antibody at 37° C. 19*S* antibodies may also be haemolysins, for example anti-Le^a and anti-P+-P_1 (Kekwick and Mollison, 1961), and some examples of anti-A haemolysin (Rawson and Abelson, 1960a).

Immune incomplete antibodies appear to be composed as a rule of 7*S* globulin; for example, instances of incomplete anti-Rh, anti-K and anti-Fy^a (Fudenberg *et al.*, 1959; Kekwick and Mollison, 1961). As expected the serological activity of incomplete anti-Rh is not abolished by treatment with 2-mercapto-ethanol (Grubb and Swahn, 1958). Occasional examples of incomplete anti-Rh and anti-K appear to be composed partly of 19*S* globulin (Kekwick and Mollison, 1961).

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Synthesis of Gamma Globulin in Newborn Infants

In the normal newborn infant the level of gamma globulin (7*S*) in the serum at birth is about the same as that in the mother's serum. Thereafter the level declines because gamma globulin is not normally produced until the infant is a few weeks old. Zak and Good (1959) studied two normal infants born to mothers with hypogammaglobulinaemia and found that the gamma-globulin concentration in the infant's serum which was negligible at the time of birth started to increase between 35 and 42 days after birth in one case and between 20 and 28 days in the other. Antibodies did not appear for a further 18 days, by which time the gamma globulin level had reached 200 mg./100 ml.

The level of 19*S* gamma globulin in the cord serum is only about one-tenth to one-twentieth of that in normal adult serum (Franklin and Kunkel, 1958; Polley *et al.*, 1961). According to Hitzig (1957) the β_{2M} line (i.e. 19*S* gamma globulin) is recognizable on immuno-electrophoresis from about the eighth week of life onwards.

Although gamma-globulin synthesis does not normally occur within the first few weeks of life and it has been considered that the capacity to form antibodies is developed only during the second month after birth (see Osborne *et al.*, 1952), it appears that if an appropriate antigenic stimulus is given, synthesis can occur. Thus Smith (1960) injected TAB vaccine intradermally into thirty-eight premature infants in the first 3 days of life; 2 weeks later twenty-nine of these had formed 'H' agglutinins which were shown to be macroglobulins. Possibly this was different from 19*S* globulin formed by adults because infants' serum treated with 2-mercapto-ethanol retained its serological activity, suggesting that the globulin had been converted into antibody molecules of lower molecular weight which retained their serological activity.

Naturally-occurring Blood Group Antibodies

Of the naturally-occurring blood group antibodies which are active *in vitro* at 37° C., anti-A and anti-B are overwhelmingly the most important, not only because they are relatively potent, but also because the corresponding antigens are very frequent. For example, in the United Kingdom, 45 per cent of random blood samples contain the antigen A, and 55 per cent of random blood samples contain anti-A. Thus, if transfusions were given without any blood group tests $\left(\frac{45}{100}\times\frac{55}{100}\times 100\right)$ per cent, or approximately 25 per cent, would be incompatible because of anti-A alone. Anti-B would cause incompatibility in about 10 per cent of random transfusions. By contrast, anti-Le^a, which ranks next in importance, is expected to cause incompatibility in only about 0.1 per cent of random transfusions.

The antibodies of the ABO system are considered in a separate table in the following chapter. Table XIII shows the other blood group systems in which naturally-occurring antibodies are relatively common. Anti-P_1 is found more commonly than anti-Le^a but whereas anti-Le^a is usually active at 37° C. and therefore capable of bringing about rapid red cell destruction *in vivo*, anti-P_1 is usually not active *in vitro* above a temperature of about 25° C. and is seldom capable of causing significant red cell destruction.

TABLE XIII

BLOOD GROUP SYSTEMS (EXCLUDING ABO SYSTEM) IN WHICH NATURALLY-OCCURRING ANTIBODIES ARE MOST OFTEN FOUND

Blood group system	*Antibody*			*Notes*
	Specificity	*Frequency in those lacking in antigen*	*Overall frequency in population*	
MNSs	Anti-M		? 0.01%	
	Anti-N		even	
	Anti-S		rarer	
	Anti-Vw	About 2%	About 2%	Usually accompanies anti-Mi^a
	Anti-Mi^a			Sometimes accompanies anti-Vw
	Anti-M^g	About 3%	About 3%	
P	Anti-P_1	Common	Common	If tests are made at 0° C. most sera from P— subjects agglutinate P+ cells (Henningsen, 1949b). If tests are made at above 20° C., the incidence is about 1% of P— subjects, an overall incidence of 0.2% of the population
	Anti-P+-P_1	100%	Extremely rare	Formerly known as anti-Tj^a
Lewis	Anti-Le^a	5 to 20% of Le(a—b—) subjects	0.3 to 1%	The lower figure is based on Kissmeyer-Nielsen *et al.*, 1955; the higher on Jordal (1956).
	Anti-Le^b	Much less	Less than 0.1%	Anti-Le^b alone is perhaps ten times less common than anti-Le^a but it quite commonly accompanies anti-Le^a.
Wright	Anti-Wr^a	About 1%	About 1%	Incidence based on Dunsford (1954)

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Antibodies such as anti-Wr^a, anti-Vw and anti-M^g are relatively common (each of them occurring in between 1 per cent and 3 per cent of human sera), but are very unimportant in transfusion practice because the corresponding antigens are so rare. For example, the Wr^a antigen occurs in approximately 0.1 per cent of the English population (see Chapter X). Incompatibility from anti-Wr^a is thus expected in only $\left(\frac{0{\cdot}1}{100}\times\frac{1}{100}\times 100\right)$ per cent, or 0.001 per cent, of all transfusions.

Origin of Natural Antibodies

Some authors believe that the so-called naturally-occurring antibodies are in fact hetero-agglutinins produced as an immune response to substances which are antigenically similar to the human blood-group substances, and which are ingested or inhaled (Wiener, 1951b).

The idea that hetero-agglutinins are formed as a response to ingested antigens has recently received experimental support, for it has been shown that their production can be prevented by rearing animals in a germ-free environment.

Thus in 'germ-free' chicks, no hetero-agglutinins against group B human red cells developed in the first 60 days of life whereas in ordinary chicks such agglutinins usually develop within the first 30 days. In germ-free chicks anti-B developed promptly after the admission by mouth of *Escherichia coli* O_{86}; the antibodies had serological characteristics similar to the 'naturally-occurring' agglutinins, that is they reacted slightly more strongly at 0° C. than at 37° C. In germ-free chicks very weak agglutinins developed 2-3 months after hatching but this was attributed to traces of non-living antigenic contaminants.

Although the stimulus to the formation of this 'naturally-occurring' agglutinin was traced to the environment, the authors pointed out that there was evidence that the time of onset and extent of the response depended upon genetic factors (Springer *et al.*, 1959).

Despite this convincing evidence it is still uncertain whether the occurrence of all 'naturally-occurring' antibodies in human sera can be so explained. It is not clear why in man, naturally-occurring antibodies like anti-P_1 and anti-Le^a should react more strongly at low temperatures than at 37° C. and be composed of 19*S* γ-globulin whereas 'immune' antibodies, that is, those antibodies arising after an identifiable stimulus such as anti-Rh, should react more strongly at 37° C. and usually be composed of 7*S* γ-globulin.

Seasonal Fluctuations in 'Naturally-occurring' Antibodies

It was first observed in cattle that naturally-occurring antibodies might fluctuate in titre, according to the season; thus, the anti-J titre is lowest in January-March and highest in August-October (Stone, 1956). The same is true of anti-R in sheep (Miss E. M. Tucker, personal communication). In both these species it is thought that there may be a relation between the animal's general condition and its agglutinin titre, since the animals are in their best condition in September after summer pasture.

In man, few observations have yet been made, although Stone and Shaw (1957) found that anti-A and anti-B were at a minimum in February-June (mean titre 16) and at a maximum in August (512).

Immune Blood Group Antibodies; Characteristics of Immune Responses

If an antigen is injected into an animal, the response depends upon whether the animal has previously encountered the antigen. If it has not, antibody production is slight and occurs relatively slowly; this is termed the primary response. The secondary response, observed after a further injection, is characterized by the brisk production of a relatively large amount of antibody; the titre may begin to rise as early as 48 hours after the injection, and may reach a peak at 6 days (for further discussion see Burnet and Fenner, 1949, p. 12).

After a first injection of antigen has been given, there is usually a period during which the animal will not respond well to a second injection. Thus in horses injected with tetanus toxoid, an interval of less than 3 months between the first two doses usually resulted in the production of non-avid antitoxin, but when the interval was over 12 months 'only high-value avid antitoxin was produced' (Barr and Glenny, 1945).

There is evidence that the dose of antigen which will provoke an optimal primary antibody response is greater than the dose needed to produce an optimal secondary response. Thus, in one series of experiments, in mice injected with sheep red cells the best primary response was obtained with the largest dose tested, which was equivalent to injecting 1.5 ml. of packed red cells into a 60 kg. human. One hundredth of this dose was definitely less effective in stimulating a primary response, but was almost as effective as the maximum dose in eliciting a secondary response. One-thousandth of the maximum dose was almost ineffective in eliciting a primary response but was not far below the maximum dose in eliciting a secondary response (Sobey *et al.*, 1956).

Qualitative differences are often observed between antibodies produced

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early in the immune response and antibodies produced subsequently. Foster (1957) found that in rabbits injected with human group A red cells the primary response consisted in the production of agglutinating antibody only. In the secondary response the agglutinin did not increase in potency but now haemolytic antibody was produced.

In man the formation of anti-Rh illustrates some of the features referred to above. For example, following a first transfusion of Rh-positive blood into an Rh-negative person antibody is often not found for several months. There is some evidence that a large dose of blood (500 ml.) is a more effective stimulus than a small dose (2-4 ml.); see Chapter IX. In a subject who has received a dose of Rh-positive blood some months previously, a further dose may cause a brisk rise in titre within 3 or 4 days (see Chapter XIII). Early in the immune response the subject tends to form an agglutinating antibody whereas, later, incomplete antibody predominates (Diamond and Denton, 1945; Waller, 1949).

Persistence of Immunity

Antibodies to polysaccharide antigens tend to persist at a high level for many months after the last injection of antigen. Thus Allen and Kabat (1958) injected 1 mg. of hog A substance or of a mixture of hog A substance with horse A and B substance, subcutaneously, on each of 2 successive days, into human volunteers. There was an initial rise in titre equivalent to a twenty-fold increase in the amount of precipitable nitrogen. In some subjects this level was maintained for 2 to 8 months. In all subjects the antibody concentration had fallen to a stable level by the end of 2 years but this level was four to five times as high as that before immunization. In some subjects followed for as long as 10 years there was no further fall in the level of antibody. By contrast, in subjects injected with diphtheria toxoid, antibody levels fell to 50 per cent of peak values within 2 weeks.

Incomplete Rh antibodies disappear only very slowly from the circulation and have been demonstrated in the blood more than 40 years after the last known antigenic stimulus. By contrast, the titre of complete Rh antibody ('saline agglutinin') declines relatively rapidly; see Chapter IX.

Effect on 'Naturally-occurring' Antibodies of an Antigenic Stimulus

Those naturally-occurring antibodies which behave as saline agglutinins acting more strongly at temperatures near 0° C. and inactive at 37° C., do not normally change in titre following an antigenic stimulus. For example, if P-positive blood is transfused to a patient whose serum contains anti-P_1 active at temperatures up to 25° C. but not above, and the patient's serum is tested at weekly intervals during the following month, the titre of anti-P_1 is found to remain constant (see Chapter X). The same result is usually observed when incompatible red cells are transfused to a patient whose serum contains anti-A_1 (without anti-A, i.e. in an A_2 or A_2B subject) or anti-M, or anti-Le^a or anti-Le^b, although there have been occasional reports of an increase in thermal amplitude or titre of these antibodies — see, for example, the anti-A_1 described by Boorman *et al.* (1946) or the anti-M described by Freisleben and Knudsen (1957).

Occasionally the production of predominantly incomplete anti-Le^a appears to be stimulated by transfusion. Thus in one case investigated in the author's laboratory, a patient who had had transfusions and injections of Le(a+) blood was found to have a potent anti-Le^a giving strong reactions in the indirect globulin test but giving only weak agglutination reactions. The antibody was composed partly of 7*S* and partly of 19*S* globulin (Kekwick and Mollison, 1961).

When group A or B blood is transfused to a person whose serum contains anti-A or anti-B, an immune response is almost always observed. Since anti-A and anti-B are usually regarded as naturally-occurring antibodies, this might appear to be a flagrant exception to the rule stated above, but there are two differences. First, anti-A and anti-B, unlike most other naturally occurring agglutinins, are active at 37° C., although they usually react more strongly at lower temperatures; secondly, the response to an antigenic stimulus consists not only in an enhancement of titre but in the production of antibody with characteristics not possessed by the original naturally occurring agglutinin. Thus, immune anti-A and anti-B sera may agglutinate cells as strongly at 37° C. as at lower temperatures. They also haemolyse cells and have a slightly different specificity (see Chapter VIII). Thus, it seems possible that in this case the immune response consists in the development of a new antibody or the 'recall' of an antibody which was previously present only in trace amounts, rather than in the increased production of the naturally occurring agglutinin.

A rather similar response has been observed in rabbits stimulated with T antigen (Fraser, 1955). Thus normally rabbit serum contains a cold agglutinin, anti-T, which cannot be absorbed at 40° C. by 'treated' red cells (i.e. cells treated with receptor-destroying enzyme). If the animal is immunized with heterologous treated red cells, potent anti-T agglutinins are produced which can be completely absorbed at 40° C. by treated cells. One year later the animal has reverted to the production of cold anti-T agglutinins.

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Perhaps the main conclusion from a clinical point of view is that unless a cold agglutinin is active *in vivo* at or near 37° C., an antigenic stimulus is unlikely to produce an increase in titre or thermal range of the antibody.

Transient fall in antibody titre following a large dose of antigen. Following the transfusion of incompatible blood, there may be a transient fall in the titre of antibody due to the adsorption of the antibody on to incompatible red cells. This is discussed in Chapter XIII.

Individual Differences in Response to Antigens

There is evidence that some individuals form antibodies much more readily than others. For example, Creger *et al.* (1951) injected twenty-five medical students with incompatible blood and found that two had far greater responses than all the rest. These same two medical students were the ones who had had the greatest responses to a previous injection of influenza vaccine. Carlinfanti and Cavalli (1947) found no relation between the ability to make anti-A (and anti-B) and the ability to make anti-typhoid antibodies, but they may by chance have dealt with a group of subjects none of whom was particularly sensitive to antigens.

The idea that certain subjects are hypersensitive from the point of view of antibody production receives some support from other observations made by Creger *et al.* (1951). They tested nine subjects with diseases which are sometimes regarded as manifestations of hypersensitivity, namely, subjects with disseminated lupus erythematosus, rheumatic fever, rheumatoid arthritis, etc. They found that all except the subjects with rheumatoid arthritis showed an exceptional response to the injection of incompatible red cells. Race and Sanger (1954, p. 359) noted that among five subjects whose sera contained the greatest number of blood group antibodies, two were suffering from some form of 'collagen disease'. One of these patients, previously described by Collins *et al.* (1950), formed most of the blood group antibodies that she was theoretically capable of forming. Thus, having the probable Rh genotype *CDe/cde*, she formed both anti-E and anti-C^w; being N-negative and S-negative, she formed both anti-N and anti-S; being K-negative, she formed anti-K and being Le(a—b—), she formed anti-Le^a and anti-Le^b. These antibodies were found after the patient had received twenty blood transfusions in the course of 12 years, although the Lewis antibodies at least may have been present beforehand.

Relatively little is known about the importance of the spleen in the production of blood group antibodies in man. Rowley (1950) injected

1 ml. of a 2 per cent suspension of sheep red cells into fourteen splenectomized patients and sixteen controls and found that whereas, in all the controls, there was at least a four-fold increase in antibody, there was no significant rise in thirteen out of fourteen of the splenectomized subjects. McFadzean and Tsang (1956) also found that the response to washed sheep red cells was lower in splenectomized subjects. However, the spleen is almost certainly not essential in the formation of antibodies against red cell antigens. For example the patient described in the previous paragraph who formed so many blood group antibodies had had her spleen removed before receiving the series of blood transfusions which immunized her.

Subjects with hypogammaglobulinaemia have a greatly diminished capacity to form certain antibodies. As described in Chapter VII their serum may lack anti-A and anti-B. In one case a patient of group A whose serum lacked anti-B was given a series of injections of B substance of animal origin but failed to form anti-B (Barandun, Büchler and Hässig, 1956).

Relative 'Potency' of Different Blood Group Antigens

Giblett (1961) estimated the relative 'potency' (antigenicity) of different blood group antigens in the following way. She assembled figures from four laboratories for the frequency with which different blood group antibodies were encountered (see Table XIV). She then calculated the frequency of the opportunity for immunization by each antigen by simply multiplying the proportion of the population lacking the antigen by the proportion having it; for example, for anti-K, approximately $0.09 \times 0.91 = 0.082$.

The assumption was then made that a single transfusion of K-positive blood to a K-negative person induced antibody formation in 5 per cent (0.05) and from this the relative antigenicity of the other factors was calculated. For example, the potency of E, taking that of K to be 0.05,

$$= 0.05 \times \left(\frac{137}{163}\right)^{*} \times \left(\frac{0.082}{0.204}\right)^{\dagger} = 0.017$$

Giblett considered whether the transfusion of Negro blood to Caucasians carried any greater risk than that of transfusing Caucasian blood to Caucasians or Caucasian blood to Negroes. She concluded that any difference was of no practical importance and there was no justification for taking the race of the donor into account in blood transfusion.

* = relative incidence of the two antibodies (see Table XIV).

† = relative frequency of opportunity of immunization, i.e. $\frac{K(-)\times K(+)}{E(-)\times E(+)}$

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Immunological Paralysis

In normal adult mice, the administration of a sufficiently large dose of pneumococcal polysaccharide results in a life-long failure to produce antibody, a state known as 'immunological paralysis' (Felton *et al.*, 1955). The massive injection of purified proteins into rabbits produces a more temporary immunological unresponsiveness (Dixon and Maurer, 1955); the difference may be due to the fact that protein antigens are more rapidly

TABLE XIV

LIST OF ANTIBODIES (EXCLUDING ANTI-D, ANTI-D + ANTI-C AND ANTI-D + ANTI-E) REPORTED FROM FOUR LABORATORIES, TOGETHER WITH ESTIMATES OF THE RELATIVE POTENCY OF THE ANTIGENS CONCERNED (FROM GIBLETT, 1961)

Specificity of antibody	*Number of examples found*	*Relative potency of the antigen*
Anti-K	163	0.0500*
(Anti-Le^a and Le^b	162	—)†
Anti-E	137	0.0169
Anti-c	124	0.0205
Anti-Fy^a	20	0.0023
(Anti-P_1	17	—)†
(Anti-M	12	—)†
Anti-C (or Ce)	9	0.0011
Anti-e	5	0.0056
Anti-Jk^a	5	0.0007
Anti-S	4	0.0004
Anti-Jk^b	2	0.0003
Anti-s	1	0.0003
Anti-k	1	0.0150
Others (including anti-V, anti-U, anti-Js^a and anti-V^w)	9	—

* Although Giblett took the figure of 5 per cent, the estimate of Kornstad and Heistö (1957) was that a single transfusion of K+ blood would induce the formation of anti-K in 10 per cent of K− subjects.

† No estimates of potency made in these cases because antibodies thought to be 'naturally-occurring'.

'digested' by the reticulo-endothelial system (personal communication from Dr J. H. Humphrey).

This failure to form antibodies after too large a dose of antigen seems to be due to the destruction of antibody-forming cells rather than to the continuous neutralization of antibody (Sercarz and Coons, 1959). With some antigens, for example bovine serum albumin, a daily dose as small as 10 mg. given to a mouse is inhibitory (Sercarz and Coons, 1959) and this should serve as a warning that in trying to produce anti-sera in animals, the best response will not necessarily be achieved by giving a large dose of

antigen. As mentioned in Chapter XII amounts of protein as small as 5×10^{-3} μg. are antigenic in rabbits.

Tolerance or Relative Tolerance

An individual is said to be tolerant of an antigen, when, following exposure to the antigen, he fails to form a corresponding antibody. During embryonic life the immunological system is regarded as going through a period of development during which it becomes tolerant of all the antigens to which it is exposed, that is to say, its own tissue antigens. If foreign antigens are introduced into an animal during its embryonic life, it may, as an adult, fail to recognize them as foreign; this phenomenon, which is known as acquired immunological tolerance, was postulated by Burnet and Fenner (1949, p. 103) and demonstrated experimentally by Billingham, Brent and Medawar (1953). It is now recognized that transient or casual exposure to antigens during foetal life is not sufficient to secure tolerance (see review by Brent and Medawar, 1958). Tolerance can be secured either by introducing into the embryo a graft which survives throughout the animal's life or by giving repeated injections of cells (Mitchison, 1959) or of soluble antigen (Smith and Bridges, 1958).

An example of a permanent graft is seen often in twin cattle, and very rarely in twin humans. Thus, in dizygotic cattle twins, the circulations may become joined during intra-uterine life, and blood-forming tissue is exchanged; the resulting twins, throughout their life, have two populations of red cells of different antigenic composition, each twin being tolerant of the red cells of the other (Owen, 1945). This phenomenon, which is known as 'red cell chimaerism', can be recognized in 80 per cent of cattle twins of unlike sex (Rendel, 1958) and has been recognized several times in human dizygotic twins (Dunsford *et al.*, 1953; Booth *et al.*, 1957; Nicholas *et al.*, 1957).

In the first case recognized in man, a blood sample from a particular donor appeared at first sight to contain very weakly reacting group A red cells. The possibility was then considered that the blood might really contain a mixture of A and O cells. It was soon demonstrated that this was so, and that the two populations of red cells differed in antigenic composition in many other ways. Although the donor had not revealed that she was a twin, it was confidently forecast that she must be one, and when questioned she admitted that she had had a twin brother who had died 25 years previously (Dunsford *et al.*, 1953).

Blood group chimaerism has been produced in animals by transfusion of blood-forming tissue after first irradiating the recipient to prevent an immune response (see Chapter IV).

RCPE0002068_0126

The first case of this phenomenon in a human was described by Beilby *et al.* (1960) and is referred to in Chapter IV.

Tolerance is not an all or none phenomenon, and it has been suggested that, in man, exposure of the foetus to maternal antigens may modify subsequent immune responses. For example, it might be expected that Rh-negative women with Rh-positive mothers would be more tolerant of Rh antigen in later life than Rh-negative women with Rh-negative mothers. Evidence on this point is conflicting and is considered in Chapter IX.

Similarly, it has been claimed that O subjects with A mothers respond less well in later life to an A stimulus than O subjects with O mothers (see Chapter VIII).

Auto-Antibodies

Under some circumstances antibodies develop which are capable of reacting with the body's own constituents. In some cases the antigen concerned is one which may not normally come into contact with the body's own antibody-forming cells. For example, antibodies against sperm or thyroglobulin sometimes develop in adults, and it may be that the antigens responsible are ones which do not normally gain access to the circulation. In other cases it seems that the mechanism responsible is the emergence of a new 'line' of antibody-forming cells, perhaps as a result of mutation, which regard normal red cells as foreign. This may be the case in lymphosarcoma in which a haemolytic anaemia associated with auto-antibodies is often recognized. Auto-antibodies are considered further in Chapter XI.

Clinical Effects of Blood Group Antibodies

The effects of antibodies on transfused incompatible red cells are considered in Chapter XIII; and the passage of antibodies across the placenta and the effects they produce on the foetus are considered in Chapter XVII.

There is one further way in which incompatible antibodies may possibly produce pathological effects, namely by reacting with spermatozoa. Gershowitz *et al.* (1958) found that secretions obtained from the cervix uteri contained 'expected' agglutinins (according to the ABO group of the subject) in seventeen out of seventy cases. It is known that spermatozoa of appropriate ABO group are agglutinated by the corresponding agglutinins and it has been demonstrated directly (by agglutination) that an AB subject produces A and B sperms (Gulbring, 1957). It may be therefore that when a group O woman is impregnated with sperm from a man of group *AO* there is a selective destruction of A sperms which would

account for the shortage of A infants in matings between O women and A men (Levine, 1943; Waterhouse and Hogben, 1947).

Leucocytes and Platelets: Antigen and Antibodies

Leucocytes

Leucocytes have the same ABO, MN and P antigens as the red cells but appear to lack Rh antigens (Gurner and Coombs, 1958). They certainly contains numerous antigens not detectable in red cells. For example, in studies with the sera of polytransfused patients, Lalezari and Spaet (1959) noted that no two unrelated subjects had leucocytes which gave the same serological reactions. Medawar (1958) has pointed out that the complexity of leucocyte antigens recalls that of the antigens which determine the incompatibility of skin homografts.

Iso-antibodies against leucocytes are found in patients who have been transfused repeatedly and in women who have been pregnant.

Usually many transfusions are required to stimulate the form of leuco-agglutinins – more than six according to van Rood *et al.* (1961) and many more than this according to some other authors. Brittingham and Chaplin (1961) suggested that the leucocytes in stored blood, being degenerate, might be less antigenic than fresh leucocytes. They succeeded in producing a leuco-agglutinin in a volunteer after giving only five injections, at weekly intervals, of absolutely fresh leucocytes from a leukaemic donor.

The presence of potent leuco-agglutinins in the serum is often associated with severe transfusion reactions and this is considered further in Chapter XV.

The formation of leuco-agglutinins during pregnancy was described by Payne and Rolfs (1958) and van Rood *et al.* (1959). Payne and Rolfs found leuco-agglutinins in twenty-five out of 144 pregnant multiparae; seventeen of these women had not been transfused previously. In most cases the leuco-agglutinin in the mother's serum reacts with the infant's leucocytes. In a further paper Payne (1960) pointed out that at least two pregnancies seem to be needed for the production of leuco-agglutinins. Payne also pointed out that women who had had previous pregnancies might have transfusion reactions (due to leuco-agglutinins) following their very first transfusion. Many of the same points emerged in the study of van Rood *et al.* although the incidence of leuco-agglutinins was distinctly lower in their series. Thus leuco-agglutinins were found in none out of 101 women who were pregnant for the first time, in three out of 134 women in their second or third pregnancy, and in eight out of 142 women who were in their third or a later pregnancy. In a further series

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of patients with leuco-agglutinins, all were found to have had previous blood transfusions but in those who had had only one to five blood transfusions, fourteen out of fifteen had also had previous pregnancies. In all cases in which leuco-agglutinins were associated with the stimulus of pregnancy alone, the leuco-agglutinin reacted with the husband's white cells and usually with the infant's cells.

Some authors believe that severe neutropenia in newborn infants is due to the passage of leuco-agglutinins from mother to foetus; see Chapter XV.

Auto-antibodies against leucocytes have been clearly demonstrated on at least one occasion (Chalmers *et al.*, 1959).

Platelets

Platelets can be shown to contain the A and B antigens (Coombs and Bedford, 1955) and the 'Tj^a' antigen (Ashhurst *et al.*, 1956), but, as in leucocytes, Rh antigens cannot be demonstrated (Ashhurst *et al.*, 1956; Gurevitch and Nelken, 1957).

As mentioned in Chapter I, in patients transfused repeatedly the survival of transfused platelets may diminish progressively, suggesting that iso-antibodies have been formed. Platelet iso-antibodies may also play some part in transfusion reactions (see Chapter XV) and may be responsible for some cases of neo-natal thrombocytopenia (see Chapter XVII).

Methods of demonstrating platelet antibodies are very unsatisfactory because of the tendency of platelets to clump; for example, the presence of traces of thrombin may cause platelet agglutination. It has proved difficult to obtain reproducible results with platelet agglutination tests and some workers have avoided direct agglutination as a criterion and have instead used the mixed antiglobulin reaction or the antiglobulin consumption test.

Most people believe that in idiopathic thrombocytopenia purpura the serum contains auto-antibodies against platelets but their demonstration is difficult; according to Professor Jean Dausset (personal communication) antibodies can be demonstrated by the direct antiglobulin consumption test in about 50 per cent of patients.

COMPLEMENT

Complement is a substance present in normal serum which may be adsorbed on to 'sensitized' bacteria or red cells and bring about their lysis or accelerated removal from the circulation.

Four components of complement are recognized and are designated as C'_1, C'_2, C'_3 and C'_4. (The division of C'_3 into sub-components will not be discussed here.) It has been suggested that these four components interact with one another and with the sensitized red cells (i.e. red cells coated with antibody) according to the following scheme (Mayer *et al.*, 1954), in which E = erythrocyte and A = antibody:

$$EA+C'_1+C'_4 \longrightarrow EAC'_{14}$$
(in the presence of Ca^{++})

$$EAC'_{14}+C'_2 \longrightarrow EAC'_{142}$$
(in the presence of Mg^{++})

$$EAC'_{142}+C'_3 \longrightarrow E^* \text{ (activated cell)} \longrightarrow \text{ghost}+Hb.$$

C'_1 is the precursor of an esterase (Becker, 1955; Lepow *et al.*, 1956). It is thought that this precursor, which is a normal constituent of serum, is activated by contact with the antigen-antibody complex and that the enzyme then acts upon C'_4 and C'_2 causing the development of binding sites and enzymic sites. The binding sites enable C'_4 and C'_2 to join on to EAC'_1 and the enzymic sites cause the formation of the active complex C'_{142} which in turn is capable of reacting with C'_3.

At the present time the components of complement have not been isolated in a pure state and most investigations have been carried out with preparations which contain mixtures of more than one component. For example, when serum is dialysed against hypotonic phosphate buffer, $I = 0.02$, pH 5.4, a precipitate is formed containing C'_1, the supernatant containing C'_4 and C'_2 (Ecker *et al.* (1943)).

Heating serum to 56° C. for 30 minutes completely inactivates C'_1 and C'_2. Heating to 56° C. damages C'_4 to a lesser extent, although after 20 minutes at 56° C., C'_4 activity may be reduced to 20 per cent of the initial value (Bier *et al.*, 1945; Heidelberger and Mayer, 1948).

C'_3 can be selectively inactivated by treatment with zymosan. For example, if 1 ml. of human serum is treated with 3 mg. of one particular preparation,[1] all C'_3 activity is removed. Zymosan does in fact remove other components of complement to a lesser extent (Bier *et al.*, 1945; Nelson, 1958).

In some species, e.g. rabbit, treatment with zymosan appears to remove all components of C′ and cannot be used to remove C'_3 selectively. The difficulty can be overcome by absorbing the rabbit serum with a complex of zymosan and properdin from human serum (Hinz *et al.*, 1961).

[1] Obtainable from Fleischman Laboratories.

RCPE0002068_0128

C'_4 is selectively destroyed by treatment with ammonia or with hydrazine.

To inactivate C'_4 1 ml. of serum is treated with 0.25 ml. of 0.15 M-ammonia at 37° C. for 60 minutes, and is then neutralized with 0.15 M-HCl. Alternatively 1 ml. of serum may be treated with 0.1 ml. of 0.16 M-hydrazine at 37° C. for 1 hour.

The binding of complement to sensitized red cells can be prevented by the addition of chelating agents. For example, the addition of 2 mg. Na_2H_2 EDTA to 1 ml. of serum completely prevents its complementary activity, by preventing the step $EA+C'_1 \longrightarrow EAC'_1$, which requires Ca^{++}.

The effect of EDTA on fresh serum is reversible if the period of contact between serum and EDTA is not too long — it appears that in the absence of ionized calcium, C'_1 is irreversibly inactivated. Thus incubation of serum at 45° C. for 5 hours with EDTA destroys all C'_1 (Young and Lepow, 1959).

Heparin is also anti-complementary. According to Andersen (1957) the addition of 12 international units (i.u.) of heparin to 1 ml. of serum abolishes its complementary property, but personal observations suggest that very much greater quantities — up to 1000 i.u. per millilitre, may be required.

Andersen (1957) found that when 1 volume of fresh serum to which heparin had been added in a concentration of 12 i.u. per ml. was mixed with 1 volume of heated anti-Le^a the mixture failed to sensitize Le(a+) red cells to agglutination by an antiglobulin serum. In the present author's laboratory it has been found that if Le(a+) red cells are first sensitized with heated anti-Le^a, then treated with fresh serum to which various amounts of heparin have been added, approximately 1000 i.u. per ml. must be added to obtain a negative antiglobulin test. Even when the Le(a+) cells are treated with heated anti-Le^a and heparinized serum simultaneously, amounts of the order of 100 i.u./ml. have been required in our hands. The discrepancy may lie in the varying potency of different anti-Le^a sera. (In this passage 'antiglobulin' refers to a serum reacting with adsorbed complement.)

Complementoid

Ehrlich and Sachs (1905) found that serum which had been heated contained partly altered complement which they called complementoid (by analogy with toxoid). Thus complementoid would bind on to antigen-antibody complexes but did not produce lysis of cells.

Sera stored at −20° C. seem regularly to contain complementoid (Polley and Mollison, 1961). Unless steps are taken to prevent complementoid from binding, it may block the adsorption of normal complement and thus lead to false negative results. Addition of EDTA to stored serum will block the adsorption of complementoid and, provided antibody binding is satisfactory in the absence of complement, good results are then obtained by adding fresh serum as a separate stage either in the indirect antiglobulin test or in haemolytic tests (see Chapter XII).

Complement and complementoid may be removed from serum by absorption with kaolin (see Chapter XII), although such treatment sometimes results in absorption of blood group antibodies (Polley and Mollison, 1961).

Properdin

Properdin, first described by Pillemer *et al.* (1954), is a β_1 globulin with a molecular weight of about 1,000,000 which plays a role in non-specific immunity (for references, see review by Isliker, 1959). Although properdin was at first considered to be part of the complement system, it may equally be regarded as a naturally-occurring antibody, with a broad spectrum of activity (see Isliker, 1959).

Properdin does not react with normal red cells but it is involved in the lysis of red cells from patients with paroxysmal nocturnal haemoglobinuria (PNH) (Pillemer *et al.*, 1954) and it has been shown that PNH red cells directly fix properdin (Martin and Voss, 1957).

Properdin may be removed from serum by absorbing the serum with zymosan at 17° C. (Pillemer *et al.*, 1954).

PRINCIPLES OF DETECTING BLOOD GROUP ANTIBODIES

Complete or Agglutinating Antibodies

When red cells containing a given blood group antigen are brought into contact, at a suitable temperature, with serum containing the corresponding antibody, antibody becomes attached to the red cells. This attachment may be detected in a number of ways. Some antisera will bring about the agglutination of red cells suspended in saline and they are then said to contain saline-agglutinins. For the first 40 years or so in human blood grouping work all tests were performed with red cells suspended in saline.

Almost all naturally occurring antibodies will agglutinate red cells suspended in saline; this suggests the possibility that it is a property of 19*S* antibodies to bring about agglutination of this kind. The only 7*S* antibodies known to be capable of bringing about the agglutination of red

RCPE0002068_0129

cells in saline are some examples of anti-A and anti-B; possibly the difference here may be the greater number of A and B antigen sites compared with those of other systems. The importance of the number of antigen sites per red cell is suggested by the observation that D^u red cells suspended in saline may fail to be agglutinated by an anti-D serum which will agglutinate ordinary D-positive cells in saline.

Incomplete Antibodies

Race (1944) and Wiener (1944a) independently discovered that anti-Rh might exist in a form which failed to bring about the agglutination of red cells suspended in saline. When Rh-positive red cells were exposed to a serum containing this kind of anti-Rh they became 'blocked'; that is to say, they were no longer agglutinated by a serum containing anti-Rh saline agglutinin.

It was soon realized that this kind of antibody, to which Race had given the name 'incomplete' and Wiener the name 'blocking', was the form in which anti-Rh most commonly occurred. For a short time a test based on the 'blocking' phenomenon was used to reveal the presence of incomplete anti-Rh but this was soon replaced by a variety of more sensitive tests.

The Use of Colloid Media

Sera containing incomplete anti-Rh will usually agglutinate red cells suspended in concentrated protein solutions (Cameron and Diamond, 1945). The serum fraction containing concentrated albumin is very suitable (Diamond and Denton, 1945), although it appears that this is due to the effect of alpha globulin present as a contaminant rather than to the albumin itself (McCulloch, 1950).

A mixture of human plasma and bovine albumin gives stronger reactions than albumin alone (Wiener, Hurst and Sonn-Gordon, 1947; Pickles, 1949). A mixture of equal parts of human AB serum and 30 per cent bovine albumin was found to be very satisfactory by Stratton and Dimond (1955); for further details see Chapter XII.

Grubb (1949) found that dextran could be used as a suitable medium for red cells in detecting incomplete antibodies. Dextran has not been widely used in detecting anti-Rh partly because of the introduction of more sensitive tests and partly because of the tendency of dextran to cause rouleaux formation. Dextran seems to be more useful in detecting incomplete anti-A and anti-B; see Chapter XII.

Wiener (1945a) used the term 'conglutination' to describe the clumping of Rh-positive red cells suspended in plasma as opposed to agglutination occurring in red cells suspended in saline; and he used the term univalent antibody or glutinin for the kind of Rh antibody responsible for 'conglutination' (Wiener, 1945b). In view of the fact that the term 'conglutination' had previously been used for a quite different phenomenon involving the clumping of red cells which had adsorbed antibody *and complement* (see review by Hole and Coombs, 1947), Coombs *et al.* (1945) objected to Wiener's use of the term to describe a different phenomenon; this objection has gained weight with time and it seems quite inadmissible to describe as 'conglutination' a reaction in which complement is not involved.

The Use of Enzymes

Morton and Pickles (1947) demonstrated that Rh-positive red cells which had previously been incubated with trypsin were agglutinable by incomplete anti-Rh sera in a saline medium. Various modifications of this original method, including the use of papain, ficin and bromelin, instead of trypsin, and direct mixture of enzyme, cells and serum to avoid pretreatment of the cells, are described in Chapter XII. As explained there, enzyme treatment destroys some blood group antigens but despite this limitation, enzymes are of great value in blood grouping.

The reason why enzyme treatment sometimes enhances blood group reactions is not yet understood, although some progress in elucidating the mechanism has been made recently. Mäkelä *et al.* (1960) found that when red cells were treated with trypsin, 50 per cent of the sialic acid content of the cells was lost. Similarly Cook *et al.* (1960) found that treatment of red cells with trypsin (50 μg./ml. cells) resulted in the release of a sialomucopeptide and they suggested that trypsin acts in the same way as sialidase in releasing bound sialic acid. Thus the action of trypsin on red cells seems to be similar to that of receptor-destroying enzyme which has been shown to be a sialidase (sometimes described as a neuraminidase).

The observation of Dodd *et al.* (1960) that various sialic acid preparations inhibit the reaction between anti-D and D-positive red cells does not at the moment seem to fit in well with these observations, because trypsin treatment of red cells, which liberates sialic acid, *enhances* the reaction between incomplete anti-D and D-positive red cells.

The Antiglobulin Sensitization Test

It would be difficult to convey to the uninitiated the enthusiasm which the blood-grouper must feel for the antiglobulin test! Introduced originally for the detection of anti-Rh it has proved capable of detecting almost

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every known incomplete blood group antibody. More recently it has been shown to detect adsorbed complement on red cells and to indicate whether an adsorbed antibody is composed of 7*S* globulin or of 19*S* globulin. It is undoubtedly the best single available test for the detection of blood group antibodies.

The principle of the antiglobulin test can be illustrated by an example. To detect human blood group antibody adsorbed on to red cells, an antiserum directed against human globulin is used. This antiserum is prepared by injecting human globulin into an animal.

The principle of this test was first applied by Moreschi in 1908 but it only came into general use after Coombs *et al.* (1945) had shown that it was an invaluable method of detecting Rh antibody of the incomplete type. In the original form of the test – 'the indirect antiglobulin test' – Rh-positive cells were incubated with incomplete anti-Rh, then washed and mixed with an anti-human globulin serum, whereupon strong agglutination occurred. The same authors (Coombs *et al.*, 1946) showed that the red cells of infants with haemolytic disease of the newborn due to anti-Rh (or in one case anti-K), after washing, were directly agglutinated by antiglobulin serum, the so-called 'direct antiglobulin test'.

Coombs and Mourant (1947) showed that so far as the reaction with cells sensitized with anti-Rh was concerned, the essential component in the antiglobulin serum was anti-gamma globulin since the reaction could be inhibited by adding purified gamma globulin to the antiglobulin serum.

The fact that an antiglobulin serum might react with adsorbed proteins other than gamma globulins was described by Dacie (1951) who showed that antiglobulin serum to which gamma globulin had been added would still react with the red cells of patients with the cold antibody type of acquired haemolytic anaemia and also with red cells sensitized with normal incomplete cold antibody. Similarly, Crawford and Mollison (1951) showed that antiglobulin serum absorbed with one type of sensitized cell (for example Rh-sensitized) would still react with other types of sensitized cells (for example cells sensitized with the normal incomplete cold antibody); and Renton (1952) showed that two fractions could be prepared from an antiglobulin serum, first by absorption with Rh-sensitized cells or by adding gamma globulin to the serum and second by absorbing with cells agglutinated with anti-A or anti-B.

It soon became clear that red cells sensitized with some blood group antibodies did not react with anti-gamma globulin but only with anti-'non-gamma' globulin, that is to say, with a serum containing anti-alpha and anti-beta globulin, and it was concluded that such antibodies (e.g.

some examples of anti-Le^a) were composed of beta, or perhaps alpha, globulin, rather than gamma globulin (Cutbush *et al.*, 1956). It was also found that some antibodies of this kind (e.g. anti-Le^a) could be detected by the antiglobulin test only if the red cells were incubated with complement as well as with antibody (Mollison and Cutbush, 1955). The correct interpretation of these and other observations was provided by Dacie *et al.* (1957) when they showed that the 'non-gamma' globulin detected on the red cells was not antibody but complement.

Evidence that it is the fourth component of complement (C'_4) which reacts with the antiglobulin serum has been produced by Jenkins *et al.* (1960) and Pondman *et al.* (1960). Jenkins *et al.* first sensitized red cells with EDTA-treated anti-Le^a serum to allow the uptake of anti-Le^a but not of complement; the red cells were then incubated with hydrazine-treated rabbit serum. This allowed the uptake of C'_1 but not of C'_4. The red cells were then incubated with human serum which had been heated to 56° C. so as to inactivate C'_1 and C'_2 and to leave only C'_4 (and C'_3). Although the only component of complement of human origin adsorbed by the cells was C'_4 the cells gave a strong reaction with anti-human globulin serum.

If red cells which have taken up anti-Le^a and complement are incubated for 24 hours, they still give a strong antiglobulin reaction (Gandini, 1959; Mollison and Polley, 1960); this suggests that the antiglobulin serum can react with the product C'_{14} since C'_{142} is known to be very unstable and to break down at 37° C. with a half-time of about 8 minutes leaving the product C'_{14} (ref. Mayer *et al.*, 1954).

It appears that C'_4 is either composed of β_1 globulin or at least that it brings about the adsorption on to red cells of β_1 globulin for if antiglobulin serum is absorbed with red cells which have taken up complement an anti-β_1 line is removed from the immuno-electrophoretic pattern (Peetom *et al.*, 1959; Jenkins *et al.*, 1960).

Müller-Eberhard and Nilsson (1960) found that a serum protein β-1C globulin was related to the complement system. After treatment with immune precipitates or with zymosan, or after storage, β-1C was converted to β-1A. Although β-1C could replace guinea-pig C'_3 it could not replace human C'_3. The β_1 globulin described by Müller-Eberhard and Nilsson appears to be the same protein as the one studied by Peetom *et al.* (see Burtin 1960, pp. 102-10b) but there are some discrepancies which have not yet been explained. Thus, whereas Peetom *et al.* (1959) like Jenkins *et al.* (1960) and Pondman *et al.* (1960) thought that the β_1-globulin was associated with C'_4 activity, Müller-Eberhard and Nilsson considered that β-1C globulin could not be C'_4, because β-1C precipitates with the euglobulin fraction of serum whereas C'_4 does not. Thus, if serum is dialysed against a buffer of ionic strength 0.02, pH 5.4, a precipitate is formed which contains β-1C as well as C'_1 (Müller-Eberhard and Nilsson, 1960); on the other hand, Jenkins *et al.* found that the supernatant, which contains C'_4 (as well as C'_2) would act as a source of the β_1 globulin reacting in the antiglobulin test.

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β-$_{1C}$ globulin has recently been identified as C'_{3a}, a subcomponent of C'_3; C'_{3a} is inactivated by hydrazine and is not completely inactivated by zymosan (4 mg./ml.) (Dr H. J. Müller-Eberhard, personal communication).

The most likely explanation for the discrepancies reported above is that antisera prepared by injecting adsorbed human complement into rabbits (see p. 403) contain antibodies against at least two components of complement, both of which are associated with β_1 globulins. From the work of Dr H. J. Müller-Eberhard it is now clear that the most prominent antibody in anti-human complement sera is anti-β-$_{1C}$, that is anti-C'_{3a}. However, as mentioned above, there seems to be good evidence that anti-complement sera also react with red cells which have taken up C'_4 without C'_2 and therefore, presumably, without C'_{3a}.

In summary, red cells which have taken up complement react with anti-globulin sera containing antibodies against β_1 globulins. It is probable that two different β_1 globulins are involved, one associated with C'_4 and the other with C'_{3a}. Anti-globulin sera prepared by injecting whole human serum into rabbits (see p. 403) normally react with 'complement-coated' red cells.

Role of complement in promoting the adsorption of antibodies and of preventing their elution. When red cells combine with a complement-binding antibody, the ease with which the antibody may subsequently be eluted may depend upon whether or not complement has also been bound. For example, if Le(a+) cells are incubated with anti-Le^a serum which has been treated with EDTA to prevent the binding of C'_1, more anti-Le^a can subsequently be eluted from the cells than from cells which have been incubated with the same serum to which EDTA has not been added. It can be shown that the amount of antibody adsorbed on to the cells is the same in the two cases: that is, in this case, complement does not promote the adsorption of anti-Le^a but it does interfere with its elution (Stratton, 1959; Polley and Mollison, 1961); antibody and complement can thus be adsorbed on to the cells in two separate stages (Vaughan and Waller, 1957; Andersen, 1957). This is particularly convenient if the serum containing anti-Le^a is anti-complementary. In the first stage of the test suitable red cells are incubated with EDTA-treated anti-Le^a serum. In this stage, anti-Le^a is taken up but the binding of complement and 'complementoid' (see above) is prevented. The cells are now washed and incubated with fresh normal serum as a source of complement and then after further washing tested with a suitable antiglobulin serum, that is one containing anti-complement-globulin. (For details, see Polley and Mollison, 1961, and Chapter XII.)

By no means all blood group antibodies give good reactions when a two-stage test is used; in these cases it seems that antibody-binding is less satisfactory in the absence of complement. In such cases the best results

are obtained by incubating the red cells with antibody and complement together. This applies to some examples of anti-K and to most examples of anti-Jk^a (Polley and Mollison, 1961), provided the sera are fresh. If the sera have been stored they may be anti-complementary and in that case two-stage tests are likely to give stronger reactions.

The extreme case is the normal incomplete cold antibody which can be demonstrated only in the presence of complement (Dacie, 1950b). It can be shown by absorption experiments that the antibody is not taken up by red cells even at 0° C. unless complement is present (Polley and Mollison, 1961).

Hinz *et al.* (1961) produced evidence that C'_1 may play an essential role in bringing about the binding of the antibody associated with paroxysmal cold haemoglobinuria ('the Donath-Landsteiner antibody'). Nevertheless it appears that there are differences between different cases because Dacie (1954b, p. 277) found that some antibodies of this kind could be adsorbed in the absence of complement; and in a case investigated in the author's laboratory, a positive indirect antiglobulin test could be produced by first incubating normal red cells in the cold with EDTA-treated serum (from a patient with the syndrome) and then, after washing the cells, incubating them at 37° C. with fresh normal serum to allow the uptake of complement (unpublished observations with Miss Polley).

Detection of Incomplete 19S Antibodies by the Antiglobulin Test

Occasionally, sera containing anti-Le^a, even after being heated to 56° C. for 30 minutes, will sensitize red cells to agglutination by an antiglobulin serum (see Thölen *et al.*, 1957). This reaction is due to the presence of small amounts of anti-19*S* globulin in 'ordinary' antiglobulin sera. By using a potent, specific anti-19*S* serum it is possible to obtain quite strong agglutination of Le(a+) red cells which have been incubated with anti-Le^a in the absence of complement (Stratton, 1961; Polley *et al.*, 1961).

Summary of Reactions of Antiglobulin Sera with Sensitized Cells

Table XV shows the reactions with different antiglobulin sera of red cells sensitized with various incomplete blood group antibodies, alone or in the presence of complement.

Almost all the immune antibodies except anti-Jk^a readily sensitize red cells to agglutination by an anti-7*S* gamma-globulin serum. In view of the fact that anti-Jk^a is composed predominantly of 7*S* globulin as judged by the fact that it is not inactivated by treatment with sulphydryl compounds (Hunter and Mollison, 1961) the negative reaction with anti-7*S* may be

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presumed to be due to the small amount of antibody adsorbed by Jk(a+) red cells because very potent anti-7*S* gamma globulin sera do in fact weakly agglutinate red cells sensitized with anti-Jk^a (personal communication from Miss Carolyn Giles; see also Polley *et al.*, 1961). Possibly each antibody molecule binds several molecules of complement thus accounting for the fact that the adsorbed complement can be readily detected whereas the adsorbed antibody cannot. In any case the adsorption of antibody on to the red cells can be detected indirectly by testing for the adsorption of complement using a suitable antiglobulin serum.

TABLE XV

REACTIONS WITH DIFFERENT ANTIGLOBULIN SERA OF RED CELLS SENSITIZED WITH VARIOUS INCOMPLETE BLOOD GROUP ANTIBODIES

Blood group antibody		*Reactions with*		
System	*Example*	*Anti-7S gamma globulin*	*Anti-19S gamma globulin**	*Anti-complement globulin (anti-beta$_1$)***
ABO	immune anti-A†	+	−	+
Rh	anti-D	+	−	−
MNSs	anti-S	+	−	+ (some)
P	anti-P$_1$	−	+ (rare)	+ (rare)
K	anti-K	+ (most)	⊕ (some)	+ (some)
Le	anti-Le^a	−	+	+
Fy	anti-Fy^a	+ (most)	−	+ (some)
Jk	anti-Jk^a	−††	+ (some)	+
Di	anti-Di^a‡	+	−	−
Js	anti-Js^a‡	+	−	−
Ii	anti-I	−	+	+

* Results from Polley *et al.* (1961).

** Red cells tested after being given an opportunity of adsorbing complement as well as antibody.

† Selected; from group O mothers of infants with ABO haemolytic disease; sera tested after 'partial neutralization' with A substance.

†† Positive with potent anti-7*S*.

‡ Only one example of each.

Most incomplete naturally occurring antibodies sensitize red cells to agglutination by an anti-19*S* globulin serum. Polley *et al.* (1961) obtained positive results with all anti-Le^a sera they tested, as well as with an incomplete anti-H(O), active at 37° C., one incomplete anti-P$_1$ serum and several examples of anti-I.

Many incomplete blood group antibodies bind complement and provided that the red cells have an opportunity of adsorbing complement as well as antibody, positive results are obtained with anti-complement globulin (anti-β_1 globulin) serum. Rh antibodies are peculiar in that they seem never to bind complement and although relatively few examples of

Lutheran antibody have been tested, it appears that they behave in the same way. Some examples of anti-S, anti-K and anti-Fy^a bind complement and some do not. Apparently all examples of anti-Jk^a bind complement but it must be pointed out that an example of anti-Jk^a which did not bind complement would probably not be detected by the antiglobulin test and might well be overlooked. Too few examples of anti-Di^a and anti-Js^a have been tested to make any generalizations about their behaviour.

Antibodies of the Lewis and P systems when they are active at 37° C. seem always to bind complement.

Discrepancies between various tests for incomplete antibodies. As described in Chapter XII, enzyme treatment destroys the antigens of the MNSs and the Fy systems. The corresponding antibodies can therefore not be detected with enzyme-treated cells. With this exception, there is usually good agreement between results with enzyme-treated cells and results with the indirect antiglobulin test. Although the antiglobulin test is more versatile, tests with enzyme-treated cells are a little more sensitive, particularly with antibodies of the Rh system. There is no doubt that occasional antibodies can be demonstrated with enzyme-treated cells but not with the indirect antiglobulin test. Thus when Rh antibodies develop during pregnancy, they may at first be demonstrable only with enzyme-treated cells and only later, when they are a little more potent, with the indirect antiglobulin test.

Tests for Haemolysis

Most human blood group antibodies do not lyse untreated red cells *in vitro*. In fact the only lytic antibodies in this sense are certain anti-A and anti-B sera, some anti-Le^a sera, occasional anti-Le^b sera and a few other rarities such as anti-P+−P$_1$ (anti Tj^a) and anti-Vel. The number of antibodies which will cause lysis *in vitro* is increased if enzyme-treated cells are used. For instance, almost all examples of anti-Le^a and anti-Le^b and anti-Jk^a will lyse ficin-treated red cells (Haber and Rosenfield, 1957).

Sometimes the animal's own complement may fail to bring about haemolysis, although the complement of another animal will do so. Thus, Todd and White (1911) found that fresh bovine serum containing isoantibody would not lyse bovine red cells unless fresh guinea-pig serum was added as a source of complement. Many human complement-binding antibodies fail to haemolyse untreated human red cells in the presence of human complement, but produce haemolysis in the present of rabbit complement (see Chapter XII).

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The Conglutination Reaction

Bordet and Streng (1909) showed that normal bovine serum would clump red cells which had been sensitized with an antibody and had also adsorbed complement; they described the clumping as conglutination, and used the term conglutinin to describe the substance in bovine serum that was responsible for this effect. Streng (1930) immunized rabbits with cells or bacteria which had adsorbed human complement. He obtained a serum which would agglutinate complement-coated bacteria or cells, and he described the antibody produced as immuno-conglutinin. Wartiovaara (1932) showed that immuno-conglutinin was also produced when bacteria alone were injected, i.e. the animal apparently produced the antibody as a result of stimulation by its own adsorbed complement. This was confirmed by Coombs and Coombs (1953), who described this kind of antibody as 'immuno-conglutinin (auto-stimulation)'.

Coombs, Blomfield and Roberts (1950) showed that C'_1, C'_2, and C'_4 were essential for conglutination and that they had to be adsorbed in a particular sequence. Thus, C'_1 had first to be adsorbed by sensitized cells, then C'_2 and C'_4 were adsorbed together. The role of C'_3 in conglutination was considered to be uncertain, but in all probability it is not needed, since zymosan-treated serum seems to supply all the necessary factors (Mollison and Polley, 1960). Thus:

EAC'_{142}+conglutinin (or immuno-conglutinin)——→agglutination.

Sensitized red cells which have been exposed to complement for more than 30 minutes react more weakly with immuno-conglutinin, and after about 4 hours fail altogether to react, suggesting that 'active' C'_{142} is needed for the reaction (Mollison and Polley, 1960; see also Gandini, 1959).

An immuno-conglutinin serum prepared in a rabbit is quite a useful reagent for demonstrating complement-binding by human blood group antibodies. The preparation and use of this reagent are described in Chapter XII.

Differences between the reactions of antiglobulin sera and I-K sera with red cells which have adsorbed complement. (1) The reactions of I-K serum are not inhibited by mixture with human serum; for instance, Le(a+) red cells sensitized with fresh serum containing anti-Le^a will react with a rabbit I-K serum to which undiluted human serum has been added. On the other hand, the reactions of an anti-human globulin serum with such cells will be inhibited by the addition of a 1 in 100 dilution of human serum. (2) An I-K serum will react with any adsorbed complement, whereas an antiglobulin serum is directed against the proteins of one particular species. (3) As already described, an I-K serum reacts only with red cells in the state 'EAC'_{142}' whereas an antiglobulin serum reacts with 'EAC'_{14}'.

Agreement between Different Tests for Complement-Binding by Blood Group Antibodies

Evidently the ability of an antibody to bring about haemolysis and to sensitize red cells to agglutination by anti-complement globulin (anti-β_1 globulin) serum or to agglutination by an immuno-conglutinin serum should all go together since all depend upon the ability of antibody to bind complement. Anti-Le^a is a good example of an antibody which gives positive results with all these tests, and anti-Rh is a good example of an antibody which fails to bind complement and so gives negative results. The results of testing a number of antibodies for complement-binding by different methods are shown in Table XVI.

TABLE XVI

TESTS FOR COMPLEMENT-BINDING BY HUMAN BLOOD GROUP ANTIBODIES

		Haemolysis			*Positive indirect antiglobulin reaction (anti-beta$_1$ globulin serum)*	*Positive immuno-conglutinin reaction*
		Normal red cells		*Enzyme-treated red cells*		
System	*Antibody*	*Human complement*	*Rabbit complement*	*Human complement*	*Human complement*	*Human complement*
ABO	anti-A	Some	Most*	Most	Some	...
Rh	anti-D	None	None	None	None	None
P	anti-P_1	Few	Few	Few	Few	? Few
K	anti-K	None	Some	? None†	Some‡	Some
Le	anti-Le^a	All	All	All	All	All
Fy	anti-Fy^a	None	Some	? None†	Some‡	Some
Jk	anti-Jk^a	Few	Most	Most	All	Most

* Dennis and Konugres (1959).
† Enzyme-treatment damages the K and Fy^a antigens.
‡ About 50 per cent of anti-K and anti-Fy^a sera sensitize cells to an anti-beta$_1$ globulin serum, i.e. bind complement.
... not tested.

Although with potent antibodies, complete agreement is obtained between the results of the different tests, with weak antibodies there may

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be discrepancies: the I-K test is the least sensitive and the antiglobulin test probably the most sensitive.

Occurrence of Immuno-conglutinin (I-K) in Human Sera

Marks and Coombs (1957) tested sera from more than 2000 apparently healthy subjects for the presence of I-K, using sheep cells sensitized with bovine antibody and treated with fresh horse serum as a source of complement. 64 per cent of the sera had a titre of only 1 or had no I-K; 13 per cent had a titre greater than 4. By contrast in patients with 'acute active rheumatoid arthritis', 88 per cent of the samples had a titre of 8 or more. I-K titres were also raised in acute virus infections and in acute rheumatic fever. In acute infections the I-K titre rose progressively during the first 14 days of the illness; that is to say, the results were compatible with the idea that I-K is an antibody produced in response to the presence of absorbed complement on bacteria or virus in the circulation.

Gm Groups and Antibodies against Gamma Globulin

Patients with Rheumatoid Arthritis

The serum of some patients with rheumatoid arthritis agglutinates Rh-positive red cells 'coated' with particular examples of potent, incomplete anti-D (Grubb, 1956b; Waller and Vaughan, 1956). Grubb and Laurell (1956) showed that the agglutination could be inhibited by adding the serum of about 60 per cent of European adults and these were said to be Gm(a+); the gene which in single or double dose determined the presence of the Gm^a antigen was termed Gm^a. The agglutination brought about by rheumatoid serum in these cases is therefore due to anti-Gm^a and in tests for the agglutination of Rh-sensitized cells the donor of the anti-D serum must be Gm(a+).

Harboe (1959) showed that those individuals who lack the Gm^a factor invariably possess another factor, Gm^b, whose respective gene in European subjects is allelic to Gm^a. At least two additional factors, Gm^x (Harboe and Lundevall, 1959) and Gm-like (Steinberg *et al.*, 1960), also belong to the Gm system. The inheritance of another gamma globulin factor, InV, is independent of the known Gm factors (Ropartz *et al.*, 1960) although it is detected in the same manner (i.e. inhibition of agglutination, by selected 'rheumatoid' sera, of cells coated with selected Rh antibodies).

The Gm antigens are 7*S* gamma globulin whereas rheumatoid factor is a 19*S* gamma globulin (Kunkel *et al.*, 1959). It appears that in the serum of patients with rheumatoid arthritis the rheumatoid factor combines

with 7*S* gamma globulin to form a dissociable complex, having a sedimentation constant of 22*S*. According to Harboe (1960) this *in vivo* combination of protein probably explains the frequent prozones exhibited by the serum of such patients when tested for Gm specificity. Sera containing the rheumatoid factor will precipitate with gamma globulin which has been aggregated by being heated at 63° C. for 10 minutes (Franklin *et al.*, 1957; Edelman *et al.*, 1958); they will agglutinate not only cells coated with human gamma globulin but also cells coated with gamma globulin from many other species (Glynn *et al.*, 1957a). There is an obvious parallel with sera containing conglutinin which will react with complement derived from many different species, provided it is absorbed on to an antigen-antibody complex.

Normal Subjects

Occasionally, samples of serum from healthy subjects will agglutinate Rh-sensitized red cells. In examining 2000 sera Milgrom *et al.* (1956) found 10 (0.5 per cent) with a titre of 8 or more. Similarly, Unger *et al.* (1958) in testing 3500 normal sera found 0.6 per cent with a titre of at least 2 (usually 10-40) and Ropartz *et al.* (1958) found about 0.3 per cent, among 400 normal subjects, with a titre of 4 or more.

Milgrom *et al.* (1956) showed that the reactions of the normal sera were not inhibited by the addition of normal human serum (see also Grubb, 1957). Similarly the sera were shown to react with Rh-positive red cells which had been coated with any anti-Rh serum (Unger *et al.*, 1958). In order to explain this effect, Milgrom *et al.* postulated that the gamma globulin became altered after being adsorbed on to the red cells and they showed that antibody eluted from Rh-coated red cells would inhibit the agglutination even though normal serum would not.

Ropartz *et al.* (1960) pointed out that the serum of a very small number of apparently normal subjects contains anti-Gm antibodies which are specific for a single Gm factor not possessed by the individual himself. Thus there is iso-antibody activity in some healthy individuals as opposed to apparent auto-antibody activity in arthritic subjects. On the other hand, this phenomenon has not been clearly differentiated from the behaviour of those normal sera described by Milgrom *et al.* (1956) which agglutinate red cells coated with anti-D regardless of the Gm type, and cannot be inhibited by the addition of human serum.

Milgrom and Dubiski (1957), have shown that antibodies against adsorbed homologous gamma globulin can be prepared in rabbits. Thus, rabbits injected with various bacteria agglutinated by rabbit immune sera produced an agglutinin

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which would react with sheep cells sensitized with antibody produced in a rabbit. Dubiski (1958) showed that the agglutinin would also react with Rh-positive red cells sensitized with human anti-Rh serum (cf. Glynn *et al.*, 1957a, see above).

The Serum Haptoglobin and Transferrin Groups

In 1955, Smithies demonstrated that haptoglobin, a seromucoid fraction of alpha-2 globulin with the property of combining with haemoglobin, could be differentiated by starch-gel electrophoresis into three different patterns. Subsequently, Smithies and Walker (1956) showed that the inheritance of these patterns is controlled by two allelic autosomal genes called Hp^1 and Hp^2. The resultant phenotypes are Hp 1-1, Hp 2-2, Hp 2-1 and, occasionally, Hp 0 (apparent ahaptoglobinaemia). Inheritance of Hp 0 is not well understood. Mutations of both Hp 1 and Hp 2 alleles have been described.

The concentration of haptoglobin in the serum varies according to phenotype in normal individuals, being greatest in those of type 1-1 and least in those of type 2-2; see Nyman (1959). It is increased in the presence of inflammatory disease, and decreases when red cell destruction is accelerated (see Chapter XIV). Only 5-10 per cent of newborn infants have a more or less distinct Hp pattern (Galatius-Jensen, 1958).

A second serum protein with molecular characteristics under genetic control is the iron-binding β1 globulin, transferrin; it appears that there are at least eight human transferrins which differ in their electrophoretic mobility (Smithies and Hiller, 1959; Giblett *et al.*, 1959). Each of these transferrins presumably represents a single autosomal gene, of which every individual possesses a pair. Thus, since most people have the single beta globulin called transferrin C, they are presumed to be homozygous for the gene, Tf^C. Turnbull and Giblett (1961) showed that the various transferrins do not differ in their ability to bind and transport iron.

Other Serum Groups

Allison and Blumberg (1961) examined the serum of sixty-one patients who had received many transfusions and in three of them they found an antibody directed against a serum protein outside the Gm, Hp and Tf systems. In the one case described in detail the antigen was shown to be an α_2 macroglobulin (but apparently not the 19*S* component of α_2). Sera which precipitated with the antibody were designated Ag(a+) and the gene determining the antigen was termed Ag^A. Heterozygotes as well as homozygotes were shown to be Ag(a+). The antibody, anti-Ag^A, was shown to be a 7*S* gamma globulin. The authors mentioned that the

specificity of the other two antibodies they encountered was different but they gave no further details.

Papers by Smithies (1959), Fraser *et al.* (1959) and others suggest that there are yet other serum proteins which have variable characteristics under genetic control. It is conceivable that as new techniques of investigation are developed the serum groups will be found to approach the red cell groups in number and complexity.

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CHAPTER EIGHT

ABO AND LEWIS GROUPS

It is now recognized that the blood group substances which characterize the ABO and Lewis blood group systems are of a closely similar composition, and it is thought that the genes which determine their inheritance, although inherited independently, act on a common precursor substance.

The A, B, H and Le^a blood group substances are mucopolysaccarides, containing the same four sugars and the same eleven amino acids (see Morgan, 1960). The close similarity between the substances is shown, for example, by the observation that the simple sugar L-fucose will inhibit both anti-H and anti-Le^a although serologically the antigens H and Le^a are quite distinct. It is thought that the differences between the blood group substances lie only in a few end-groupings, and that the genes determining the group characters act at a relatively late stage in the development of the substances (see Watkins and Morgan, 1957, 1959).

ABO GROUPS

The ABO blood group system, which was the first human blood group system to be discovered, remains the most important in transfusion practice. This is because of the regular occurrence of the antibodies anti-A and anti-B in persons whose red cells lack the corresponding antigens, (see Table XVII), so that if transfusions were to be given without regard to the ABO groups a considerable proportion would be incompatible. Table XVIII shows that in the British population the proportion would be about 1 in 3.

Subgroups of A

In Europeans about four-fifths of group A bloods belong to subgroup A_1, and one-fifth to subgroup A_2; similarly, four-fifths of AB samples are A_1B and one-fifth A_2B. For many purposes A_2 can simply be regarded as a weak form of A, but the difference is not simply quantitative, since there is an antibody, anti-A_1, which reacts predominantly with A_1 samples and which is found in the serum of a proportion of A_2B and A_2 people (see Table XVII).

Although A_1 red cells may undergo partial destruction after transfusion

to a recipient of subgroup A_2B whose serum contains a relatively potent anti-A_1 (see Chapter XIII), the subgroups of A can normally be ignored in selecting donors for transfusion. It appears that infants of subgroup A_2 have so weak an A antigen that they are protected from haemolytic disease due to anti-A (see Chapter XVI).

The main practical importance of the A subgroups lies in the fact that bloods of subgroup A_2, and particularly of subgroup A_2B, react weakly *in vitro* with anti-A sera and thus more care is required for their detection.

There are several ways of deciding whether a blood sample should be classified as A_1 or A_2. The oldest method is to use a group B serum

TABLE XVII

ANTIGENS AND ANTIBODIES IN THE ABO SYSTEM

Group	*Subgroups*	*Antigens in red cells*	*Antibodies (agglutinins) in serum*†
O	—	None*	Anti-A, Anti-A_1, Anti-B
A	A_1	A, A_1	Anti-B
	A_2	A	(Anti-A_1 in about 1% of subjects)
B	—	B	Anti-A, Anti-A_1
AB	A_1B	A+B, A_1	Nil
	A_2B	A+B	(Anti-A_1 in about 25% of subjects)

* With very rare exceptions human red cells contain the antigen H and the amount of this is influenced by the ABO group; thus, O cells contain most and A_1B cells least (see text).

† For incidence of anti-H(O), see Table XX.

absorbed with A_2 cells (see below). Another method is to partially neutralize an immune anti-A serum by adding A substance to it. When titrated in AB serum, such a partially neutralized anti-A serum will agglutinate A_1 cells but not A_2 cells (see Chapter XII). The third, and possibly the best, method of detecting A subgroups is to use an extract of the plant *Dolichos biflorus*. When diluted appropriately, such an extract will agglutinate only A_1 cells (Bird, 1952).

Forms of A even weaker than A_2 are occasionally recognized. Thus, the red cells of A_3 blood are only partially clumped by anti-A serum from a group B donor, many free cells being present (Friedenreich, 1936); the

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donor's serum sometimes contains anti-A_1 (Friedenreich, 1936; Dunsford, 1959; Salmon, Schwartzenberg and André, 1959a). About 1 in 1000 group A bloods belong to sub-group A_3 (Gamelgaard, 1942). The character A_3 was shown by Friedenreich (1936) to be inherited, but some blood samples which can be shown by family studies to be genetically A_2B behave as A_3B in grouping tests; that is to say they give only partial agglutination with anti-A sera (Salmon, Schwartzenberg and André, 1959b).

Other still weaker forms (A_x, A_4, A_5, A_0, A_m) have been described. It appears that these can be divided into two main classes. Red cells which are not agglutinated by serum from a group B donor but are agglutinated by serum from a group O donor may be called A_x, following the description of Fischer and Hahn (1935).

TABLE XVIII

CHANCE OF GIVING INCOMPATIBLE RED CELLS IF ABO GROUPS OF DONOR AND RECIPIENT ARE IGNORED IN TRANSFUSION

Recipient's blood group		*Blood groups of incompatible donors*		*Chance of incompatible transfusion*
	(i) Frequency		*(ii) Combined frequencies of donors*	*(i)×(ii)*
O	0.47	A, B, AB	0.53	0.25
A	0.42	B, AB	0.11	0.05
B	0.08	A, AB	0.45	0.04
AB	0.03	None	—	—
				0.34

Group A_m refers to cases in which the red cells are not agglutinated by anti-A although the saliva of the same individual contains approximately the normal amount of A. This kind of blood was originally described by Gamelgaard (1942), but since he used the name A_x, which had previously been used for another kind of blood (see above), the name A_m suggested by Wiener and Gordon (1956) is to be preferred.

Race (1957) has suggested that some very weakly reacting group A blood samples may be the result of the action of suppressor genes (see below) although there is as yet no proof of this. In a family investigated by Salmon, Borin and André (1958) the character A_m was found in two generations. The postulated suppressor gene (y) would have had to have been carried by three unrelated people in this family to have explained the finding. This seemed so unlikely that the authors preferred the explanation that in this family A_m was being inherited as an allele.

It seems likely that the character A_m may develop as an acquired change since

Salmon, André and Dreyfus (1959) reported that of four A_m samples they had tested, two came from patients with acute leukaemia.

Bloods with a Weak B antigen

It seems that only very rarely does a blood sample from an adult of group B or AB have a B antigen which is distinctly weaker than average.

In the case described by Yokoyama *et al.* (1957) the red cells were not agglutinated by anti-B or by most group O sera; occasional selected group O sera with immune anti-B produced very weak agglutination. The patient's saliva and serum contained B substance and the serum did not contain anti-B. The sample was therefore analogous to A_x (see above). A kind of B which the authors called B_3 was described by Moullec *et al.* (1955) and Sussman *et al.* (1969). The red cells reacted only with potent anti-B sera and with some group O sera. There was no B substance in the serum and the saliva contained H but not B.

The B_w of Levine, Celano and Griset (1958) differed slightly, in that agglutination of the red cells by potent anti-B was extremely weak and the saliva contained B substance.

Differences between group B samples from newborn infents were found by Engasser and Witebsky (1948) using partially neutralized immune anti-B sera.

In detecting weak B antigens, as in detecting weak A antigens it may be very helpful to expose the red cells to anti-B (or anti-A) and then to prepare eluates; it appears that on the whole, the weaker the antigen the more potent the eluate (see Celano *et al.*, 1957).

H Substance in Red Cells

Almost all human red cells contain H substance; this may be regarded as the basic substance from which the A and B substances are made under the influence of the *A* and *B* genes. It is thought that the *O* gene does not compete for this basic substance, and so group O red cells contain the most H substance, H substance cannot be a product of the *O* gene because H substance may be present in the secretion of individuals of the genotype A_1B (Watkins and Morgan, 1955). This point of view is only slightly different from that of Hirzfeld and Amzel (1940), who regarded the basic substance in red cells as a product of the O gene and considered that the *A* and *B* genes were mutations from *O*.

When tested with anti-H, human red cells tend to arrange themselves in the following order, according to the strength of their reactions: $O > A_2 > A_2B > B > A_1 > A_1B$. There are considerable variations between different samples of the same ABO group and a particular sample of A_2B red cells may react more strongly than a sample of A_2 cells.

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A and B Substances in Serum

Moss (1910) showed that group A serum contains a substance which will inhibit the haemolytic properties of an anti-A serum and Schiff (1924) demonstrated the presence of A substance in group A serum by immunization experiments in rabbits. Aubert, Boorman and Dodd (1942) tested samples from over 300 subjects and found that the sera of about 50 per cent of group A or AB subjects would inhibit anti-A and that the sera of almost 90 per cent of subjects of group B and AB would inhibit anti-B. This inhibitory effect of serum was found to be rather weak, being equivalent very approximately to that of a 1 per cent suspension of red cells, although the amount varied from one subject to another; the amount of B substance tended to be greater than that of A substance. There is a little evidence that the amount of A substance in the serum of A_2 subjects is less than in that of A_1 subjects (Jakobowicz and Bryce, 1941). The amount of A and B substances in the serum of 'secretors' is definitely greater than that in the serum of 'non-secretors' (Dr J. F. Mohn, personal communication). Patients with an increased amount of A substance in their sera have been described: one had a gastric carcinoma (Barber and Dunsford, 1959) and the other an ovarian tumor (Moullec *et al.*, 1960); in the latter case the ascitic fluid was even richer in A substance than the serum.

Possible Somatic Mutation

Atwood (1958) believes he has demonstrated a small proportion of A_2 or O cells in A_1 blood or A_2B cells in A_1B blood and has suggested that they may be the result of somatic mutation in the proliferating marrow. Further details of the method used have been published by Atwood and Scheinberg (1959).

Two types of experiment were performed. In the simplest, red cells from an A_1B donor were agglutinated by an extract of lima beans. The inagglutinable cells were extracted and found to react with anti-A but not with anti-A_1; they reacted more strongly with anti-H than did unselected cells from the same donor. In the second type of experiment the red cells of an A_1 donor were labelled with ^{51}Cr. After agglutination with, for example, anti-A_1 lectin, the inagglutinable cells were treated with unlabelled 'carrier' A_1 cells and more lectin. Eventually it was found that there was no further fall in the amount of ^{51}Cr in the supernatant cell suspension, and this was taken to indicate the existence of truly inagglutinable cells, i.e. not A_1.

Although the observations seem to show clearly the existence of cells relatively deficient in A in a 'population' of red cells from an A_1 donor, it does not seem necessary to postulate that such cells are *genetically* different.

Development of the ABO Antigens

The A and B receptors can be detected in the red cells of 5 or 6 week old embryos. Even at the time of birth the receptors are not fully developed (Kemp, 1930).

With anti-A agglutinating sera the difference between the reactions of infants' and adults' red cells is usually slight; with 'partially neutralized' immune anti-A sera the difference is considerable (Witebsky and Engasser, 1949). The difference can also be demonstrated by comparing the reactions with a serum containing anti-A haemolysin (Crawford *et al.*, 1953a).

A comparison between the reactions of adult A_1 cells, adult A_2 cells and infant A_1 cells with a group O serum is shown in Table XIX.

Infants may appear to belong to subgroup A_2 at the time of birth although they really belong to subgroup A_1; not only do they react more weakly as

TABLE XIX

THE REACTIONS OF CELLS FROM AN INFANT (SUBGROUP A_1) AND FROM ADULTS OF SUBGROUPS A_1 AND A_2, WITH GROUP O SERUM (DATA FROM CRAWFORD *et al.*, 1953a)

	Agglutinin titre	*Haemolysin titre*	*Indirect antiglobulin test*
Adult A_1	512	60	+++
Adult A_2	256	48	++
Infant A_1	512	18	(+)

judged by the indirect antiglobulin test but they also react more weakly with anti-A_1 (alpha-1) agglutinating sera (Witebsky and Engasser, 1949; Crawford *et al.*, 1953a).

According to Thomsen and Kettel (1929) the strength of A and B agglutinogens increases steadily during childhood and becomes maximal in adolescence; thereafter the strength decreases.

The red cells of newborn infants also react weakly with anti-H sera; by the end of 1 month the reactions are stronger and by the end of 6–12 months are as strong as the reactions of adult cells (Watkins, 1952).

Weakening of A Receptor in Acute Leukaemia

Van Loghem *et al.* in 1957 reported that a patient whose red cells had previously been grouped as normal A was later found to have red cells which were only partially agglutinated by anti-A. The patient had acute myeloblastic leukaemia, and the authors mentioned the possibility that the change in red cell reactivity might be due to this.

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Since then there have been several reports of weakening of A antigens in patients with acute leukaemia. Sometimes the blood has appeared to contain a mixture of group A and group O cells (Salmon, Dreyfus and André, 1958; Gold *et al.*, 1959) or of A_1 and weak A (Salmon, André and Dreyfus, 1959). In other cases the red cells have reacted weakly with anti-A, even behaving like A_3 or A_m samples (Salmon, André and Dreyfus, 1959). These latter authors pointed out that in the course of testing some 300,000 blood samples they had encountered twenty-two weak A samples (ten A_x, four A_m and eight A_3). All the ten A_x samples came from normal subjects but two of the four A_m and two of the eight A_3 came from patients with acute leukaemia. Like Gold *et al.*, they concluded that somatic mutation of the A gene in acute leukaemia was the most likely explanation.

B-like Antigen Acquired by A_1 Subjects

Cameron *et al.* (1959) described the finding of seven A_1 blood samples with an acquired B-like antigen. That is to say, the serum of each subject contained normal anti-B but the red cells were agglutinated by some anti-B serum. The reactions of the red cells with anti-B were never as strong as those of normal group B red cells. Five of the seven subjects had cancer and six of the seven were over 60 years old. In two of these seven cases the red cells were polyagglutinable.

Marsh *et al.* (1959) investigated a similar case. They showed that normal red cells treated with a powerful 'T'-activating bacterial enzyme behaved in the same way as the cells of their patient – that is the cells would take up anti-B and then yield an anti-B eluate.

Marsh (1960) extended these observations and showed that the substance on which the bacterial enzyme acts was not one of the ABH or Lewis substances. He concluded that the enzyme confers B specificity on some 'non-specific' substance present in all red cells. He also showed that normal serum inhibits the T-activating part of the enzyme but not the B-producing activity and suggested that this explains why only the cells with the strongest acquired B were polyagglutinable.

Andersen (1960) also encountered an example of this phenomenon. The anti-B serum with which the cells reacted was used to 'screen' 50,000 blood samples and five further cases were found. Subsequently two further cases were discovered and these, like the previous six, were all in group A_1 subjects.

In two of Andersen's eight cases, family studies were carried out; in one, there was no evidence of inheritance of the abnormal B, but in the other, two children of the propositus as well as one grandchild typed as A_3^b. Although Andersen classed this family as showing a variation of the phenomenon reported above (i.e. Pseudo-B), it seems more likely to the present author that in fact this family had a B gene determining a weak kind of B antigen. This supposition is supported by Andersen's finding that no anti-B was demonstrable in these A_2^b cases.

Secretors and Non-Secretors

The fact that A and B substances are present in the saliva of most individuals was first discovered in 1930 by Lehrs and by Putkonen (for reference to these and other early papers see Wiener, 1943, p. 275).

Approximately 80 per cent of subjects are 'secretors', and these secrete H substance irrespective of their ABO group; thus the saliva of group O secretors contains H and that of group A secretors contains A and H (Morgan and Watkins, 1948). The 20 per cent of subjects who do not secrete H substance ('non-secretors') do not secrete A or B either; if they are either homozygous or heterozygous for the Lewis (*Le*) gene, their saliva contains Le^a substance.

The amount of A substance in the saliva of group A 'secretors' follows a log-normal distribution. The amounts of A and H in the saliva appear to vary independently of one another, although there is a significant correlation between the ratio of A to H amongst the sibs in a particular family (Clarke *et al.*, 1960). Although McNeil *et al.* (1957) have described as 'aberrant secretors' subjects who secrete A without H or H without A, it seems more likely that the individuals concerned are at one end of the normal distribution curve described by Clarke *et al.*

Group specific substances have been found in seminal fluid, tears, sweat, urine, digestive juices, bile, milk, pleural, pericardial and peritoneal fluids, amniotic fluid and in the fluids of hydroceles and ovarian cysts (Wiener, 1943, p. 274). The relative amounts in different secretions are not necessarily constant for different blood group substances; for instance lactating women with a lot of A or H in the saliva have a lot of A or H in their milk but those with a lot of B in their saliva may secrete no B substance in the milk (Morganti *et al.*, 1959).

The ability to secrete A, B or H substances is inherited as a Mendelian dominant character. The relevant gene was formerly called *S* but is nowadays usually called *Se* (allele *se*) to distinguish it from *S* of the MNSs system. It should be noted that the term 'secretor' is applied only to those who secrete A, B or H substances in their saliva; it does not take into account the presence of Lewis substances in the saliva.

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Suppressor Genes

Xx. The first clear evidence of the existence of genes interfering with the expression of the ABO genes was provided by a case studied by Levine, Robinson, Celano *et al.* (1955). In their family the red cells of the propositus behaved like the 'Bombay' blood sample described earlier by Bhende *et al.* (1952), that is to say, the red cells were not agglutinated by anti-A, anti-B or anti-H; the serum contained anti-A, anti-B and anti-H, all active at 37° C. Family studies showed that the propositus was a carrier of *B* and *Se* (secretor gene), that is to say, these genes were passed on to her offspring, although she herself behaved like a group O non-secretor.

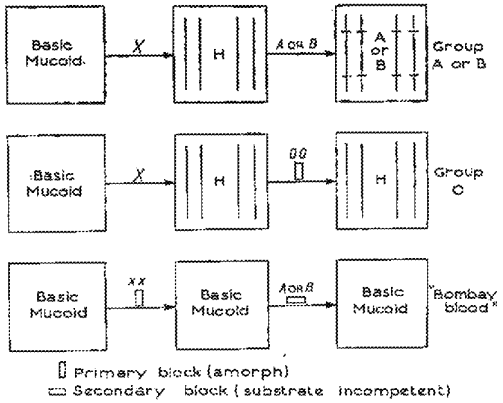

FIG. 55. A hypothetical scheme to illustrate the influence of the *ABO* and *Xx* genes on the development of red cell characteristics. The *X* gene is responsible for the conversion of 'Basic Mucoid' to H substance, which the *A* and *B* genes can then convert to A or B. The genes *x* and O are 'amorphs', being without effect. It is assumed that the *Se-se* locus has no detectable effect on the specificity of red cell antigens and that Lewis antigens are acquired from the plasma (after Ceppellini *et al.*, 1959).

Levine, Robinson, Celano *et al.* (1955) suggested that the suppressor gene responsible for the effect should be termed *x* (allele *X*); the gene, which is evidently very rare, when present in double dose prevented the development of A, B and H in the red cells and in the saliva.

The possibility that the behaviour of 'Bombay' blood samples was due to the operation of a suppressor gene was forecast by Ceppellini (1952); a suggested solution to explain the site of activity of the suppressor gene, based on Ceppellini *et al.* (1959) is shown in Fig. 55.

A blood sample with red cells containing A but almost no H was described by Levine (1960); the red cells were not agglutinated by anti-A_1

and Levine suggested that, as in the Bombay type of blood, H was suppressed by *x* or by an allele, x_1 which had only partially suppressed the agglutinogen A. Levine further suggested that a new type of blood should be referred to as A_h and the original Bombay type as O_h.

Yy. Weiner *et al.* (1957) have suggested giving the name *y* (allele *Y*) to the very rare gene which, in double dose, inhibits the development of the A antigen in red cells and, to a very much smaller extent, interferes with the development of A in the saliva. The case described by Wiener and Gordon (1956) as A_m, in which A substance was present in the saliva but the red cells were not agglutinated by anti-A, might then be regarded as A, *yy*.

Sese. se, the allele of *Se*, the secretor gene, may, in a similar way, be regarded as a gene which, in double dose, prevents the development of the water-soluble form of A, B and H (Race, 1957).

ABO Antigens in White Cells, Platelets and other Cells

White cells. Thomsen (1930a) found that although white cells (from patients with leukaemia) were only occasionally agglutinated by anti-A or anti-B sera, they would usually absorb the appropriate iso-agglutinin. Similarly, anti-A or anti-B can be eluted from white cells of group A or B subjects (Archer and Kooptzoff, 1958). Dausset (1954) found that white cells could be specifically agglutinated by using sufficiently potent antisera.

Bakemeier and Swisher (1957) pointed out that there is a tendency for leucocytes and platelets to adhere to red cells which have adsorbed antibody and complement. They found that in some preparations in which the leucocytes appeared under an ordinary microscope to be firmly agglutinated, examination with a phase-contrast microscope showed that the leucocytes were in fact adhering to red cells. They considered that their findings threw doubt on the previous claim that A and B were present in leucocytes. This doubt seems to have been resolved by Gurner and Coombs (1958), using the technique of specific mixed agglutination. Red cells were first exposed to anti-A (for example), then washed and exposed to leucocytes from subjects of different groups; only leucocytes from group A subjects formed mixed agglutinates.

Platelets. Gurevitch and Nelken (1954) found that A and B antigens were present in platelets: in many cases the platelets were specifically agglutinated by potent antisera, but in some cases the presence of the

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antigen could be demonstrated only by absorption experiments. The presence of A and B in platelets was also demonstrated by Coombs and Bedford (1955) using 'specific mixed agglutination'.

Other cells. The technique of specific mixed agglutination has been used to demonstrate the presence of A and B antigens in epidermal cells (Coombs, Bedford and Rouillard, 1956), cells of amniotic fluid (Fuchs *et al.*, 1956) and spermatozoa (Gullbring, 1957). Gullbring was able to demonstrate both A and B sperm in an AB subject.

Antibodies of the ABO System

Iso-agglutinins

If A is absent from a person's red cells, anti-A agglutinins are found in the serum, and if B is absent from a person's red cells his serum contains anti-B agglutinins (see Table XVII). The isoagglutinin titre in healthy subjects shows a normal distribution; thus a few subjects have only a trace of isoagglutinin (see below), a few have a titre of 1000 or more, and two-thirds of subjects have a moderate titre in the approximate range 16 to 128. The actual titration value ascribed to a serum is to some extent abitrary, since it is affected by technique. The effect of various factors is discussed in Chapter XII, and examples are given there of the very different titres reported by different authors (see Table XXXVI in Chapter XII). Anti-A and anti-B normally react more strongly at 4° C. than at 37° C.

Absence of 'expected' iso-agglutinins is very rare; Dobson and Ikin (1946) found only sixteen cases among 199,221 samples of blood. The sera from twelve group A and three group O persons failed to agglutinate B cells, and the serum of one group O person failed to agglutinate A cells. Halvorsen (1944) mentions the finding of one group O serum devoid of agglutinins in a series of about 5000. Dunsford (1953) described three group O bloods in which anti-A (or anti-B) seemed at first to be missing but were detected on closer examination; in one instance the serum contained normal anti-A but a weak incomplete anti-B; the undiluted serum weakly haemolysed, but did not agglutinate, B cells suspended in saline.

In hypogammaglobulinaemia the iso-agglutinins may be absent or present in only very low titre. In eighteen cases of congenital agammaglobulinaemia studied by Gitlin *et al.* (1959) only two had definite iso-agglutinins. These two patients were group A and their undiluted serum weakly agglutinated B cells. Out of forty-two patients whose serum gamma-globulin concentration was less than 200 mg./100 ml., and whose serum was tested in the author's laboratory (on behalf of the Medical Research Council Working Party on Hypogammaglobulinaemia) twenty-six had no iso-agglutinins and fourteen had iso-agglutinins with a titre of 4 or less. Only two out of the forty-two had iso-agglutinins with a normal titre and both these patients, despite having a low gamma-globulin concentration, had a normal or raised concentration of 19*S* globulin (Dr J. F. Soothill, personal communication).

It should be mentioned that in hypogammaglobulinaemia when the serum is examined by immuno-electrophoresis at least three different proteins are found to be missing or present only in very low concentrations, namely, gamma (7*S*) globulin, β-2M (19*S*) globulin and β-2A globulin (see Gitlin *et al.*, 1956; Grabar *et al.*, 1958).

Iso-agglutinins are also weak or absent in chronic leukaemia (Davidsohn, 1938) — perhaps only in chronic lymphatic leukaemia (Teitelbaum *et al.*, 1959) — and in multiple myelomatosis (Lawson *et al.*, 1955; Wiener *et al.*, 1958).

Anti-A titres tend to be a little higher than anti-B titres, either when comparing group B with group A subjects or when comparing the titres of the two antibodies in group O subjects. The anti-A titre in group O subjects tends to be higher than that of anti-A in group B subjects, although there is little difference between the anti-B titre of group O and group A sera (Thomsen and Kettel, 1929). Ichikawa (1959) confirmed all of these findings except that he found the titre of anti-B to be higher in group O than in group A subjects.

In group O subjects some antibody molecules behave as if they have the specificity anti-AB. This was first described by Landsteiner and Witt (1926), who found that if A cells were incubated with group O serum the antibody subsequently eluted from the A cells would react with B cells as well as with A cells. Dodd (1952) confirmed this observation, using immune group O sera.

Moss (1910) put forward a slightly different theory to explain why absorption with either A or B cells would reduce the titre of a group O serum against both A and B cells. He suggested that both A and B cells contained an antigen C and that group O serum contained anti-C. Wiener (1953) concluded that there was evidence in favour of the existence of such an antibody, although unlike Moss he considered that it was *additional* to anti-A and anti-B. Wiener and Wexler (1954) suggested that anti-C might play an important role in the causation of haemolytic disease of the newborn and explain why the disease occurs mainly in the offspring of group O mothers. Nevertheless, the existence of anti-C is not generally accepted (see Dodd, 1957).

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Anti-A_1. If serum from a group O or a group B person is absorbed with A_2 cells it will no longer agglutinate A_2 cells but will still agglutinate A_1 cells. On the other hand, if a group B or group O serum is absorbed with A_1 cells, it will no longer react either with A_1 cells or with A_2 cells. Accordingly, group B (or group O) serum is regarded as containing two separate populations of antibody molecules: anti-A, acting both with A_2 and with A_1 cells, and anti-A_1, acting only with A_1 cells. The anti-A_1 has a lower thermal amplitude than the anti-A (Friedenreich, 1931).

Anti-A_1 occurring as a separate antibody was found in 1–2 per cent of A_2 bloods and 25 per cent of A_2B bloods by Taylor *et al.* (1942). The figures refer to tests made at room temperature. Anti-A_1 occurring in A_2 and A_2B blood samples is usually inactive at 37° C. A rather higher incidence of anti-A_1 was reported by Speiser *et al.* (1951), namely 7.9 per cent in A_2 bloods and 35 per cent in A_2B bloods. The figures of Juel (1959) for the incidence of anti-A_1 in A_2B bloods, namely 22 per cent, in tests made at room temperature, are closer to those of Taylor *et al.*

Juel (1959) showed that if conditions were made more sensitive the serum of all A_2B blood samples could be shown to contain anti-A_1. Thus, he tested forty samples; the serum was absorbed at 4° C. for 2 hours with an equal volume of group O, P-positive red cells, and then tested against ficin-treated A_1 cells. At 5° C. all the sera agglutinated A_1 cells and at 18° C. thirty-six out of forty did so. More surprising was the fact that twenty-six out of the forty agglutinated ficin-treated A_2 cells at 5° C. and thirteen of them did so at 18° C. None of the sera reacted with ficin-treated O cells at 18° C. The fact that A_2 cells will react, even though only weakly and at low temperatures, with anti-A_1 was demonstrated by Lattes and Cavazutti (1924). Recognition of A_1 samples using a lectin from *Dolichos biflorus* is described in Chapter XII.

According to Speiser (1956a) anti-A_1 is very rare in infants; one example was found in examining 10,000 blood samples. Speiser produced some evidence that the presence of anti-A_1 in the serum is genetically determined. Thus the antibody was present in a pair of non-identical twins, in two mother and child combinations and in two siblings.

Clinical significance of anti-A_1. Examples, in patients of weak sub-groups, of anti-A_1 agglutinins which are active *in vitro* at 30° C. but only dubiously active at 37° C. bring about the destruction of only a proportion of A_1 cells *in vivo* (see Chapter XIII). Very occasionally anti-A_1 is clearly active at 37° C. *in vitro* and then can cause the destruction of a complete population of transfused A_1 cells.

Boorman *et al.* (1946) reported a case in which a patient of sub-group A_2 was transfused with at least seven bottles of A_1 blood within a period of 4 days. Seven days after the last transfusion the patient became jaundiced and anaemic and was found to have anti-A_1 in her serum active at 37° C. All the A_1 cells had been eliminated from her circulation although group O cells, which had been transfused 4 days before the A cells, were still present.

Another case was described by Salmon, Schwartzenberg and André (1959a). A patient of sub-group A_3 whose serum contained anti-A_1, with a titre of 2 at 37° C., was transfused with nine bottles of A_1 blood and one of group A of unknown sub-group. Three days after transfusion the patient's serum bilirubin concentration was 7 mg./100 ml.; the PCV was 42 per cent. Fourteen days later the PCV was only 22 per cent. The anti-A_1 titre showed no change during the 4 weeks after transfusion.

Anti-H (*O*). Schiff (1927) discovered an antibody which preferentially agglutinated O and A_2 cells, and found that the saliva of some group O individuals would inhibit the reaction. It was at first thought that the serum must be reacting with a product of the O gene, and the agglutinin was termed anti-O, but in the course of time evidence accumulated that the serum could not be reacting with a product of the O gene (see above). Morgan and Watkins (1948) reviewed this evidence and suggested that the substance secreted by O secretors should be called H. They later postulated that the *A* and *B* genes converted H, with varying degrees of efficiency into A and B substances, and that the O gene was without effect (Watkins and Morgan, 1955).

Anti-H is found as a cold agglutinin in the serum of a small proportion of A_1, A_1B and B subjects (see Table XX). It is recognized by the fact that it reacts preferentially with O, A_2 and A_2B cells (in that order) and less well with B and A_1 cells, and least of all with A_1B cells. Sometimes, an agglutinin giving these reactions is found not to be neutralized by secretor saliva (containing H). By convention those sera which are neutralized by secretor saliva are called anti-H and those that are not neutralized are called anti-O, although this terminology is not a very happy one and it is perhaps less confusing to think simply of two sorts of anti-H. Sanger (1952) pointed out that the kind of anti-H which is neutralized by secretor (H-containing) saliva is found in the serum of subjects with Le(a+) red cells, i.e. non-secretors of H, whereas the kind of anti-H which is not neutralized by secretor saliva is found in subjects who themselves secrete H (Sanger, 1952); see Table XX.

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TABLE XX

ANTIBODIES WITH THE SPECIFICITY ANTI-H(O) OCCURRING IN HUMAN SERA

Thermal range; active at: 0°	10°	25°	37°	*Serological characteristics* *Agglutinating*	*C′-binding*	*Inhibited by H substance*	*Occurring in* *Secretors or non-secretors*	*ABO groups*	*Notes*
+	(+)	–	–	No	Yes	Yes	Both	All	Normal incomplete cold antibody
+	+	+	±	Yes	Sometimes	Yes	Non-secretors	A_1, B, or A_1B	Anti-H
+	+	+	±	Yes	Sometimes	No	Secretors	A_1, B, or A_1B	Sometimes called anti-O because not inhibited by H substance
+	+	+	+	Yes	Yes	Yes	Non-secretors	...*	'Bombay' bloods

* In 'Bombay' bloods the expression of the ABO groups is suppressed; see Fig. 55.
+ = agglutination; (+) = weaker agglutination; ± = agglutination only in some cases; – = no agglutination.

An example of anti-O(H) which agglutinated cells only when they were suspended in citrate and not when they were suspended in saline was described by Jakobowicz and Bryce (1949); this is discussed briefly in Chapter XI.

Anti-H(O) occurring in group A_1 subjects may react with group O red cells at 37° C. and is then capable of causing rapid red cell destruction *in vivo*. In a case investigated by the author the serum agglutinated group O red cells strongly at 20-25° C. but only dubiously at 37° C.; at 37° C. the serum sensitized red cells to agglutination by an anti-$beta_1$ globulin serum, that is, the antibody fixed complement (Mollison, 1959d); some details of the effects which this antibody produced *in vivo* will be found in Chapter XIII.

Anti-H agglutinins almost as active at 37° C. as at 0° C. have been found in all examples of the very rare type of blood known as 'Bombay'.

Incomplete cold antibodies with a specificity anti-H are found in all normal sera. Dacie (1950b) showed that if red cells of any normal subject are exposed at 0° C. to a relatively large volume of the subject's own fresh serum and the cells are then washed in warm saline, they are found to be agglutinated by antiglobulin serum. The antibody was later shown to behave like anti-H in that it reacted more strongly with group O red cells, and less well with A_1B cells, and was neutralized by secretor saliva, but not by the saliva of non-secretors (Crawford *et al.*, 1953b).

Reagents with the specificity anti-H can be obtained from two plants: *Ulex europaeus* and *Lotus tetragonolobus*, or from the blood of the eel, *Anguilla anguilla*; the preparation and use of these reagents is described in Chapter XII.

Development of Iso-agglutinins

As explained in Chapter VII, the synthesis of gamma globulin normally begins only after the first few weeks of life, and the only antibodies found in newborn infants are those which have been derived by placental transfer from the mother. The question of the permeability of the placenta to different blood group antibodies is considered in Chapter XVII in connection with haemolytic disease of the newborn.

The infant starts to produce its own iso-agglutinins between the age of about 3 and 6 months (Morville, 1929). In some infants it is evidently a little difficult to say exactly when this begins because iso-agglutinins derived by placental transfer may be present for some weeks after birth, disappearing from the circulation with a half-time of the order of 20 days (see Chapter XVII). Yliruokanen (1948) tested various infants at intervals

RCPE0002068_0144

after birth and found that the proportion whose sera contained iso-agglutinins was as follows:

Birth	53 per cent
7–14 days	40 per cent
1–2 months	35 per cent
2–3 months	35 per cent
4–5 months	55 per cent
5–6 months	85 per cent

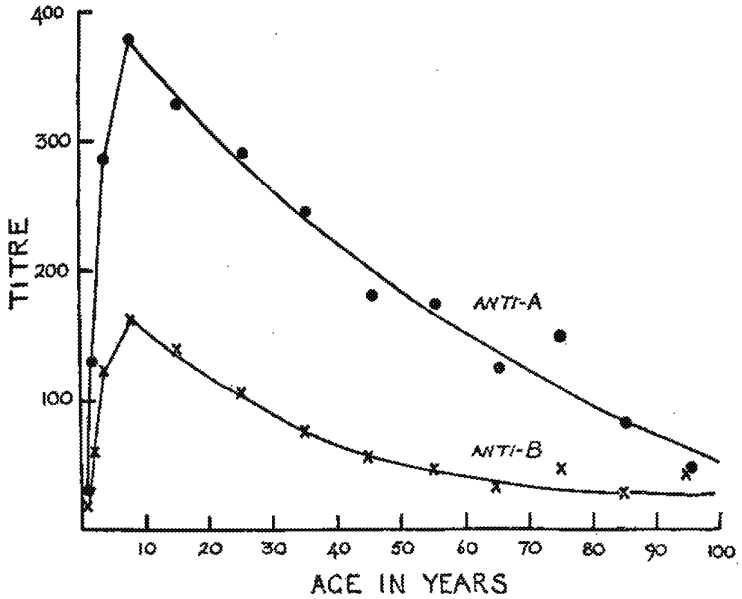

FIGURE 56. Anti-A and anti-B iso-agglutinin titres in group O subjects of different ages (from the data of Thomsen and Kettel, 1929).

These figures do not represent tests on the same infants and thus only indicate the general trend. However, they support the contention that the formation of iso-agglutinins commonly begins at 3 to 6 months.

The titre of iso-agglutinins reaches its maximum at the age of 5 to 10 years and thereafter falls gradually (see Fig. 56).

The amount of iso-agglutinin in the serum of an individual appears to be determined partly by heredity, for Carlinfanti (1948) has shown that there is a positive correlation between the iso-agglutinin titres of children and their parents.

'Immune' Characteristics of Antibodies of the ABO System

Some anti-A and anti-B sera are capable simply of agglutinating A and B cells suspended in saline; such sera are said to contain saline agglutinins, or 'naturally-occurring' anti-A or anti-B. Other anti-A or anti-B sera, particularly those from subjects who have recently received a known antigenic stimulus, are capable of haemolysing A or B cells; these sera usually display other characteristics, for example, after being mixed with A or B substance, although they will no longer agglutinate A or B red cells suspended in saline they will agglutinate the same cells in a medium of serum (Witebsky, 1948). These various characteristics are often referred to as 'immune'. Immune (haemolytic) anti-A and anti-B can apparently be either 19*S* or 7*S* γ globulin (Rawson and Abelson, 1960a) but it appears that 7*S* anti-A and anti-B are only rarely formed by group A or B subjects (Rawson and Abelson, 1960b; Kochwa *et al.*, 1961).

The immune characteristics will be considered in turn before discussing the antigenic stimuli which are known to stimulate their development.

Haemolysis (using Untreated Red Cells and Human Complement)

Anti-A or anti-B sera will haemolyse red cells only in the presence of complement. Accordingly, sera which have been inactivated by heating to 56° C. will not cause haemolysis.

Sera which are haemolytic can be shown to fix complement. Witebsky (1948) used amniotic fluid, previously shown to contain A or B substance, as a source of antigen for the test. Ervin and Young (1950) also noted that immune anti-A sera fix complement. They found that complement is fixed when an immune anti-A serum is added to A_2 or A_1 cells. This agrees with the observation that an anti-A serum with haemolytic properties will haemolyse A_2 as well as A_1 cells.

If increasing amounts of A substance are added to an immune anti-A serum, the haemolytic property is the first to be abolished, followed by the ability to agglutinate cells in saline and finally the ability to sensitize A_1 red cells to agglutination by an antiglobulin serum.

The ability to agglutinate cells in serum or to sensitize cells to an anti-human globulin serum (see below) is unaffected by a dose of A substance that will completely abolish the haemolytic property of the serum (Ervin *et al.*, 1950; personal observations).

Effect of increasing the sensitivity of the haemolysin test. The division of anti-A sera into immune and naturally-occurring is rather artificial and is probably best regarded as quantitative; that is to say, probably all anti-A sera contain at least

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traces of 'immune' anti-A although unless very sensitive tests are employed, only those sera which contain a relatively large amount of such antibody are commonly regarded as 'immune'.

Many anti-A and anti-B sera which are not otherwise lytic will produce lysis if they are absolutely fresh (tested within 2 hours of bleeding) and if a very weak (1 per cent) suspension of red cells is used (Thomsen and Thisted, 1928b). Similarly the number of anti-A sera found to be lytic is much greater if a 2 per cent cell suspension rather than a 5 per cent suspension is used (Chaplin *et al.*, 1956).

All anti-A sera will lyse group A red cells which have been left as a saline suspension for 1-4 days (Hesser, 1924; Thomsen and Thisted, 1928a). Similarly group A red cells stored as clotted blood or with oxalate for 1 week, or as ACD blood for 3 weeks, are more easily lysed than fresh red cells (Chaplin *et al.*, 1956).

Dacie (1949) found that all anti-A sera would lyse red cells from a group A patient with paroxysmal nocturnal haemoglobinuria. Some anti-A sera which are non-haemolytic when human, horse or guinea-pig complement is used become haemolytic when rabbit complement is used (Dennis and Konugres, 1959).

Reactions after Partial Neutralization

Witebsky (1948) first observed that if an immune anti-A serum is treated with a suitable amount of 'AB' substance (a mixed preparation of A and B substance) it may lose its ability to agglutinate cells suspended in saline. He added two parts of AB substance to one part of a particular 'immune' serum and found that the serum would then only weakly agglutinate A_1 cells suspended in saline and would not agglutinate A_2 cells at all. However, if the test cells were now suspended in serum instead of saline, the titre was actually increased.

Some of the characteristics of an immune anti-A serum are shown in Table XXI. Ervin, Christian and Young (1950) observed that an immune anti-A serum, after 'neutralization' with AB substance, would sensitize A_1 cells to an anti-human globulin serum, although it would no longer agglutinate cells suspended in saline.

Sometimes the presence of the incomplete form of anti-A may be overlooked if an excess of A substance is added to the serum. The technique of adding just sufficient A substance to abolish agglutination in saline is therefore recommended (see Chapter XII).

Resistance to Heating

Boorman and Dodd (1946) investigated the properties of an immune anti-A serum which exhibited zoning. They found that A cells exposed to the undiluted serum were 'blocked'; that is to say, they could not be fully agglutinated by subsequent exposure to anti-A agglutinin. Moreover,

TABLE XXI

CHANGES IN CHARACTERISTICS OF ANTI-A FOLLOWING THE INJECTION OF ANTI-TETANUS SERUM IN A TEN-YEAR-OLD BOY (GROUP O)

Dilutions of Group O Serum

			1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/8192	1/16384	
(-) 'partially neutralized'	Saline AB serum		++++ ++++	++++ ++++	++++ ++++	++++ ++++	++ ++	++	++	(++) (++)	W W	- -						A1 cells
	Saline AB serum		- -	- -	- -													A2 cells
(+) 'partially neutralized'	Saline		haemolysis	haemolysis	haemolysis slight	++++ ++++	++++ ++++	+++ +++	+++ +++	++ +++	++ +++	+ +++	(+) ++	W ++	- +			A1 cells
	AB serum		haemolysis	haemolysis	haemolysis	++++ ++++	++++ ++++	+++ +++	+++ +++	+++ +++	+++ +++	+++ +++	++ ++	++ ++	+ +	+	(+)	
	Saline AB serum				(+) +++	W ++	- ++	++	++	++	+	(+)						A1 cells

[a] One volume of serum was 'partially neutralized' by adding two volumes of saliva from a group A 'secretor'.

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these cells were agglutinated by an anti-human globulin serum. The original serum was heated to 75° C. for 20 minutes to destroy agglutinins. The serum now failed to agglutinate A cells suspended in saline but it did sensitize them to an anti-human globulin serum.

Personal experience suggests that it is best to dilute the serum one in three (in saline) to avoid precipitation of proteins, and that it may be necessary to try a range of conditions in order to demonstrate incomplete anti-A. In some sera in which incomplete anti-A can be demonstrated by 'partial neutralization' (see above), the presence of the antibody cannot be demonstrated by heating since it proves impossible to find conditions under which the agglutinin is destroyed without destroying the incomplete antibody.

Effect of Heating on Haemolytic Property

If sera containing anti-A haemolysin are heated to 56° C. for 30 minutes, the haemolytic property cannot always be completely restored by adding fresh complement. Tovey (1959) found that in about 20 per cent of sera, the haemolytic property could not be restored at all and in the remainder there was usually a fall in haemolysin titre.

It is uncertain whether the effect of heating is due to damage to the antibody or to the production of anti-complementary properties in the serum. If sera containing anti-Le^a are heated to 56° C. for 30 minutes and then tested after adding fresh Le(a−b−) serum as a source of complement, the titre is found to be lower than before heating; but if the heated serum is absorbed with kaolin and then mixed with fresh Le(a−b−) serum, its haemolysin titre is found to be the same as before heating. It is concluded that the effect of heating is to produce a substance — perhaps denatured gamma globulin — which deviates complement and that the antibody (haemolysin) is not appreciably damaged by heating to 56° C. for 30 minutes (Polley and Mollison, 1961). It is not known whether the same applies to anti-A haemolysin.

Enhancement of Titre by Using Serum as a Diluent

The agglutination of A and B cells by naturally occurring anti-A and anti-B is not intensified by carrying out titrations in serum instead of in saline, but the reactions of immune sera may be distinctly better in serum (see Table XXI). Not only is agglutination firmer, but the titre is greater (Boorman, Dodd and Morgan, 1945). The use of serum diluted one in three is just as efficient as the use of undiluted serum as a titrating medium.

The ability of different sera to enhance agglutination varies widely. Some are virtually ineffective, whilst others will increase the titre of an

immune serum fourfold or eightfold. Boorman and Dodd (1947) recommended that different samples should be tested to find one with the maximum enhancing power. At the same time the serum must be examined for the presence of A or B substances, since if these are present in appreciable amounts they will inhibit the agglutinating activity of the antibody.

Ervin *et al.* (1950) have shown that many anti-A sera which are not enhanced by titration in serum exhibit other characteristics which are probably to be regarded as 'immune' (see above). 'Enhancement by serum' must therefore be regarded as a comparatively crude test and a negative result does not exclude the possibility that the serum under test is immune.

The phenomenon of enhancement of agglutination resembles the agglutination by certain normal sera of red cells sensitized with Rh antibody, although the relationship between the two phenomena does not seem to have been investigated.

Replacement of serum by concentrated albumin does not enhance the reactions of anti-A or anti-B sera, whether they contain naturally occurring or immune antibodies. Indeed, the titre of an anti-A serum is often lower when albumin is used as the diluting medium than when saline is used.

Alterations in Thermal Amplitude

Naturally-occurring anti-A and anti-B sera react more strongly at 4° C. than at 37° C. By contrast, immune sera react equally well at 37° C. as at 4° C. (Wiener, 1941a), or even better at 37° C. (Hubinont, 1949).

As already explained, anti-A_1 is occasionally found as a cold agglutinin in the sera of subjects of subgroups A_2 and A_2B; when A_1 blood is transfused to such subjects there is normally no increase in the avidity of the antibody and no change in thermal amplitude (Boorman *et al.*, 1946, Cases 1 and 2). In one subject of group A_2B described by Stratton (1955a), the agglutinin did not become active at 37° C. even after repeated injections of A_1 blood.

Occasionally, an anti-A_1 agglutinin active at 37° C. is found in the sera of subjects of subgroup A_2 or A_2B; in four instances described in the literature the subjects concerned were affected with acquired haemolytic anaemia (Wiener, 1941a; Young, 1945; Boorman *et al.*, 1946). In some of these cases there was further evidence, apart from the mere finding of an anti-A_1 agglutinin active at 37° C., that the antibody was immune. For example, in one of the two cases described by Boorman *et al.*, the titre of anti-A_1 at 37° C. rose to a peak some 2 weeks after transfusion and then

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fell again. In the other case the agglutinin, having become active at 37° C. after the transfusion of A_1 blood, later fell in titre and was then active at room temperature and no longer active at 37° C.

Reactions in Saline with A_1 and A_2 Cells

Several authors have noted that A_2 cells are as strongly agglutinated as A_1 cells by immune anti-A sera, whereas naturally occurring anti-A sera usually react distinctly more strongly with A_1 cells (Wiener, 1941a; Hubinont, 1949). In the author's experience this phenomenon is variable. Sera obtained from volunteers immunized with A substance sometimes continue to react more strongly with A_1 cells than with A_2 cells.

Reactions of 'Immune' Sera with A^p Cells

Winstanley *et al.* (1957) showed that anti-A sera with immune characteristics, unlike anti-A saline agglutinins, would react with group A pig cells. They postulated that immune anti-A sera contained an extra antibody, anti-A^p, and they showed that the titre of this antibody was correlated with the anti-A haemolytic titre of the same serum. They further showed that absorption of immune anti-A serum with pig A cells removed the anti-A haemolysin but left the titre of saline agglutinins unaltered. In a further paper, Konugres and Coombs (1958) showed that absorption of immune anti-A serum with pig A cells removed the antibody detected by Witebsky's partial neutralization test (see above). Moreover, eluates from pig A cells were not easily neutralized by soluble A substance. Konugres and Coombs showed that the A^p component is present on group A red cells both of adults and of newborn infants.

Reaction of immune anti-B with B substance. There seem to be differences in specificity between naturally-occurring and immune anti-B, for Gibbons and Morgan (1952) found that B substance prepared from human ovarian cyst fluid was highly active in neutralizing immune anti-B (from persons who had been transfused with incompatible red cells or immunized with human B substance) but had negligible activity against anti-B agglutinins.

Relation of 'Immune Characteristics' to Known Antigenic Stimuli

If a parenteral injection of A substance is given to a person whose serum contains anti-A the serum will usually develop the immune characteristics described above, if it did not possess them beforehand. Some examples are described in the following section. Immune characteristics are quite frequently displayed by the serum of subjects who have received no

known antigenic stimulus. It seems very likely that in these cases the stimulus has come from A-like substances present in the tissues of many animals and, probably, in bacteria. It is known that the ingestion of antigenic material by mouth can stimulate antibody formation (Powell, 1925). Moreover, as described below, many prophylactic vaccines contain substances closely related to human A. It would therefore not be surprising if subjects who had recently received injections showed an increased incidence of immune characteristics in their sera. Crawford *et al.* (1952) found that group O donors who had received such injections were significantly more likely to have immune anti-A (or anti-B) in their serum than those who had not received recent injections. This could not be confirmed by Tovey (1958), who suggested rather that all individuals were subjected to A stimuli and that the development of haemolysin depended more on the response of the individual than on the stimulus received.

Correlation between Iso-Agglutinin Titre and Immune Characteristics

Most sera with moderate or low agglutinin titres will not haemolyse normal A or B cells; thus Crawford *et al.* (1952) found that among seventy-two group O sera with agglutinin titres below 200, only four haemolysed A cells. By contrast, of the nine sera with the highest agglutinin titres, five were haemolytic.

Most sera which are capable of haemolysing cells will also sensitize them to an antiglobulin serum. Thus Crawford *et al.* (1952) found that among twenty-two haemolytic sera, sixteen gave good positive indirect antiglobulin reactions.

Gardner and Tovey (1954), in testing the sera of nearly 1000 group O donors, found that the antibodies detected by the indirect antiglobulin test were most often found in association with anti-A haemolysins; the antibodies which most strongly sensitized red cells to an antiglobulin serum were confined to sera containing strong anti-A haemolysins.

As mentioned above, discrepancies between the agglutinin titre and the presence or absence of immune characteristics are not uncommon. Some examples are shown in Table XXII. In devising a screening test for group O donors whose blood may be transfused to recipients of other groups a knowledge that these discrepancies exist is important.

Injections of Incompatible Blood and Group Specific Substances

Incompatible transfusions. If A blood is injected intravenously into a person of group B or group O, or B blood is injected into a person of

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group A or group O, the titre of anti-A or anti-B increases after a few days. Moreover the serum usually acquires immune characteristics.

Perhaps the first record of such a response is that of Thalheimer (1921). His patient, a boy aged 6, received a first transfusion from his father without incident; however a second transfusion from his father 18 days later produced a severe haemolytic reaction. It was now found that the father was group B and the child group O. Direct matching before the first transfusion had not revealed incompatibility, and it may be presumed that at that time the child had only a low titre of anti-B. Because of the success of the first transfusion, it was considered unnecessary to carry out a direct matching test before the second transfusion, although had the test been repeated the incompatibility would presumably have been obvious.

Rø (1937) studied a group O patient who received 500 ml. of group A

TABLE XXII

DISCREPANCIES BETWEEN PRESENCE OF POTENT ANTI-A AGGLUTININ AND IMMUNE FORMS OF ANTI-A (FROM CRAWFORD *et al.*, 1952)

Group O donors	*Titre in saline solution*	*Indirect antiglobulin test*	*Haemolysis*
Case 98	64	+++	+++
Case 49	512	0	0
Case 26	64	(+)	++

blood. The anti-A titre rose after transfusion and subsequently fell again. Wiener (1941a) showed that there might be an initial fall in titre, immediately after an incompatible transfusion, presumably due to absorption of antibody by the transfused erythrocytes. This was also shown by Mollison and Young's (1941b) patient (group O) who received 360 ml. of group B blood. Eight hours after transfusion the anti-B titre was only 2 compared with an anti-A titre of 128. After 48 hours the anti-B titre started to increase, and between the sixth and tenth days it rose very rapidly to a peak. It then fell slowly and, 3 weeks after transfusion, had still not reached a stable level (see 'anti-B' in Fig. 57).

In the five cases studied by Boorman, Dodd and Mollison (1945) the peak of the increase in iso-agglutinin titre occurred between 7 and 14 days after transfusion. In Mollison and Young's case (1941b), daily estimations were made for the first 12 days; the peak was reached between 9 and 10 days after the incompatible transfusion.

Evidently the initial decrease in titre is less likely to occur when only small amounts of incompatible blood are transfused. For example, Fig. 58 records the changes in titre of anti-A and anti-B in a group O patient who received 45 ml. of group A blood; 24 hours after transfusion the titre of anti-A was 512 (titre of anti-B, 128).

Nevertheless, Wiener, Samwick *et al.* (1953b) found a considerable drop in titre in some group O subjects after the intravenous injection of as

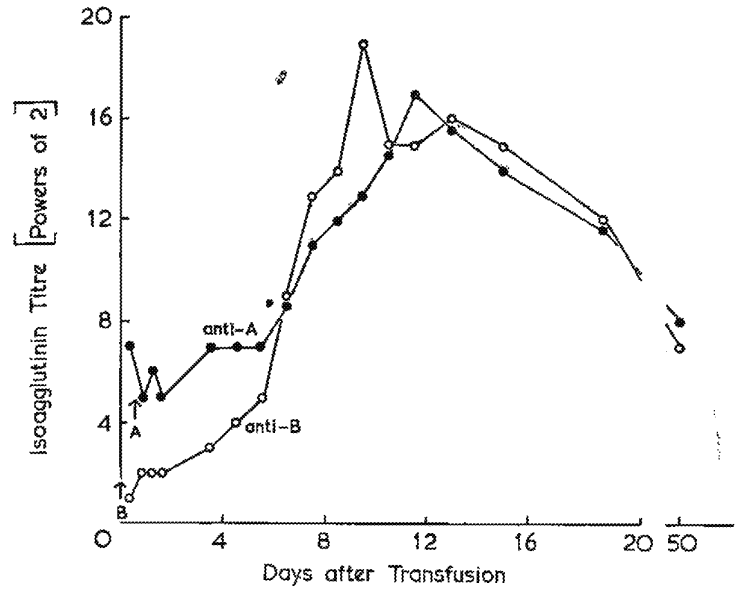

FIGURE 57. Changes in iso-agglutinin titre in a group O patient following the transfusion of 360 ml. of group B blood (at 'B') and of group A serum (at 'A'). These titrations and those recorded in Fig. 58 were performed by the standard technique of making doubling dilutions, using the same pipette, and were not checked by making direct dilutions (from the data of Mollison and Young, 1941b).

little as 25 ml. of A_1 or A_2 blood to fifteen subjects; 10 days later only six of the fifteen subjects showed a significant increase in titre. No tests appear to have been made for subsequent changes.

Dunsford (1952) injected small amounts (25 ml.) of washed red cells from a donor of group A_4 into volunteers of group B and group O. Ten days after the injection there was little change in the anti-A titre of the group B person, but serum from the group O person now haemolysed A_1

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cells and the saline agglutinin titre had risen from 64 to more than 4096 with A_1 cells and from 16 to 256 with A_2 cells.

Intramuscular injections of blood. Thomsen (1930b) gave intramuscular injections of group A or B red cells to forty-seven subjects of other groups. Four to six injections were given, each of 10–15 ml. of a 50 per cent suspension of twice-washed red cells. The intervals between the injections were not stated, nor were details given of the days on which the subject's

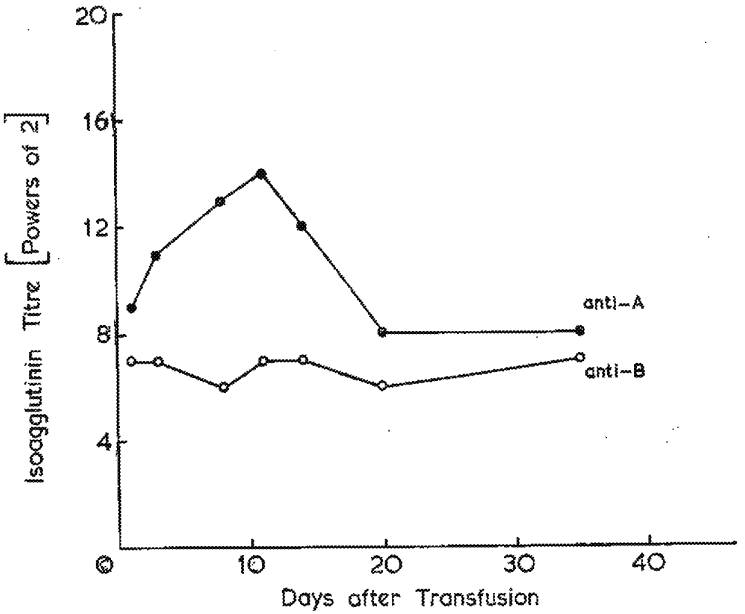

FIGURE 58. Changes in iso-agglutinin titre in a group O patient following the transfusion of 45 ml. of group A blood (data from Mollison, 1943a).

serum was tested. No significant alterations in iso-agglutinin titre were observed, but in eight cases in which group A blood was injected into group O or group B subjects there was a considerable increase in the haemolytic power of the subject's serum. Before the injection in these cases the serum failed to haemolyse A cells at room temperature or did so only very weakly. After the injections the serum caused almost complete haemolysis at room temperature, even at a dilution of one in two. The serum also haemolysed group B cells and this led Thomsen to conclude

that there was no evidence of specific stimulation; however, as described below, it is not uncommon for anti-B to be stimulated by an A antigen.

Wiener (see Wiener, Soble and Polivka, 1945) injected 'substantial amounts of red cell stromata without producing any appreciable change in iso-agglutinin titre', but did not refer to the development of haemolysins.

The observations of Rapoport and Stokes (1937) like those of Thomsen, suggest that the production of haemolysins may be provoked by intramuscular injections.

During an epidemic of poliomyelitis Rapoport and Stokes carried out an investigation of the prophylactic value of intramuscular injections of blood. Sixty ml. of blood were taken from one or other of a child's parents and injected into the child's buttocks. Amongst 1341 children, fifty-two showed a reaction which followed a characteristic pattern: some 6 days after the injection the child became acutely ill and febrile; the sites of injection became hot, red and painful. The reaction subsided after 3 to 4 days. In seventeen cases the blood groups of the child and its parents were investigated; in every case the parent's cells were incompatible with the child's serum. In thirteen out of seventeen cases in which the test was made within 4 weeks of injection, the child's serum haemolysed the parent's cells. In one case a child who had had a reaction was given a second injection of blood some months later; in this case the second reaction developed only 28 hours after the injection.

Skin grafting. Immune responses have been observed following skin grafting in cases in which the donor and recipient have different blood groups. Thus Dr D. M. Parkin (personal communication) has observed the development of anti-A haemolysin in a group B infant grafted with skin from a group A donor. It appears that the development of red cell antibodies does not depend on the presence of red cells in the donor's skin since, in mice, homografts comprising only superficial dermis stimulate the production of red cell antibodies (Amos *et al.*, 1954).

The presence of A and B antigens in human epidermal cells has recently been demonstrated by Coombs, Bedford and Rouillard (1956) using the technique of 'specific mixed agglutination'. An example will illustrate the method: epidermal cells from a group A subject were first exposed to anti-A serum, then washed and mixed with group A red cells. The red cells were found to adhere to the epidermal cells, forming 'specific mixed agglutinates'. The reaction was strictly specific; for example, no mixed agglutinates were found between group B epidermal cells and group A red cells. Epidermal cells have also been shown to be capable of adsorbing anti-A and anti-B by Nelken *et al.* (1957).

Injections of human serum. The A and B group-specific substances are present in small amounts in the plasma and serum, and the transfusion of

RCPE0002068_0150

group A or group B serum, freed from all red cells, to persons whose plasma contains anti-A or anti-B, provokes an immune response (Aubert, Boorman and Dodd, 1942). If A serum is injected intravenously, the titre of anti-A agglutinin rises, and if B serum, that of anti-B. Sometimes there is a non-specific response, i.e. after giving group A serum to a group O subject, both the anti-A and the anti-B titre may rise; the non-specific response is usually much smaller than the specific response.

The peak of the immune response is usually reached at about 10 to 15 days after the transfusion, although occasionally it is reached earlier.

Some of these observations were confirmed by Mollison and Young (1941b) in the course of investigations made on a patient of group O who had received incompatible blood (group B); the titre of anti-A as well as that of anti-B rose to a very high level (see Fig. 57). The increase in anti-A titre could be ascribed to a transfusion of group A serum that the patient had received immediately after the blood transfusion. In two further cases group A serum was given to two group O patients and shown to produce a rise in the titre of anti-A but not of anti-B. The highest titres were reached between the seventh and twelfth days after transfusion.

Aubert, Boorman and Dodd (1942) succeeded in producing immune responses to samples of A serum which contained so little A substance that its presence could not be demonstrated by tests *in vitro*.

Injections of human saliva. Wiener, Soble and Polivka (1945) found that the intramuscular injection of the saliva of A and B secretors would produce immune responses. Wiener, Samwick *et al.* (1953a) found that the response to the intramuscular injection of 1 ml. of boiled, autoclaved saliva was much smaller than the response to the intramuscular injection of 2 ml. of commercial blood group substances. Thus for purposes of immunizing volunteers, the group-specific substances of animal origin are to be preferred.

Injections of human urine. Freda *et al.* (1957) found that following injections of pooled urine (used for the content of gonadotrophin) considerable increases in anti-A occurred in two out of four group O subjects.

Injections of human group-specific substances. Loutit and Morgan (1946) gave intramuscular injections of purified A substance from human pseudomucinous ovarian cysts and observed immune responses. Sickles and Murdick (1953) prepared purified material from selected human meconium; the responses to injections of this material seemed to be at least as good as responses to animal substances.

Injections of purified group-specific substances of animal origin. An experiment made by Forssman (1911) revealed the existence of serologically related substances in animals widely separated in the zoological system. Forssman showed that the injection of extracts of guinea-pig organs into rabbits provoked the formation of lysins active against sheep red cells. Subsequently the name 'Forssman antigen' was used for any substance which would stimulate the formation of sheep haemolysin. Schiff and Adelsberger (1924) showed that the Forssman antigen was allied to the human A substance; Andersen (1931) found that the Forssman antibody could not be completely absorbed by human A cells and the position was summarized by Landsteiner (1947, p. 99): 'Forssman reactions ... are due to similarity rather than identity of the respective substances.' Landsteiner suggested that the polysaccharides which determine the specificity of these substances are made up of relatively few 'building-stones', so that there is considerable likelihood of overlapping reactions due to the presence of similar specific groupings. The relation between human A substance and a similar substance in some animals is referred to below in discussing the role of vaccines in stimulating group-specific antibodies in man.

The fact that polysaccharides occurring in some animals closely resemble the human blood group substances was put to practical use by Witebsky and his colleagues (Witebsky *et al.*, 1944; Witebsky, 1946) who prepared A substance from hog stomach and AB substance from horse stomach. Unless otherwise specified, these preparations are meant when the term 'group-specific substance' is used below.

Witebsky *et al.* (1944) found that the intravenous injection of as little as 0.1 mg. of purified group-specific substance to human subjects might produce a very large increase in iso-agglutinin titre and in the 'avidity' of the serum. In one case a slightly larger injection was given intramuscularly and this also produced a good response.

Boyd and Lowell (1946) gave intramuscular injections of 1.0 mg. group A specific substance to twenty-two volunteers of group O and noted that in sixteen there was a fourfold increase in anti-A titre. Several subjects had small rises in anti-B titre, but this seemed to be due to the contamination of the 'purified A substance' with traces of B substance. Many other workers have used injections of group-specific substances to prepare potent sera for grouping purposes. Thus Stanbury (1949) observed a sixteenfold increase in anti-A titre 7 days after injection in subjects given 0.1 ml. of commercial A and B substances intravenously. Fourteen days after a second injection, the titre showed a further eightfold increase. A few subjects responded poorly. Sickles and Murdick (1953) found that when 0.2 ml.

RCPE0002068_0151

of animal A and B substance was injected intravenously a substantial rise in titre occurred within 10 days in most subjects; further injections at 10-day intervals caused no further rise.

The relative blandness of commercial preparations of A and B substance was demonstrated by McNeil *et al.* (1950). On 3 successive days, two volunteers received 10 ml. (10 mg.) of combined A and B substance. The injections were completed in 5 minutes without reaction. After each injection the recipient's iso-agglutinin titre fell to 0. Two days after the last of the three injections, the iso-agglutinin titre had risen to about 10,000 having been only 28 to 56 before injection.

In ten other subjects who were given 5 mg. of group-specific substance intravenously 1 day and 7 mg. intravenously 2 days later, there were also no important ill-effects. However McNeil *et al.* refer to a case, brought to their attention, of a boy who developed severe signs following an intravenous injection of 2 ml. of commercial A and B substance. Itching, urticaria and dyspnoea were followed by stupor and a fall of blood pressure to 60 mm. of mercury. The signs were relieved by the intravenous injection of adrenalin. The boy was shown to be sensitive to horse serum.

Dr R. E. Rosenfield (personal communication) has seen very bad collapse with flushing after an intravenous injection of as little as ½ ml. of a preparation of animal group specific substance, and recommends injecting 10 ml. intramuscularly to stimulate agglutinins.

The group-specific substances are sometimes used to neutralize anti-A and anti-B in group O plasma, when using group O blood for transfusion to other groups. It is important to remember that in such 'neutralized plasma' there is sufficient 'free' group-specific substance to act as a stimulus. Tisdall, Garland and Wiener (1946) made observations on twelve subjects who had received 'neutralized plasma' and found an increase in iso-agglutinin titre in all cases; on the average the titre increased sixfold. They pointed out that this implied that it might not be a good practice to add group-specific substances to blood that was going to be transfused to female recipients, for it might increase the risks of damage to A and B foetuses during a subsequent pregnancy.

Injections of vaccines. Ottensooser (1932) noted that preparations of diphtheria toxoid contained A substance, and showed that this came from the peptone used, which in turn was derived from hog stomach. Ottensooser and Willenegger (1938) pointed out that this explained the rise in anti-A seen in humans after the injection of diphtheria toxoid. Balgairies and Christiaens (1937) showed that tetanus toxoid and diphtheria toxoid (but not TAB) injections produced rises in iso-agglutinin titre and this was attributed by Moullec (1947) to hog pepsin present in the preparation.

At least some preparations of TAB stimulate anti-A and anti-B and cause not only a rise in agglutinin titre but also the development of immune characteristics (Crawford *et al.*, 1952). The same authors showed that immune anti-A and anti-B were produced following the injection of tetanus toxoid. Similar findings were reported by Montandon (1952).

Jakobowicz *et al.* (1959) tested the hypothesis that individuals exposed to A substance in foetal life might be partially tolerant to A. The anti-A response to a preparation of tetanus toxoid rich in A substance was investigated in relation to the ABO group of the subject's mother, and was found to be significantly higher in subjects with group O mothers than in those with group A mothers.

Hendry and Sickles (1951) showed that if tetanus toxoid was prepared in a medium free from peptone of animal origin it did not cause any rise in iso-agglutinin titre.

The practical importance of injections of vaccine containing hog pepsin in rendering group O donors 'dangerous' was shown by Dausset and Vidal (1951); they described six haemolytic reactions following the transfusion of group O blood to group A recipients; in every case the donor had received injections of vaccine known to contain hog pepsin. In most cases the donors had received the injections within the previous 3 months, but in two instances it was shown that the immune characteristics of the donor's serum persisted for at least 7 years after the last injection.

Injections of horse serum. Davidsohn (1938) found that the injection of horse serum might stimulate anti-A and anti-B and observed that such responses were most pronounced in subjects who developed serum sickness. The injection of anti-tetanus (horse serum) usually produces not only a rise in iso-agglutinin titre but also the development of immune characteristics (Crawford *et al.*, 1952; see also Table XXI). However, the most important source of A substance in anti-tetanus serum is not horse A substance but hog A substance from the pepsin used in digesting the serum (André *et al.*, 1952). Although substances resembling human A and B are found in horse saliva (Friedenreich and Thyssen, 1937) and in horse serum (Zoutendyk, 1952), horse A substance is not so closely related to human A as is hog A substance (Kabat, 1952).

Iso-Immunization to the A and B antigens during Pregnancy

When A or B substances are present in the foetus, but not in the mother, it is convenient to speak of the pregnancy as 'heterospecific'. Hirzfeld

RCPE0002068_0152

originally applied this term to all pregnancies in which the groups of mother and infant were different. However it is now accepted that the foetus *in utero* is incapable of forming antibodies and that the possibility of incompatibility between the red cells of the mother and the plasma of the foetus can therefore be ignored. When the red cells of the foetus contain an antigen (derived from its father) which is foreign to the mother, various interactions between this antigen and the maternal tissues may take place. Dienst (1905) first observed that, after delivery, there might be a rise in the mother's anti-A or anti-B iso-agglutinin titre if the infant belonged to group A or B.

Jonsson (1936) made a systematic study, but looked for haemolysins only. He examined the sera of 594 group O mothers, 3 weeks after parturition. 14.5 per cent of the sera haemolysed either A cells or B cells or both. By contrast, in a series of 636 group O men he found iso-haemolysins in only 3.5 per cent. The relationship of the iso-haemolysins to a previous heterospecific pregnancy was strikingly shown when the infant's blood group was also taken into account. Thus in the mothers with O children the incidence of iso-haemolysins was 7.1 per cent; in those with B children it was 20.8 per cent, and in those with A children 28.1 per cent.

Boorman, Dodd and Mollison (1945) studied the changes in iso-agglutinin titre during the 3 weeks following delivery. They found that after delivery the titre of the relevant iso-agglutinins usually increased considerably. In 80 per cent of the cases the increase occurred between 5 and 20 days after delivery (most commonly between 10 and 20 days). Only eight out of forty-four cases of heterospecific pregnancy failed to show this response. Smith (1945) added the very valuable observation that the infants who failed to stimulate their mothers' iso-agglutinins were non-secretors of the A and B substances. He found a rise in iso-agglutinin titre in forty out of forty-six cases, and in all these forty the infant secreted either A or B substance in its saliva. Unlike Boorman *et al.* (1945) and Hurst *et al.* (1946) he frequently noted a rise in the titre of anti-A or anti-B during the last month of pregnancy.

Zuelzer and Kaplan (1954a) found no change in iso-agglutinin titre and no development of immune characteristics during heterospecific pregnancy, but observed these changes within 3 weeks of delivery when the infant was a secretor.

The relationship between heterospecific pregnancy and haemolytic disease of the newborn due to ABO incompatibility is discussed in Chapter XVII.

Persistence of Immune Anti-A

Allen and Kabat (1958) found that after an injection of hog A substance or horse A and B substances the 'peak' value of antibody tended to be maintained for many months. There was then a steady fall but even after 12 months the value was still about 50 per cent of the 'peak'. They pointed out that the response to polysaccharide antigen is usually much more prolonged than that to protein antigens, for example, diphtheria toxoid.

Tovey (1958) found that in blood donors whose serum contained anti-A or anti-B haemolysins, repeated tests during a two-year period showed that these antibodies persisted in 80 per cent of donors, but he pointed out that this may have been due to the constant ingestion of A-like or B-like antigens.

THE LEWIS BLOOD GROUP SYSTEM

The Lewis blood group system differs in some important ways from some of the other human blood group systems. First, it seems that the system is primarily one of saliva antigens (Grubb, 1951) and of serum antigens rather than red cell antigens, and that the red cells acquire their Lewis phenotype simply by adsorbing Lewis substances from the serum (Sneath and Sneath, 1955). Second, the Lewis phenotype is influenced by the secretor genotype, so that if a person inherits the Lewis gene *Le* he will have the phenotype Le(a+b−) if he is a non-secretor (*sese*), but the phenotype Le(a−b+) if he is a secretor (*Sese* or *SeSe*) (Grubb, 1951; Ceppellini, 1955). Third, the Lewis phenotype is modified by the ABO phenotype; thus A_1 may interfere with the expression of the Le^b antigen (Andresen, 1948) and with the expression of the Le^a antigen (Cutbush *et al.*, 1956).

The Lewis blood group system shows many resemblances to the J blood group system in cattle and to the R blood group system in sheep. In all three systems the antigens are acquired passively by the red cells from the serum. Thus, J-negative cells can be transformed into J-positive cells by incubating them with the serum of J-positive animals (Stormont, 1949), and a similar transformation can be carried out with R-negative cells in sheep (Rendel *et al.*, 1954). J is serologically related to the human A antigen (Stormont, 1949), as is R (Neimann-Sørensen *et al.*, 1954); r, the allele of R, is related to H (Neimann-Sørensen *et al.*, 1954).

Watkins and Morgan (1959) proposed a scheme to explain the inter-relationship between the ABO and Lewis blood group systems and the influence of the secretor gene; the scheme was primarily concerned with the occurrence of blood group substances in saliva. They suggested that the Lewis gene could be regarded as responsible for transforming a

RCPE0002068_0153

precursor substance into Le^a substance which was in turn transformed (but only in 'secretors', i.e. those of genotype *SeSe* or *Sese*) into H substance and Le^b substance. These substances were then acted upon by the gene determining AB. Thus a person carrying the genes *Le, Se* and *A* would have in his saliva Le^b substance (with some unconverted Le^a substance), as well as H substance and A substance; a person with the genes *Le, se* and *O* would have only Le^a substance. A person lacking the gene *Le* would have no Lewis substance in the saliva but it was suggested that the *Se* gene could act on the theoretical precursor substance to produce H substance, thus explaining the presence of H substance (with or without A and B) in the saliva of persons of the genotype *lele, SeSe* or *Sese*. Those of the genotype *lele, sese* would not be expected to have H substance or Lewis substance in their saliva, nor would they be expected to have A or B substances since it is postulated that these substances are formed by the conversion of H substance.

Watkins (1959) proposed an extension of this scheme to explain the occurrence of these various blood group substances on red cells.

s' (*se*) is a gene which in double dose suppresses the transformation of precursor substance, or Le^a substance, to H in the formation of 'secretor' substances, e.g. H in saliva. *s'* does not affect the formation of blood-group substances on red cells and therefore does not prevent the formation of H in the red cells of non-secretors.

Genetics of the Lewis Blood Group System

It was at first thought that *Le^a* was a recessive gene, all Le(a+) subjects having the genotype *Le$^aLe^a$*, but it is now known that a mating between two such subjects can produce Lewis negative (Le(a—b—)) children (Ceppellini *et al.*, 1959). As mentioned above, the phenotypes (Le(a+) and Le(b+) are now thought to be both expressions of the gene *Le* acting either in single or in double dose, Le(b+) being the result of the combined action of the genes *Le* and *Se*, and Le(a+) being the result of *Le* alone (see Table XXIII). The independent inheritance of the Lewis genes and secretor genes was first shown by Ceppellini and Siniscalco (1955); further evidence was presented by Ceppellini (1959).

In newborn infants the Lewis groups are imperfectly expressed, and the Lewis phenotypes of adults and infants will be described separately.

The Lewis Groups of Adults (see Table XXIII)

The Phenotype Le(a—b+)

Persons belonging to this phenotype always have Le^b substance in their saliva and serum; they also have Le^a substance in their saliva, and often

have a trace of Le^a in their serum. Their saliva always contains H substance (with or without A and B). If the scheme proposed by Watkins and Morgan (1959) is correct, Le^b must invariably be accompanied by H, and no exceptions to this have been reported. The red cells of adults of phenotype O, Le(a—b+) are always agglutinated by anti-Le^b serum, but the red cells of adults of the phenotype A_1, B or A_1B, Le(a—b+) are agglutinated only by certain anti-Le^b sera (see below). According to the scheme of Watkins and Morgan (1959) the *A* and *B* genes convert Le^b and H to

TABLE XXIII

SCHEME OF THE LEWIS SYSTEM AND SECRETION OF ABH (MODIFIED FROM WATKINS AND MORGAN, 1959, AFTER CEPPELLINI, 1955)

Genotypes	*Saliva* ABH	*Saliva* Le^a	*Saliva* Le^b	*Serum* (*Lewis substances*)	*Red cells* (*Lewis phenotype*)	*Approximate frequencies (per cent) in population* *British**	*Italian†*
SeSeLeLe, *SeSeLele*, *SeseLeLe*, *SeseLele*	+	+	+	Le^b§	Le(a—b+)§	68.6	72
seseLeLe, *seseLele*	—	+	—	Le^a	Le(a+b—)	26.6	16
seselele	—	—	—	—	Le(a—b—)	4.8	2
SeSelele, *Seselele*	+	—	—‡	—	Le(a—b—)	4.8	9

* From Marshall (1960).
† From Ceppellini (1955).
‡ The saliva of those individuals of the red cell phenotype Le(a—b—) who are secretors of ABH inhibits the reaction of certain anti-Le^b sera. It is believed that this is due to the similarity between H and Le^b.
§ The serum of subjects of the phenotype Le(a—b+) may contain some Le^a substance and red cells of the same subjects may react with anti-Le^a (see text).

A and B. This presumably explains why it is only the red cells of group O subjects which have an appreciable amount of Le^b antigen.

The phenotype Le(a+b+). Occasional samples of red cells from adults of group O and A_2 which are strongly agglutinated by anti-Le^b serum may be weakly agglutinated by anti-Le^a serum (Andresen *et al.*, 1950; Grubb, 1951; Simmons and Jakobowicz, 1951). If a suitable incomplete anti-Le^a is used to sensitize ficin-treated Le(b+) cells to an antiglobulin serum it will be found that most if not all samples of group O and A_2

RCPE0002068_0154

appear to belong to the phenotype Le(a+b+); such red cells may undergo partial destruction *in vivo* by anti-Le^a (Cutbush *et al.*, 1956). The phenotype *Le(a+b+)* cannot be recognized in *Le(b+)* samples from adults belonging to group A_1. Thus, the character A_1 seems to interfere with the expression of Le^a just as it interferes with the expression of Le^b.

The Phenotype Le(a+b−)

Persons belonging to this phenotype have Le^a substance in their serum and in their saliva, and their red cells are agglutinated by anti-Le^a serum. Their saliva may partially inhibit some anti-Le^b sera, perhaps due to a cross-reaction (Miller, Rosenfield *et al.*, 1954).

Adults of the phenotype Le(a+b−) never secrete ABH substances in their saliva (Grubb, 1948) but they have a large amount of Le^a substance. Thus, the phenotype Le(a+b−) may be recognized by testing the saliva against an anti-Le^a precipitating serum produced in rabbits (Glynn *et al.*, 1956). Although the saliva of Le(a−b+) persons will inhibit an anti-Le^a agglutinating serum (the inhibition being one-quarter as great as that of Le(a+b−) saliva), only Le(a+b−) saliva forms a precipitate with an anti-Le^a precipitating serum. Perhaps this is because a higher proportion of the molecular surface is occupied by Le^a when A, B and H are not competing (Brown *et al.*, 1959).

The Phenotype Le(a−b−)

The incidence of this phenotype varies in Europeans from 1.5 per cent in Sweden (Grubb, 1951) to 11.8 per cent in Italy (Ceppellini, 1955) and has been found to be 4.8 per cent in the British Isles (Marshall, 1960). In negroes the incidence is much higher; for example 22 per cent according to Miller, Rosenfield *et al.* (1954).

Persons of the phenotype Le(a−b−) are thought to have the genotype *lele* and do not have Lewis substances in their saliva or serum (but see note below). Since the inheritance of a secretor gene *Se* is independent of the inheritance of the Lewis genes, about 75 per cent of Le(a−b−) adults are secretors of ABH substance.

The saliva of Le(a−b−) individuals who are secretors of ABH inhibits the reaction of certain anti-Le^b sera (Grubb, 1951). This at first sight seems incompatible with the genetical theory outlined above, but according to Ceppellini the reaction is to be thought of as a cross-reaction between H and anti-Le^b and does not therefore demonstrate the presence of Le^b in the saliva (see Ceppellini *et al.*, 1959).

There are a few individuals who, despite having Lewis substances in their saliva, have red cells of the phenotype Le(a−b−) (Ceppellini *et al.*, 1959). This seems to be a further reason for regarding the Lewis groups as being primarily a system of antigens in secretions.

The Lewis Groups of Infants

Andresen (1948) showed that the receptors Le^a and Le^b (L_1 and L_2 in his original terminology) are absent or extremely weak at birth and that thereafter Le^a develops more rapidly than Le^b. It has been suggested that cord blood specimens are essentially Le(a−b−) and that 'the Lewis phenotype appears as an agglutinable characteristic of the red cells only after a few weeks of age' (Rosenfield and Ohno, 1954). Nevertheless, if the indirect antiglobulin test is used, Le^a can be shown to be present on the red cells of about 50 per cent of cord blood samples (Cutbush *et al.*, 1956).

Andresen (1947) found that the frequency of Le(a+) samples was higher in infants under the age of 1 year than in adults, and Jordal and Lyndrup (1952) showed that if infants more than a few weeks old and less than 6 months old were tested, about 80 per cent of them were Le(a+). Similarly, Jordal (1956) found that at the age of 1-2 months 90 per cent of infants were Le(a+), and at the age of 12 months only 45 per cent were positive, and by 2-3 years only 22 per cent, that is to say, approximately the same frequency as in adults. Among infants of the phenotype Le(a+b−) those who are going to retain this phenotype as adults can be distinguished by their failure to secrete ABH substances.

The red cells of an infant which is destined to become an Le(a−b+) adult may react successively as Le(a−b−), Le(a+b−), Le(a+b+) and finally Le(a−b+) during the first 15 months of its life (Cutbush *et al.*, 1956).

Jordal (1956) found that the red cells of 22 per cent of infants aged 1-2 months behaved as Le(a+b+); at 1-2 years only 14 per cent behaved as Le(a+b+); and at 7-14 years only 2 per cent.

Lewis Groups in Pregnant Women

The agglutinability of red cells by anti-Le^a and anti-Le^b is reduced during pregnancy. Thus a woman of the phenotype *Le(a−b+)* may appear during pregnancy to have the phenotype *Le(a−b−)* (Brendemoen, 1952).

Adsorption of Lewis Substances on to Red Cells

Sneath and Sneath (1955) found that when Le(a+b−) cells were transfused to an Le(a−b+) recipient the cells adsorbed Le^b substance from the

RCPE0002068_0155

recipient's plasma and then reacted as Le(a+b+). The transformation could also be produced *in vitro* by incubating Le(a+b—) cells in Le(a—b+) plasma at 35° C. for 24 hours. Le(a+b—) or Le(a—b+) cells incubated with Le(a—b—) plasma lost their antigen to the plasma. This observation recalls the findings of Grubb and Morgan in 1949 that, after washing, Le(a+) cells reacted more weakly with anti-Le^a sera, although it is worth emphasizing that even after washing Le(a+) cells twenty times in saline they still react just as well with anti-Le^a sera when the indirect antiglobulin test is used (Cutbush *et al.*, 1956).

Mäkelä and Mäkelä (1956) found that exposure of O, Le(b—) cells to saliva from an O, Le(b+) person for a period as long as 5 days did not result in their change to Le(b+), although exposure to plasma from an O, Le(b+) person changed them after 2 or 3 days (at 37° C.). The same authors showed that the plasma of newborn infants was unable to transform red cells although the red cells of newborn infants could be transformed by plasma from adults.

Levine and Celano (1960) found that Le(a—b—) red cells treated with tannic acid would take up Le^a substance from saliva and would then react as Le(a+) red cells.

As already mentioned, Sneath and Sneath (1955) suggested that the red cells might simply adsorb Lewis antigens *in vivo* and that the fundamental expression of the system might be found in the serum antigen. The correctness of this view seems to be established by the findings of Nicholas *et al.* (1957). These authors describe twins displaying red cell chimaerism. One twin had 49 per cent of O cells and 51 per cent of A cells. The genotype of this twin was shown to be *OO* by the finding of H substance but not A substance in the saliva; all the red cells in this twin behaved as Le(a—b+). In the other twin, 61 per cent of the cells were A_1 and 39 per cent O. The saliva of this twin contained Le^a substance but not A or H; all the red cells behaved as O, Le(a+b—). Thus, red cells of the twin who secreted Le^a substance behaved as Le(a+) in that twin, but as Le(a—b+) in the twin who secreted H but not Le^a. The red cells from the twin who secreted H but not Le^a behaved as Le(a—b+) in their own circulation and as Le(a+b—) in the circulation of the twin who secreted Le^a.

Although the theory that Lewis groups of red cells are determined solely by adsorption of serum antigens seems to be very well grounded, a few observations remain to be explained. For example, in newborn infants the cells may react well with anti-Le^a serum at a time when Le^a substance cannot be demonstrated in the infant's serum (Cutbush *et al.*, 1956).

Antibodies of the Lewis System

Systematic work on the Lewis blood group system stemmed from the discovery by Mourant in 1946 of an antibody which was subsequently given the name anti-Le^a. The second antibody belonging to the Lewis blood group system was discovered by Andresen in 1948 and subsequently named anti-Le^b.

Anti-Le^a

There is little doubt that isolated examples of anti-Le^a had been encountered by earlier workers. Landsteiner and Levine (1929) described five sera which gave parallel reactions and agglutinated about 16 per cent of samples. The agglutination reactions were 'of a special sort' — a few rather large clumps, but mostly free cells. Parr and Krischner (1932) described an antibody which at 37° C. failed to agglutinate red cells but caused a slow lysis; Neter (1936) described an antibody which showed agglutinating and haemolysing activity, acting on about 25 per cent of blood samples. Levine and Polayes (1941) also described an antibody which agglutinated 25 per cent of red cell samples at 20° C. and caused lysis at 37° C. As described below, anti-Le^a sera react with about 20 per cent of samples and, unlike most other human blood group antibodies, cause haemolysis *in vitro*.

Anti-Le^a occurs relatively commonly in human sera. For example, in one series of 17,000 samples tested at room temperature, 57 contained anti-Le^a, an incidence of 0.3 per cent (Kissmeyer-Nielsen *et al.*, 1955). The antibody is found only in persons who 'secrete' ABH substances (Miller, Rosenfield *et al.*, 1954; Pettenkofer and Hoffbauer, 1954).

Anti-Le^a, like anti-Le^b, is found only in persons who are genetically Lewis negative, that is *le le* and who therefore have the red cell phenotype Le(a—b—). Jordal (1956) examined the sera of 121 adults of this phenotype and found 14 examples of anti-Le^a and 9 of anti-X (see below). This is an overall incidence of about 20 per cent of Lewis antibodies in Le(a—b—) subjects and would correspond to about 1 per cent in the population as a whole. This is more than three times higher than the incidence reported by Kissmeyer-Nielsen, but the difference may be partially explained by the fact that in Jordal's series a special search was being made for Lewis antibodies.

Many Lewis antibodies agglutinate weakly and some are more readily detected by the indirect antiglobulin test. Differences in technique may therefore account to some extent for different estimates of their frequency.

Donors of anti-Le^a more frequently belong to groups A, B or AB than

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would be expected in a random distribution. Thus Dr Eloise R. Giblett (personal communication) found the ABO distribution in fifty-three donors of anti Le^a to be:

O	11
A	33
B	6
AB	3

Thus only 21 per cent of the donors belonged to group O. In a series investigated in the author's laboratory, only one out of thirteen donors of anti-Le^a belonged to group O (unpublished observations).

Anti-Le^b

Anti-Le^b can often be demonstrated as a weak antibody in serum which contains a relatively potent anti-Le^a. Anti-Le^b occurs on its own relatively infrequently; for example, Pettenkofer and Hoffbauer (1954) tested the serum of 12,000 unselected pregnant or recently delivered women and found fifty-two examples of anti-Le^a but only two of anti-Le^b. Most sera which contain a potent anti-Le^b contain a weak anti-H agglutinin, but do not contain anti-Le^a. Miller, Rosenfield *et al.* (1954) found that producers of anti-Le^b (without anti-Le^a) were always of the phenotype *Le(a—b—)* and were non-secretors of ABH.

Some anti-Le^b sera fail to react with A_1 cells while reacting strongly with cells of groups O or A_2 (Andresen, 1948). There are in fact two kinds of anti-Le^b (Brendemoen, 1950; Ceppellini, 1955; Ceppellini *et al.*, 1959; Sneath and Sneath, 1959): (i) those that are neutralized by the salivas of all ABH secretors (i.e. including those of Le(a—b—) subjects; these anti-Le^b sera, called anti-Le^bH by Ceppellini *et al.* (1959), fail to react with A_1 blood samples; (ii) those which are not neutralized by the saliva of Le(a—b—) secretors of ABH but are neutralized by the saliva of Le(a—b+) persons. These anti-Le^b sera react with A_1 samples almost as well as with those of other groups, and are called anti-Le^bL (i.e. true Lewis type) by Ceppellini *et al.*

Anti-X

Occasional sera are encountered which react with all blood samples except those of the phenotype Le(a—b—). The first example was described by Andresen and Jordal (1949) and named anti-X. Many further examples have been encountered since; they are generally regarded as containing a mixture of anti-Le^a and anti-Le^b.

Antibodies Reacting with Le(a—b—) Samples

Antibodies have been described which react only with the red cells of Le(a—b—) subjects; this implies either that the *le* gene is not amorphic or that some precursor substance, which in the absence of the *Le* gene remains unconverted, produces a detectable antigen.

The Magard antibody. Andersen (1958) described an antibody which agglutinated only red cells from group A, Le(a—b—) subjects who were 'secretors' of A and H; this serum did not react with any group O samples or with any group A samples from 'non-secretors'. The donor of this serum belonged to group A_2, Le(a—b+) and was presumably a secretor of A and H.

Anti-Le^c. Iseki *et al.* (1957) injected the saliva of Le(a—b—) secretors into rabbits and then absorbed the immune serum at a low temperature with enzyme-treated Le(a+b—) and Le(a—b+) cells. The resulting serum weakly agglutinated enzyme-treated Le(a—b—) red cells in the cold, but not at 37° C. Iseki *et al.* also prepared anti-Le^c by injecting the saliva of Le(a—b—) secretors into rabbits and absorbing the immune serum first with non-secretor human saliva and then with the saliva of an Le(a—b+) subject. The resulting Le^c precipitin reacted only with Le(a—b—) saliva (of secretors and non-secretors).

Production of Lewis Antibodies in Rabbits

Glynn *et al.* (1956) succeeded in producing a precipitating serum against Le^a in rabbits. As an antigen they used a vaccine of haemolytic streptococci, group A, type IV, previously coated with Le^a substance from human ovarian cyst fluid. The resulting immune serum produced a precipitate when mixed with the saliva of Le(a+) subjects.

The precipitating serum has been tested in the author's laboratory and shown to react well with Le(a+) red cells. The tests were made by incubating Le(a+b—) red cells with the rabbit antiserum, then washing the cells and incubating them with fresh human serum as a source of complement and finally testing the cells with anti-human globulin serum to detect the adsorbed human complement.

Levine and Celano (1960) produced an anti-Le^a serum in rabbits by injecting tanned rabbit red cells, coated with human saliva containing Le^a substance. The resulting immune serum would agglutinate Le(a+) cells.

Characteristics of Lewis Antibodies

Sera containing anti-Le^a usually agglutinate Le(a+) cells suspended in saline and react slightly more strongly at room temperature than at 37° C.

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(Mourant, 1946; Brendemoen, 1949). Red cells agglutinated by anti-Le^a, when first spread on the slide, have a 'stringy' appearance (Dunsford and Bowley, 1955, p. 202). In some cases the serum may fail to agglutinate cells at 37° C. (Grubb and Morgan, 1949) and occasionally may not agglutinate saline-suspended cells at all and be detectable only by the indirect antiglobulin test.

The fact that Lewis antibodies bind complement at 37° C. can be shown in many ways. Firstly, the sera will haemolyse test cells at 37° C. (Andresen and Henningsen, 1951; Rosenfield and Vogel, 1951); Le(a+) cells from infants lyse more readily than Le(a+) cells from adults (Cutbush *et al.*, 1956). Sera which fail to lyse untreated test cells almost always lyse enzyme-treated cells (Rosenfield and Vogel, 1951). Secondly, Lewis antibodies will sensitize red cells to an immuno-conglutinin serum, provided that the serum containing Lewis antibody also contains complement or that complement is added (Mollison and Polley, 1960). Thirdly, Lewis antibodies will sensitize red cells to an anti-beta$_1$-globulin serum (see below).

The behaviour of Lewis antisera in the antiglobulin test has been discussed in Chapter VII. As explained there, most Lewis antibodies are composed of 19*S* globulin and therefore red cells sensitized with Lewis antibodies will react with anti-19*S* globulin but not with anti-7*S* globulin. Since most antiglobulin sera contain little or no anti-19*S* globulin (Polley *et al.*, 1961), it is found that Le(a+) red cells must be given an opportunity of binding complement as well as anti-Le^a before they will give a positive antiglobulin test (Mollison and Cutbush, 1955). In practice the most reliable test for Lewis antibodies is the two-stage antiglobulin test described in Chapter XII.

Great variation is found among the reactions of Le(a+b−) cells from different group O donors, if judged by their degree of sensitization to an antiglobulin serum after exposure to an incomplete anti-Le^a. Cells from some donors will give very strong reactions, while those of others give barely detectable reactions (Mollison and Cutbush, 1955). Anti-Le^a saline agglutinins apparently fail to detect these differences.

Clinical Significance of Lewis Antibodies

Lewis antibodies are frequently found in subjects who have never been transfused or received any other known antigenic stimulus, and they are therefore regarded as 'naturally-occurring'. It is not known whether a person whose serum lacks anti-Le^a may form the antibody as a result of an antigenic stimulus, nor is there much information as to whether the

potency of anti-Le^a is increased by antigenic stimuli. In the following case observed by the author, there was very suggestive evidence of the formation of immune anti-Le^a:

> Patient W. B. was investigated after he had had about eighteen transfusions, some of which had given rise to unpleasant reactions; one transfusion had been followed by severe haemoglobinuria and this was shown to be from an Le(a+) donor. The patient was subsequently transfused many times with Le(a−) blood, but he was also given many test injections of small amounts of Le(a+) blood for the purpose of investigation. Serum taken at a later date was analysed in the ultracentrifuge and it was shown that the anti-Le^a was composed partly of 7*S* globulin. Since most Lewis antibodies are probably made only of 19*S* globulin, it seems very likely that in this case immune anti-Le^a had been formed (Kekwick and Mollison, 1961).

Many haemolytic transfusion reactions due to anti-Le^a have been described since the early reports of Brendemoen and Aas, 1952; von Lauer *et al.*, 1954, and Mollison and Cutbush, 1955. Details of the pattern of red cell destruction brought about by Lewis antibodies are given in Chapter XIII.

It is uncertain whether Lewis antibodies ever cause haemolytic disease of the newborn, although one suggestive case was reported by Mellone *et al.* (1953), and another by Schwenzer and Spielmann (1957). In this latter case the mother's serum contained a relatively potent anti-Le^a (indirect antiglobulin titre 64). The infant was Le(a+) and had a negative direct antiglobulin test but its blood contained microspherocytes. Despite a small exchange transfusion with Le(a−b−) blood 2 hours after birth the infant's haemoglobin fell to 9.8 g. on the sixteenth day.

There are two reasons why Lewis antibodies normally fail to cause damage to the foetus. Firstly, these antibodies seem usually not to cross the placenta, presumably because they are usually composed of 19*S* globulin. Secondly, the red cells of newborn infants react weakly or not at all with Lewis antibodies.

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CHAPTER NINE

THE Rh BLOOD GROUP SYSTEM

Rh Antigens

Description in Terms of a Single Antigen and Antibody

For most clinical purposes it is sufficient to divide human beings into Rh-positive and Rh-negative. The distinction is made by testing their red cells with the commonest kind of Rh antibody which, in Fisher's nomenclature, is known as anti-D; this antibody divides people into Rh(D)-positive (83 per cent of the English population) and Rh(D)-negative (17 per cent). When the terms 'Rh antibody' or 'anti-Rh' are used without qualification it is usually anti-D that is meant.

The Rh blood group system is of great importance in clinical medicine because Rh-negative individuals are relatively easily stimulated, either by transfusion of Rh-positive blood or by a pregnancy with an Rh-positive foetus, to form potent anti-Rh, which may cause a severe haemolytic reaction if Rh-positive blood is subsequently transfused. When the Rh-negative individual is a female there may be a far more serious consequence, namely that any Rh-positive infant born to her subsequently will have haemolytic disease of the newborn.

During the early 1940s, when the practice of blood transfusion was increasing rapidly and Rh testing had not been introduced into routine clinical practice, very many Rh-negative individuals were immunized to Rh(D) and anti-Rh(D) was commonly encountered. During the late 1940s the practice of determining the Rh(D) group of donor and recipient as a preliminary to direct matching tests and transfusing only Rh-negative blood to Rh-negative recipients became widespread. Since that time the number of Rh-negative individuals immunized by the transfusion of Rh-positive blood has greatly declined so that in transfusion work Rh antibodies other than anti-D have become relatively important; but amongst women immunized to Rh antigens by pregnancy the dominating position of D must be expected to continue. One survey, published some years ago, showed that amongst women immunized to Rh antigens through pregnancy over 99 per cent were D-negative (van Loghem and van der Hart, 1949).

According to Fisher's original theory, the presence of the antigen D on the red cells is determined by a gene *D* which has an allele *d* so that

there are three genotypes, *DD*, *Dd* and *dd*. Of the 83 per cent of D-positives, 35 per cent are *DD* and 48 per cent *Dd*. The distinction between the homozygote (*DD*) and the heterozygote (*Dd*) is of importance when the person concerned is the husband of a woman whose serum contains anti-D, for if the man passes on *D* to his offspring it will be affected with haemolytic disease of the newborn, whereas if he passes on *d* it will be unaffected. Unfortunately the distinction between *DD* and *Dd* cannot be made directly, because the antibody anti-d does not occur; this will be considered in more detail later in relation to the other Rh antigens.

Other Rh Antigens, including Problems of Nomenclature

Fisher suggested that the inheritance of Rh antigens was determined by three pairs of allelic genes which were very closely linked. According to this theory a person inherits three Rh genes from each of his parents; that is to say, (i) *C* or *c*; (ii) *D* or *d*; (iii) *E* or *e*. For example, a person may inherit *CDe* from one parent and *cde* from another. The frequency of the commonest gene-combinations is shown in Table XXIV.

Fisher's theory was based on the observation that certain Rh antibodies gave antithetical reactions. Thus, all samples of red cells which failed to react with one Rh antibody (later called anti-C) were agglutinated by another Rh antibody (later called anti-c), and vice versa. Fisher predicted that anti-d and anti-e (not then discovered) would be found; anti-e was found soon afterwards and gave reactions which were antithetical to anti-E but anti-d has not been found.

Although Fisher's theoretical scheme is a convenient introduction to Rh genetics, it is now generally agreed that an Rh gene should be regarded as an entity and that C, D and E are not separable genes. One of the strongest pieces of evidence in favour of the idea that the Rh gene is a unit is the finding that there are some antigens which are formed only by particular combinations of the CDE 'genes'. For example, the antigen Ce is found on the cells of persons of the genotype *CDe/cde* (and *Cde/cde*) but not on those of a person of the genotype *CdE/cde* (or CDE/cde); that is to say, the genetic determinants for C and e must be inherited together, on the same chromosome, to determine Ce (Rosenfield and Haber, 1958). Thus it may be better to think of the gene *CDe* as being capable of producing more than just C, D and e; not only may it produce Ce but it may possibly produce other antigens not yet defined. It is for this reason that some workers feel that it may be more correct to use a different nomenclature for genes on the one hand and antigens on the other; for example, *CDe* may be referred to by the symbol R_1 (see Table XXIV).

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(The use of the symbol '^', as in $\hat{Ce}$ has been suggested by Professor R. Ceppelini to indicate that the joint product is different from separable C plus e.)

From the outset some confusion has arisen between the merits of Fisher's scheme as a theory for Rh genetics on the one hand and as a highly convenient method of notation on the other. As just described, Fisher's idea of separable Rh genes is now accepted with some reserve, although his idea of antithetical genes has been retained; (for example an

TABLE XXIV

FREQUENCY OF COMMON Rh GENES

*Short symbol**	*CDE nomenclature*	*Frequency†*
R_1	*CDe*	0.4076
r	*cde*	0.3886
R_2	*cDE*	0.1411
R_0	*cDe*	0.0257
$R^{w}{}_1$	*C^{w}De*	0.0129
r''	*cdE*	0.0119
r'	*Cde*	0.0098
R_z	*CDE*	rarer
r_y	*CdE*	

* These symbols are based partly on those originally introduced by Wiener (1944b) and partly on those introduced by Race *et al.* (1944). They are widely used but with minor variations. Thus Dr P. Levine uses superscripts for all gene symbols, e.g. R^1. Drs R. R. Race and Ruth Sanger at present use some subscripts and some superscripts (see, for example, Table XXVI) but a change to the use of superscripts only is contemplated. If this change is made it will be wise to use the symbol r' rather than R' for the gene complex *Cde* to avoid confusion between R' and R^1. (Dr R. R. Race, personal communication.)

† These frequencies, for an English population, are taken from Race, Mourant *et al.* (1948).

Rh gene determines C or c). Fisher's notation remains by far the most convenient because it is so easy to work out the reactions of a particular antiserum; for example, anti-e reacts with blood of the genotype *cde/cDE* but not with blood of the genotype *cDE/cDE*.

Wiener has been responsible for a different genetic theory and for a different notation. According to Wiener's theory, the inheritance of Rh antigens is determined by a series of allelic genes. This is, of course, the safer theory in the sense that it cannot be wrong. As already mentioned, one of Wiener's main assertions, namely that the inheritance of Rh antigens is determined by a single gene rather than three separable genes, is now held to be correct. On the other hand, a genetic theory which simply allots a new name to every new gene is not very helpful since it makes no predictions which can be tested and gives no insight into the relation of one gene with another.

Wiener's notation differs in many ways from the CDE notation. In Wiener's notation separate terms are used for an agglutinogen, that is the whole complex of antigens determined by the gene, and for factors, that is each separately reacting part of the antigenic structure. To illustrate the difference between this system and the CDE terminology, in the following illustration the CDE terms are given in brackets. Thus the gene R^1 (R_1) determines the agglutinogen Rh_1 (CDe) and this has multiple factors: Rh_0 (D), rh′ (C), hr″ (e), rh_1 (Ce) and Rh^A and Hr_0 (no named equivalents in CDE terminology); see Wiener (1958). The main objection to this terminology is that it is less explicit than the CDE terminology. Whereas the complex CDe obviously contains the antigen e, it is not obvious that Rh_1 contains hr″. A second objection is that it is easier to modify the symbol e (e.g. e^s) than to modify hr″.

Determination of the Rh Phenotype

The completeness with which the Rh phenotype can be determined depends on the antisera available. Thus, if anti-c is available but not anti-C, samples can be classified as c-positive (i.e. *cc* or *Cc*) and c-negative (i.e. *CC*). If anti-C is also available, *Cc* can be distinguished from *cc*.

If any excuse is needed for slipping back here into the 'original' Fisher genetical terminology, one can only say that it is convenient to distinguish between samples with a single dose and a double dose of C and since this is determined genetically, the Fisher terminology is the most direct way of expressing the idea.

A convenient notation for Rh phenotypes is that introduced by Mourant (1949). Suppose a sample is tested with anti-C, anti-c, anti-D and anti-E and gives positive reactions with all four antisera, the phenotype is written CcDE. If positive reactions are obtained with anti-C, anti-c and anti-D, but the reaction with anti-E is negative, the phenotype is written as CcDee, since an absence of E implies a double dose of e.

This notation is occasionally misleading; for example, although a negative reaction with anti-E usually implies that the cells are *ee*, they may be ee^s.

Determination of the Probable Rh Genotype

When a woman has Rh antibody in her serum it is desirable to know whether her husband is homozygous or heterozygous for the gene deter-

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mining the corresponding Rh antigen. This almost always means trying to find out whether the husband's red cells contain a single or double dose of *D*. Since anti-d is not available, a guess has to be made by determining the Rh phenotype as completely as possible and then calculating the probabilities from this. For example, suppose the Rh phenotype is CcDee, the genotype may be *CDe/cde*, *CDe/cDe* or *Cde/cDe*.

The frequencies of these Rh genotypes in an unselected English population are approximately 33 per cent, 2 per cent and less than 0.1 per cent (Race, in M.R.C. Memo. No. 27). Therefore, the chances are 16 to 1 (or 94 to 6, see Table XXV) that the genotype is *CDe/cde*. The odds are not as great as this if the person being tested is the father of an infant with haemolytic disease of the newborn, since such persons have a greater chance (about four times greater) than unselected persons of having a double dose of D. Thus, in the present case the chance that the true genotype is *CDe/cDe* is not 1 in 16 but about 1 in 4. (For further information see Table XXV.)

The Commonest Rh Genotypes and Phenotypes

As explained in Chapter VII, from a knowledge of the gene frequencies in any blood group system, the genotype (or phenotype) frequencies can readily be calculated. As Table XXIV shows, the commonest Rh gene complexes are R_1 and r. Thus the commonest three Rh genotypes are R_1r, with a frequency of $2\times(0.4076\times0.3886) = 0.31$, or 31 per cent; R_1R_1 with a frequency of $(0.4076\times0.4076) = 0.16$, or 16 per cent, and rr, with a frequency of $(0.3886\times0.3886) = 0.15$, or 15 per cent.

The phenotype of blood of the genotype R_1r is CcDee; as Table XXV shows, blood of this phenotype may also result from the genotype R_1R_0, and there is one rarer genotype which also results in the same phenotype. The overall frequency of the phenotype CcDee is 34.89 per cent; the frequencies of the next commonest phenotypes in order are: CCDee 18.51 per cent; ccddee 15.10 per cent; CcDEe 13.34 per cent and ccDEe 12.67 per cent. These together account for approximately 95 per cent of the total Rh phenotypes in Britain (figures derived from Table V of Race, R. R., in M.R.C. Memorandum No. 27, 1954b).

Further Complexities, including D^u

Wiener (1944b) noted that occasional blood samples gave 'weak or intermediate reactions with one or more of the Rh antisera' and suggested that four intermediate Rh genes existed in addition to the genes already

described. He demonstrated that the intermediate genes might be inherited.

As a result of further work, these 'intermediate genes' have now been more clearly defined. Callender and Race (1946) described the allele C^w, which can be looked on as an alternative to C; for example, a person may

TABLE XXV

TO SHOW THE ERRORS INVOLVED IN GUESSING THE Rh GENOTYPE FROM THE Rh PHENOTYPE

(*a*) in unselected persons, and (*b*) in fathers of infants with haemolytic disease of the newborn. The table includes only those examples in which the error of the guess may be fairly large; and only relatively common genotypes are shown. (Based on Table III from Race, R. R., in Medical Research Council Memo. No. 27, 1954b).

*Rh phenotype**	*Rh genotypes*		
	Alternatives	*Incidence in* (a) *Unselected Persons*	(b) *Fathers of infants with haemolytic disease of the newborn*
CcDee	*CDe/cde* *CDe/cDe*	94% 6%	79% 21%
CCDee	*CDe/CDe* *CDe/Cde*	96% 4%	99% 1%
ccDE	*cDE/cde* *cDE/cDE* } or *cDE/cDe*	80% 20%	51% 49%
CcDE	*CDe/cDE* *CDe/cdE* } or *cDE/CDe*	90% 10%	97% 3%

* Determined by tests with anti-C, anti-c, anti-D and anti-E. In the combinations not shown (e.g. ccDee), the errors involved in guessing the genotype are very small.

have the gene-combination C^wDe rather than *CDe*. A person who has the antigen C in his or her red cells can form anti-C^w as a result of transfusion with C^w blood or pregnancies with C^w foetuses. In a few cases, anti-C^w has caused haemolytic transfusion reactions and haemolytic disease of the newborn (e.g. Lawler and van Loghem, 1947). The antigen C^w is rare, occurring in less than 2 per cent of the English population.

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Stratton and Renton (1954) have described a case of haemolytic disease of the newborn due to the even rarer allele C^x.

D^u. An Rh variant of practical importance is D^u (Stratton, 1946). Race, Sanger and Lawler (1948) described a blood sample as D^u if it gave some but not all of the reactions expected of D. They recognized a whole series of variants of the D antigen and these, at present, are all considered as D^u. From the serological point of view D^u bloods are not as sharply distinguished from D as are C^w bloods from C. Thus, although D^u subjects very occasionally form anti-D, no specific anti-D^u serum has been found whereas many examples of anti-C^w are known. From most points of view D^u may be regarded simply as a very weak form of D, i.e. D^u red cells are ones which have relatively few D sites. Masouredis (1960b), using ^{131}I-labelled anti-D has shown that D^u red cells take up only 7-25 per cent as much antibody as ordinary D positive cells.

D^u blood samples sometimes cause confusion, because they are agglutinated by some anti-D sera and not by others. Thus, if one laboratory reports a sample as Rh-negative and another reports it as Rh-positive, D^u should be suspected. If a D^u blood sample from a prospective recipient of a transfusion, or from a pregnant woman, is mistakenly diagnosed as 'Rh-negative' it matters little; however, if the sample comes from a prospective donor the mistake is potentially serious.

The two commonest genotypes of blood in which the antigen D^u occurs are *CD^ue/cde* and *cD^uE/cde*. If these bloods were tested against anti-D alone, they might fail to react (unless the indirect antiglobulin test were used) and be considered to be Rh-negative.

Most transfusion centres, when selecting Rh-negative donors for blood transfusion, test the red cells against anti-C and anti-E as well as against anti-D. This procedure detects persons of the genotypes *Cde/cde* and *cdE/cde*, and these donors are usually regarded as 'Rh+' although in fact they are scarcely more 'dangerous' as donors for Rh-negative subjects than for Rh-positive subjects. C is a poor antigen and E is foreign not only to Rh-negative recipients but also to most Rh-positive recipients. Testing with anti-C and anti-E also serves to detect donors of the genotypes *CD^ue/cde* and *cD^uE/cde*. Since the discovery of the antigen D^u, it has been realized that many samples previously classified as *Cde/cde* or *cdE/cde* are in fact D^u bloods. Thus Rosenfield, Vogel *et al.* (1951) found that of 161 samples which appeared to be *Cde/cde*, seventy-two were *CD^ue/cde*; of ninety-two which appeared to be *cdE/cde*, nineteen were cD^uE/cde; and of 628 which appeared to be *cde/cde*, three were *cD^ue/cde*.

To establish the fact that a person who appears to belong to the genotype *Cde/cde* is not of the genotype *CD^ue/cde*, it is essential to expose the cells to a potent incomplete anti-D serum free of anti-C and carry out the antiglobulin test. Only those bloods which remain unsensitized can be considered as *Cde/cde* (*R'r*).

The question arises whether blood samples which fail to react with saline-agglutinating anti-C, anti-D and anti-E should also be tested with an incomplete anti-D serum, using the indirect antiglobulin test, to detect blood donors of the type *cD^ue/cde*. As noted above, Rosenfield *et al.* found three samples of this type among more than 600 samples of the apparent genotype *cde/cde*. This suggests that it is necessary to test for *cD^ue/cde* donors, contrary to the opinion expressed by Simmons and Albrey (1955).

It seems to the present author that it might be more logical not to test donors with anti-C and anti-E but simply with an anti-D serum (or perhaps better with an anti-CD serum), using the indirect antiglobulin test, and to regard as Rh-negative all those failing to react.

A D^u infant can suffer from haemolytic disease of the newborn if its mother's serum contains anti-D (Mollison and Cutbush, 1949c). In such a case the mother will usually have been immunized by a normal D antigen, for example by an earlier transfusion, since D^u itself is a poor antigen (see below).

The original kind of D^u described was shown to be inherited (Stratton, 1946; Race, Sanger and Lawler, 1948). It is now known that a blood sample can behave as D^u if a person inherits a particular combination of normal Rh genes. Thus, when the gene *Cde* is paired with a gene containing normal *D* the reaction of D is partially suppressed, and the blood behaves as D^u (Ceppellini *et al.*, 1955). This was confirmed by Levine *et al.* (1959) who suggested that the stronger reactions of *cDE/cDE* samples compared with *CDe/CDe* might be due to the same effect, that is, a suppression of D by C on the opposite chromosome (so-called *trans* position).

'Joint products' of the theoretically separable CDE genes. Lawler and Race (1950) described an anti-E serum which reacted more strongly with cDE/cde cells than with *CDe/cDE* cells. Race *et al.* (1954) subsequently showed that certain anti-E sera reacted more strongly with *CdE/cde* and *CDE/cDe* cells and less strongly with *Cde/cdE* and *CDe/cDE* cells and this led them to suggest that when C and *E* were in the *trans* position (i.e. on different members of a pair of allelic genes) E was inhibited. Similarly they found

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that the antigen C was inhibited when *C* and *E* were in the *cis* position (e.g. *CdE/cde*) and was not inhibited when *C* and *E* were in the *trans* position, e.g. *CDe/cDE*. Rosenfield and Haber (1958), suggested that these findings might be better explained by supposing that some of the sera used for the tests contained antibodies against 'joint products'.

As described earlier in this chapter, Rosenfield and Haber (1958) themselves described an antibody reacting with a product of chromosomes carrying *C* and *e*. Thus the antibody anti-Ce (or rh_1) reacted with a sample of the genotype *Cde/CdE* but not with *cde/CDE*.

Drs R. R. Race and Ruth Sanger (personal communication) have now re-interpreted their earlier observations. To take an example they point out that some anti-C sera are mixtures of anti-C and anti-Ce; those that contain mainly anti-Ce may fail to react with samples such as *CdE/cde* or *CDE/cDe* and react well with *Cde/cdE* or *CDe/cDE*.

Other 'joint products'. When the antibody originally called anti-f was first discovered it was observed that it was reacting with products of genes carrying *c* and *e* together; that is to say, blood of the genotype *cde/CDe* was f positive whereas blood of the genotype *CDe/cDE* was negative; that is determinants for c and e had to be present on the same gene to determine f (Rosenfield *et al.*, 1953); thus f is now thought of as ce.

The Rh antigen, V. This blood group antigen was described by DeNatale *et al.* (1955). It is common in negroes and rare in white persons. Of 168 New York negroes, forty-five (27 per cent) had the V antigen; of 444 New York whites, two (0.5 per cent) had V. Recently evidence has been produced that the antibody originally called anti-V can be regarded as anti-ce^s, e^s being a form of e which is commonly found in blood samples from negroes (Sanger *et al.*, 1960).

The Rh antigen G. Allen and Tippett (1958) described a sample of red cells which reacted with all anti-CD sera but not with specific anti-C or anti-D sera and suggested that the antigen so recognized should be called G, and the corresponding antibody anti-G. They found that all red cells which have the C or D antigens also have G and that most sera which apparently contain anti-C and anti-D consist either of anti-D+anti-G or anti-D+anti-G+anti-C.

They pointed out that this finding helped to explain why Rh-negative subjects quite often form an antibody reacting with C+ red cells whereas the formation of anti-C by Rh-positive subjects is very rare, the explanation being that the antibody formed by Rh-negative subjects is really

anti-G and that this cannot be formed by Rh-positive subjects since they are G+.

Tippett *et al.* (1961) found that the sample of G positive red cells described by Allen and Tippett (1958) did in fact react with some anti-C sera. They suggested that the sample might be regarded as containing 'depressed C' so that G may yet turn out to be a product of genes determining C or D or both. Tippett *et al.* (1961) summarized their present view of the relation between gene complexes and the resultant antigens and this is shown (slightly modified) as Table XXVI.

TABLE XXVI

SOME OF THE GENE SYMBOLS OF THE Rh SYSTEM AND THE RESULTING ANTIGENS: MODIFIED FROM TIPPETT *et al.* (1961)

Gene complexes *Short symbol*	*DCE* symbol*	*Resulting antigens*				
R_1	*DCe*	D	G	C	Ce	e
r	*dce*			c	ce	e
R_2	*DcE*	D	G	c	cE	E
R_0	*Dce*	D	G	c	ce	e
R''	*dcE*			c	cE	E
R'	*dCe*		G	C	Ce	e
R_z	*DCE*	D	G	C	CE	E
R_y	*dCE*		G	C	CE	E
R'^s	dCe^s		G	C	Ce^s	e^s
r^v	dce^s			c	ce^s	e^s
$R_0{}^v$	Dce^s	D	G	c	ce^s	e^s
	D——	D	G			

* The order DCE is sometimes used because there is evidence that this may be the order of the sites on the gene-complex.

Red cells lacking some or all Rh antigens. Rare samples exist which fail to react with anti-C or anti-c, and with anti-E or anti-e. The genotype of such samples is often written *—D—/—D—* to indicate that determinants for Cc and Ee are missing (Race, Sanger and Selwyn, 1951).

These samples appear to have an abnormally large amount of the D antigen as judged by their agglutination in a saline medium by sera which seem to contain only incomplete anti-D. The reaction is thought to be due to traces of saline agglutinin in certain anti-Rh sera, because it is not given by all sera containing potent incomplete antibodies (Waller *et al.*, 1953).

Samples (*cD—/cD—*) which fail to react with anti-E and with anti-e and which have an unusual type of c antigen were described by Tate *et al.* (1960). Finally a sample of blood which completely failed to react with all Rh antibodies was

RCPE0002068_0163

described by Vos *et al.* (1961) who discussed the possibility that this might be due to lack of a precursor substance for Rh antigens rather to the absence of the Rh genes themselves.

Chemical Nature of Rh Specificity

A clue to the chemical nature of the end-groups determining Rh specificity appears to be provided by the observation that certain derivatives of ribonucleic acid, namely, cytidylic acid, uridylic acid, adenylic acid and cytidine sulphate, when prepared as a 2 per cent solution in phosphate buffer at pH 6.8, inhibit anti-D, anti-C and anti-E (and incidentally anti-Lu^a and anti-Lu^b), but not anti-A, anti-B, anti-M, anti-N, anti-P or anti-Fy^a (Hackel *et al.*, 1958). Boyd *et al.* (1959) at first failed to confirm these observations and found that anti-D was inhibited by common monosaccharides. Later Boyd (1960) reported that he had found weak inhibition with nucleic acid derivatives, as reported by Hackel *et al.* (1958) but he considered that sugar units had greater significance than nucleotides in determining the specificity of the terminal groups of the Rh antigens.

Rh Substances in Tissues other than Red Cells

White cells and platelets. Using the mixed antiglobulin reaction, Gurner and Coombs (1958) failed to find the Rh antigen on leucocytes, although positive results were reported by Jankovic and Lincoln (1959), using a fluorescent antibody technique, and by Dausset *et al.* (1958) using the antiglobulin consumption test.

Using specific mixed agglutination, Ashurst *et al.* (1956) could not demonstrate the Rh antigen in platelets, and neither could Gurevitch and Nelken (1957) who tried many different techniques.

Saliva. Most workers have failed to detect Rh substances in saliva (Wiener and Forer, 1941; Levine and Katzin, 1941; Mohn and Witebsky, 1948).

Amniotic fluid. Walker and Bailey (1956) were unable to demonstrate any inhibition of anti-Rh sera by amniotic fluid from Rh-positive foetuses, although this finding disagrees with the earlier observations of Mohn and Witebsky (1948).

RH ANTIBODIES

Nomenclature

In Fisher's nomenclature the antibodies equivalent to the various antigens of the Rh system are designated by adding the prefix 'anti-' to the

antigen concerned. For example, the antibody equivalent to the antigen e is anti-e. It is easy to see that this antibody reacts with a blood sample from a person of the genotype *CDe/cDE* and, in fact, with any blood which contains the antigen e.

In Wiener's terminology (see Wiener, 1951a, and Table XXVII) the relationship between antigens and antibodies is not always so evident, as can be illustrated by translating the same example into his terms. Anti-e in Wiener's terminology is anti-hr″. The genotype *CDe/cDE* is R^1R^2. No separate letters are assigned to the individual antigens, so that these cannot be described directly. Instead it has to be learned that a series of bloods of different genotypes possess, in common, the property of reacting with anti-hr″; or in Wiener's terminology have the blood factor hr″.

TABLE XXVII

TERMINOLOGY OF THE MAIN ANTIBODIES OF THE Rh SYSTEM

Fisher-Race	*Wiener**
Anti-C	Anti-rh′
Anti-D	Anti-Rh_0
Anti-E	Anti-rh″
Anti-c	Anti-hr′
Anti-e	Anti-hr″

* Wiener (1951a).

Sera which react with more than one CDE antigen sometimes prove to contain inseparable antibody. For example, in investigating the serum of a donor of the type —D—/—D—, Hackel (1957) found that the activity against C could not be separated by absorption or by other means from the activity against c. Professor R. Ceppellini (personal communication) has suggested writing anti-Ce for such an antibody.

Reactions of Rh Antibodies

Some sera containing Rh antibodies will agglutinate Rh-positive cells suspended in saline, but the majority either fail completely to cause agglutination under these conditions or, at best, react very weakly. By contrast, almost all anti-Rh sera will agglutinate Rh-positive cells if the latter are suspended in 20 per cent albumin or in one of a variety of other colloid media. It is generally held that there are two different kinds of Rh antibody — saline agglutinins and incomplete antibodies — which are responsible for these two different reactions; and that the relative propor-

RCPE0002068_0164

tions of these two kinds of antibody in a serum determine the way in which it reacts.

When saline agglutinins occur it is usually at a fairly early stage of the immunization. Thus, Diamond (1947) found that in Rh-negative volunteers immunized with Rh-positive blood anti-Rh saline agglutinins were first produced; later the serum lost its ability to agglutinate cells suspended in saline but would still agglutinate them if they were suspended in 20 per cent albumin. He therefore called the saline agglutinin 'early' antibody and the incomplete antibody he called 'late' or 'hyper-immune'. Wiener and Sonn-Gordon (1947a) also noted that one of their volunteers first produced saline agglutinins, but later, after further stimuli, produced 'blocking' (incomplete) antibody.

Waller (1949) found that all four of his subjects who produced anti-Rh most readily first produced saline agglutinins; later the titre of the sera against cells in 20 per cent albumin increased. In five other subjects who produced anti-Rh only after five or more injections of Rh-positive blood, no saline agglutinins were detected, and the presence of anti-Rh was revealed only by the indirect antiglobulin test or by a test using cells suspended in albumin.

The existence of an incomplete form of anti-Rh was first discovered independently by Race (1944) and Wiener (1944a), who observed that there was a kind of anti-Rh which failed to agglutinate Rh-positive cells suspended in saline but which combined with Rh-positive cells in such a way that subsequently the cells could not be agglutinated by the 'saline agglutinating' type of anti-Rh. For a short period the 'blocking test' was used to detect the presence of incomplete antibody, but it soon became clear that it was an insensitive test, and it was replaced by others, such as the indirect antiglobulin test.

Coombs and Race (1945) showed that the incomplete antibody was more thermostable than the agglutinin. Heat just insufficient to cause coagulation of a serum (65-75° C. for 5-10 minutes) was found to destroy the agglutinin but to leave the incomplete antibody unchanged.

It is now known that anti-Rh saline agglutinins are composed of 19*S* globulin (Fudenberg *et al.*, 1959); as expected their serological activity is destroyed by treatment with sulphydryl compounds (Grubb and Swahn, 1958; Hunter and Mollison, 1961) and they do not pass the placenta (Wiener, 1948a; Broman, 1949).

By contrast, incomplete Rh antibodies separate in the ultra-centrifuge with a sedimentation constant of 7*S* (Fudenberg *et al.*, 1959); and are not affected by treatment with SH compounds (Grubb and Swahn, 1958),

although one exception was found by Hunter and Mollison (1961); incomplete Rh antibodies pass the placenta readily so that in Rh-negative infants born to women whose serum contains Rh antibody the titre may be the same in the cord serum as in the mother's serum (Wiener, 1948a; Mollison, 1951, p. 370).

Methods of Detecting Incomplete Rh Antibodies

Sera containing incomplete anti-Rh will usually agglutinate red cells suspended in concentrated protein solutions (Cameron and Diamond, 1945). The serum fraction containing concentrated albumin is very suitable (Diamond and Denton, 1945), although it appears that this is due to the effect of alpha globulin present as a contaminant rather than to the albumin itself (McCulloch, 1950).

A mixture of human plasma and bovine albumin gives stronger reactions than albumin alone (Wiener, Hurst and Sonn-Gordon, 1947; Pickles, 1949). A mixture of equal parts of human AB serum and 30 per cent bovine albumin was found to be very satisfactory by Stratton and Diamond (1955). When the red cells were suspended in this mixture, and the serum under test was diluted in the same mixture, 'prozones' were much less often encountered than when using concentrated albumin alone.

The indirect antiglobulin test is more sensitive than an agglutination test in albumin, and a test with enzyme-treated red cells is more sensitive still (e.g. Stratton, 1953a). Thus, occasionally in antenatal tests Rh antibody is first detectable only with enzyme-treated cells and later becomes detectable by other methods (Stratton and Stone, cited by Stratton, 1953a). The method which gives the highest 'score' of all with incomplete anti-D sera is to carry out the indirect antiglobulin test using red cells which have first been treated with papain (Vogt and Heistö, 1953).

Very occasionally an antibody reacts comparatively strongly with enzyme-treated red cells but fails to give a positive indirect antiglobulin reaction. Heistö (1955) and Vogt *et al.* (1958) have both described examples of anti-E behaving in this way.

Renton and Hancock (1958a) carried out experiments which help to explain some of the reactions of anti-Rh sera. They made mixtures of two sera, one containing a powerful saline agglutinin and one containing an incomplete antibody, and they found that the agglutinating action of anti-D depended on the proportion of incomplete antibody to agglutinin and on the amount of D antigen in the cells. They pointed out that D-positive cells could be ranged in this order: low-grade D^u, high-grade D^u,

RCPE0002068_0165

normal D, *cDE/cDE*, 'strong' D (Renton and Hancock, 1956) and *—D—/—D—*.

Even low-grade D^u cells were agglutinated by the undiluted potent agglutinating serum, but when a small amount of incomplete antibody was added, so that the ratio of complete to incomplete was less than 32 to 1, low-grade D^u cells were not agglutinated. Normal D cells were agglutinated until the ratio of complete to incomplete fell below 1 to 2. Cells whose D antigen was partly blocked by incomplete anti-D behaved like D^u cells (Renton and Hancock, 1958a).

In a further paper Renton and Hancock (1958b) showed that 'prozones' encountered in titrating anti-Rh sera might be due to excess of agglutinin or of incomplete antibody. Thus D^u cells were not agglutinated by undiluted serum containing a saline agglutinin but if the washed cells were mixed with additional untreated cells agglutination occurred. Ordinary D positive cells incubated with a mixture of 1 part saline agglutinin and 4 parts 'blocking' antibody failed to agglutinate but after being washed and mixed with excess D positive cells, they agglutinated.

Studies with ^{131}I-labelled Antibody

The first studies with ^{131}I-labelled Rh antibody were described by Boursnell *et al.* (1953). Whole serum containing anti-Rh was labelled with ^{131}I and added to suspensions of Rh-positive and Rh-negative red cells. When the cells were suspended in saline, Rh-negative cells took up just as much ^{131}I as Rh-positive cells but if the cells were first suspended for 1 hour at room temperature in antibody-free, unlabelled serum, Rh-positive cells took up slightly more ^{131}I than Rh-negative cells. The problem of non-specific uptake of ^{131}I-labelled protein was largely overcome by Masouredis (1959). He prepared gamma globulin from serum containing potent anti-Rh and labelled it with ^{131}I; the labelled protein was incubated with stroma prepared from Rh-positive cells and an eluate was then prepared. This eluate gave highly specific reactions, the amount of reactivity taken up by Rh-positive red cells being about forty times as great as that taken up by Rh-negative cells. Masouredis found that the reaction between anti-D and D positive cells could be characterized as a first order reaction with a rate constant at 37° C. of almost 6 per cent per minute, so that 50 per cent of the antibody was bound in about 12 minutes. Masouredis found that the biological activity of the labelled antibody was unaffected by heating the preparation to 60° C for 30 minutes although heating to 70° C. for 30 minutes reduced its biological activity to 60 per cent of the original figure.

In a further study, Masouredis (1960b) showed that C— D+ red cells from homozygous (*DD*) donors took up 1.6 times as much antibody as those from heterozygous (*Dd*) donors. He also showed that C+D+ cells took up 0.7 times as much antibody as C—D+ cells.

The Number of Rh Receptors (Antigen Sites) on a Single Rh-positive Cell

If the amount of Rh antibody, as gamma globulin, taken up by a given number of Rh-positive cells is estimated, then the number of antigen sites on each Rh-positive cell can be calculated. Boursnell *et al.* (1953), using ^{131}I-labelled serum containing anti-Rh, calculated the number of receptors per red cell to be 5000.

Grubb (1955) estimated the amount of gamma globulin on Rh-positive red cells fully coated with anti-Rh, by using them to absorb antiglobulin serum and comparing the inhibition with that produced by known amounts of gamma globulin. He calculated that the number of Rh antigen sites on each red cell was at least 2000.

Naturally-Occurring Rh Antibodies

The occurrence of anti-D in a person who has never received Rh-positive red cells is extremely rare. Wiener (1949a) reported that he had twice found weak 'univalent' (incomplete) Rh antibodies in the serum of AB, Rh-negative male donors, and Allen and Newell (1958) found a weak antibody in a woman who had never received an injection or transfusion and who was pregnant with an Rh-negative foetus. The antibody did not react in saline, but gave good reactions with trypsinized red cells. It also agglutinated Rh-positive red cells suspended in albumin, and this reaction tended to be better than the reaction given in the indirect antiglobulin test (personal communication from Dr F. H. Allen).

Although naturally-occurring anti-D is extremely rare, many examples of naturally-occurring anti-E are known. In the second edition of this book a personal communication from Dr I. Dunsford was quoted, describing the finding of eight examples of anti-E, all acting more strongly at 4° C. than at 37° C., and reacting better in saline than in albumin. The sera gave very poor indirect antiglobulin reactions. None of the donors appeared to have been immunized to the E antigen; two were males. Grove-Rasmussen and Levine (1954) also described two examples of naturally-occurring anti-E agglutinins in males who had never been transfused; both were active at 37° C. In one case the serum failed to give a positive indirect antiglobulin test; it reacted well with cells suspended in saline or albumin.

RCPE0002068_0166

An example of naturally-occurring anti-C^w was described by Chown and Lewis (1954); the serum agglutinated cells suspended in saline and albumin, reacting more strongly at room temperature than at 37° C. It did not sensitize cells to an antiglobulin serum. Two further cases were reported by Kornstad *et al.* (1960); in both the antibody reacted only slightly more strongly at 18° C. or 4° C. than at 37° C. The best reactions were obtained with papainized red cells and the indirect antiglobulin test was negative. The authors pointed out that immune anti-C^w, accompanying anti-C, may also fail to sensitize red cells to an antiglobulin serum and give agglutination in saline over a wide thermal range.

An example of a naturally-occurring anti-C^x was described by Plaut *et al.* (1958). According to Cleghorn (1960) anti-C^x is found not uncommonly, together with antibodies against other rare blood group antigens in the serum of patients with 'symptomatic' acquired haemolytic anaemia.

Formation of Immune Rh Antibodies Following Transfusion or Injection

Transfusion or Intravenous Injection

It appears that a single transfusion of Rh-positive blood stimulates the formation of anti-Rh in at least 50 per cent of subjects. This conclusion was first reached by Diamond (1947) as a result of examining a large number of Rh-negative males who had been transfused during war service. Similarly, Dr M. M. Pickles (personal communication) found anti-Rh in the serum of thirty out of sixty Rh-negative men who had received a single transfusion of group O, Rh-positive blood; she noticed that the antibody did not usually develop until 8 or 9 weeks after the transfusion, and sometimes only developed later. She concluded that the best time to look for antibodies was 20 weeks after transfusion.

The relatively high incidence of Rh antibody in the sera of persons who have received a single transfusion of Rh-positive blood contrasts with the somewhat lower incidence reported in volunteers deliberately immunized with small intravenous injections of Rh-positive blood. The difference may be partly accounted for by the fact that in most of the investigations to be described the subjects were not tested for 4 to 5 months after a single injection, but were given further injections in the meantime, so that the effect of a single injection could not be properly assessed. It seems likely that a more important factor was the smaller dosage of Rh-positive cells. Thus, the subjects who were deliberately immunized in most cases received only a few millilitres of Rh-positive blood intravenously, and

this may have been insufficient to obtain an optimum primary response (see Chapter VII).

One of the most complete accounts of the deliberate injection of Rh-negative volunteers is that of Wiener (1949b). Injections equivalent to 2–4 ml. of Rh-positive blood were given to a large group of subjects; the injections were given at intervals of 3 to 4 months and the recipient's serum was tested 10 to 14 days after each injection. Only a few subjects were tested after the first injection; one was found to have anti-Rh in his serum but gave a history of previous transfusion. After the second injection, anti-Rh was found in eighteen out of forty-seven cases (38 per cent). Of the twenty-nine subjects who failed to respond to two injections, twenty-three were given a third injection and six of these then produced antibody. Thus of forty cases (excluding the previously transfused patient) who had received three injections, approximately 55 per cent produced anti-Rh. Of the seventeen individuals who failed to respond to the third injection, fifteen returned for a fourth injection and three of these responded. Of the twelve who failed to respond to the fourth injection, ten returned and received a fifth injection; only one of these responded. Thus after five spaced injections about two-thirds of the subjects developed anti-Rh. From this series it may be concluded that there are great differences in the response of different individuals to the same stimulus. Some are readily sensitized, others are difficult to sensitize, and some perhaps cannot be sensitized at all.

This conclusion is supported by the experience of Jones, Diamond and Allen (1954). Twenty-two Rh-negative volunteers were given injections of Rh-positive blood every 10 weeks. Within 9 months, eighteen of the twenty-two had developed anti-D; the remaining four subjects received further injections for another 18 months but still failed to produce Rh antibody.

Intramuscular Injections

Although there have been no systematic attempts to produce anti-Rh by giving intramuscular injections of blood, it is to be expected that such attempts would succeed.

Several cases are on record in which there is strong circumstantial evidence of immunization by intramuscular injection. Thus, Bessis (1947) described the production of Rh antibodies in a first pregnancy in a woman who had previously received an intramuscular injection of blood. Similarly, in the second edition of this book a case was reported of a woman who lost her first infant from *hydrops foetalis* and was found to

RCPE0002068_0167

have Rh antibody in her serum. She had never been transfused, but 10 years previously she had been given, prophylactically, an intramuscular injection of blood from her brother who was convalescing from measles. The brother's blood was later shown to be Rh-positive.

The case reported by Thompson and Walsh (1950) suggests that intramuscular injection can at times be a very effective stimulus.

> The patient was a single woman aged 23, suffering from disseminated sclerosis. As a therapeutic measure, 2 ml. of human blood, containing malaria parasites, were injected subcutaneously and intramuscularly. The patient developed clinical malaria 7 days later. On the eighteenth day after injection, the patient had become slightly anaemic, and she was transfused with 1000 ml. of group O blood. During the blood transfusion she developed rigors and haemoglobinuria. Tests now showed that the patient was A, *CDe/cde* and that her serum contained anti-E with a titre of 16. The donor of the transfused blood was O, *cDE/cde*.

In a case described by Vaccari and Baldini (1957) a haemophiliac who had never been transfused before but who, 20 years previously, had received a series of intramuscular injections of blood, developed a severe haemolytic transfusion reaction with anuria following his first transfusion. Nine days later his serum was shown to contain potent anti-Rh.

The conclusion is that if intramuscular or subcutaneous injections of blood are to be given they must be preceded by the same grouping and compatibility tests as are usual for intravenous injections.

Influence of ABO incompatibility. If Rh-positive red cells of incompatible ABO group are injected into Rh-negative subjects, they are much less likely to stimulate the formation of antibody than Rh-positive cells of compatible ABO group. This was first shown experimentally by Stern, Davidsohn and Masaitis (1956). These investigators gave two to ten intravenous injections at intervals of 6-10 weeks (sometimes 5 months). The amounts injected were at first 5 ml., then 2.5 ml. If anti-Rh developed, only one further injection of 1 ml. was given. Only one adverse reaction was noted (flushing of the face and faintness), and this was in a subject receiving ABO incompatible cells. Of seventeen subjects injected with ABO compatible cells, ten developed anti-Rh; in five subjects the titre rose to between 16 and 128; and reached from 256 to 512 in the remainder. By contrast, anti-Rh developed in only two out of twenty-two subjects receiving ABO incompatible Rh-positive cells, and the titre was only 1 in 2 to 1 in 8. In one of these two cases seven further injections of Rh-positive cells failed to produce any increase in titre.

In a later study (Stern *et al.*, 1961) the series was extended slightly and

the total figures for the production of anti-Rh became: after ABO compatible Rh-positive cells, seventeen out of twenty-four; after ABO incompatible, five out of thirty-two. Ten subjects who had failed to form anti-Rh after receiving ABO incompatible Rh-positive cells were subsequently injected with compatible Rh-positive cells and four produced anti-Rh.

Stern *et al.* considered that their results were best explained by the theory of 'clonal competition' for antigen. Thus in an O Rh-negative subject injected with A Rh-positive cells there would be more 'anti-A-forming cells' already available to take up A antigen so that little Rh antigen would reach the few anti-Rh forming cells.

> It is well established that animals injected simultaneously with two antigens may show significantly lower responses to one of them if it has previous experience of the other (see, for example, Barr and Llewellyn-Jones, 1953).

Sixteen additional subjects were given a course of injections (in most cases five injections) of Rh-positive red cells 'coated' *in vitro* with anti-Rh and none produced anti-Rh; ten of these subjects were given subsequent injections of uncoated Rh-positive cells and five produced anti-Rh. Of eight (group A) subjects who received injections of A Rh-positive cells 'coated' with anti-A, six produced anti-Rh.

The failure of 'anti-Rh coated' Rh-positive cells to stimulate anti-Rh may have been due to the take up of the cells by the 'wrong' kind of phagocytes, i.e. a kind incapable of forming anti-Rh since anti-Rh coated Rh-positive cells are removed predominantly in the spleen (see Chapter XIII). The antigenicity of 'anti-A coated' A Rh-positive cells in A Rh-negative recipients was probably to be explained by the fact that anti-A, unlike anti-Rh, rapidly elutes from red cells *in vivo*, so that 'anti-A coated' group A red cells may escape rapid destruction; see Chapter XIII.

Failure of Salk vaccine to stimulate anti-Rh. The Salk poliomyelitis vaccine is prepared by growing virus in kidney tissue from Rhesus monkeys. The final preparation has a very low protein content and is hardly expected to be capable of stimulating Rh antibodies. Nevertheless it is reassuring that Abelson *et al.* (1955) found that Salk vaccine administered to subjects already sensitized to Rh did not cause any significant rise in antibody titre. The same subjects, after being injected intravenously with 0.25 ml. of a 50 per cent suspension of Rh-positive red cells, responded with a considerable rise in antibody titre, provided that they had not been stimulated recently and that their titre at the time of injection was low.

D^u as an antigen. Although D^u can stimulate the formation of anti-D (see van Loghem, 1947), it appears to be a less effective antigen than D.

RCPE0002068_0168

Schmidt *et al.* (1959) investigated fifteen Rh-negative patients who had received twenty-five transfusions of D^u blood; none of them produced anti-D. One patient, transfused with D^u K+ blood produced anti-K but not anti-D.

Production of Anti-D by D-Positive Subjects

Several cases have now been described in which a subject whose red cells are agglutinated by anti-D serum has been found to have an antibody in his serum behaving like anti-D. In the case described by Argall *et al.* (1953) the red cells of the subject gave the reactions of D^u. In a case described by Wiener, Geiger and Gordon (1957) the red cells of the donor reacted like ordinary D-positive cells. Wiener *et al.* suggested that the cells lacked a factor 'Rh^A' normally present in Rh-positive red cells, and they described the donor's cells as Rh_1^a, and the antibody as anti-Rh^A. In a later paper it was shown that all of a large series of Rh_o-positive bloods from white subjects were Rh^A-positive; Rh^A was absent from one D^u blood sample. In a series of negroid Rh_o-positive blood samples, 0.9 per cent were Rh^A-negative. Two subjects of type Rh_2rh were found to have other varieties of Rh antibody closely resembling anti-Rh_o (anti-D), and these were given the names anti-Rh^B and anti-Rh^C (Unger and Wiener, 1959).

The risk that a D^u subject will form anti-D following the transfusion of D-positive blood is very small, and it has been suggested that it may be safer to transfuse D^u subjects with D-positive blood than with D-negative. This is because many samples behaving as D^u come from persons of the genotype *Cde/CDe*, and such subjects, if transfused with Rh-negative blood (*cde/cde*) may form anti-c (Levine, Celano, McGee *et al.*, 1957).

Of course the risk of provoking the formation of anti-c exists in all CC recipients, not just those of type CCD^uee and if this risk is guarded against by 'screening' Rh-positive recipients with anti-c (see section on compatibility tests), then it may after all be safer to use D-negative blood for D^u recipients. A very severe haemolytic reaction following the transfusion of D-positive blood to a D-positive (D^u) woman with anti-D in her serum has been encountered by Dr Eloise R. Giblett (personal communication).

It seems that D^u subjects are more liable to form anti-D than ordinary D-positive subjects and that the risk extends to D^u subjects in whom the expression of D has been subdued by the presence of the gene *C* in the *trans* position (see above). Thus, in a case investigated in the author's laboratory a woman of the probable genotype *CDe/Cde* and the phenotype CCD^uee formed anti-D in addition to anti-c.

The conclusion seems to be that when a D^u recipient is c positive, e.g. *CD^ue/cde* then Rh-negative blood should be transfused, but when the recipient is c negative, e.g. *Cde/CDe* (behaving as D^u), then Rh-positive blood is safest.

Responses to Antigens other than D

When an Rh-negative patient is immunized with Rh-positive blood, usually only a single antibody, anti-D, is formed, despite the fact that the injected cells contain other foreign Rh antigens. For example, most Rh-positive blood belongs to type *CDe/cde* or *CDe/CDe*, but, of those people of type *cde/cde* who form anti-Rh, 60 per cent form anti-D only (Race, in M.R.C. Memo No. 27).

About 30 per cent of sera containing anti-D will also react with C+ red cells (Race, in M.R.C. Memo. No. 27; cf. the estimate of Levine, Robinson, Stroup *et al.*, 1956, that 18 per cent of sera containing anti-D also contain anti-C). It is now thought that in most of these cases anti-C is not present as a separate antibody but that the sera contains anti-G which reacts with C+ red cells because (like D+ red cells) they contain an antigen G (Allen and Tippett, 1958).

Sera which react with C+ and D+ red cells sometimes behave as if they contained incomplete anti-D and 'complete' anti-C; that is to say they will agglutinate C+ red cells suspended in saline but not C—D+ red cells suspended in saline.

Anti-D is occasionally accompanied by anti-E (in 2 per cent of cases, according to Race, see M.R.C. Memo. No. 27; in 1 per cent in the series of Levine, Robinson, Stroup *et al.*, 1956).

Wiener and Sonn-Gordon (1947a) noted that in a person who was immunized with R_2 (*cDE*) blood, anti-D saline agglutinins were produced first, then anti-D incomplete antibody, and, finally, anti-E agglutinins in addition.

Van Loghem (1947) gave injections of *CD^ue/cde* or *CD^ue/CD^ue* blood to five Rh-negative volunteers twice a week in small doses (first 2 ml., later 0.25 ml.) for 6 weeks; after an interval of 6 weeks five more twice-weekly injections were given. One recipient formed anti-C and anti-D and the remainder failed to respond.

Anti-C is very seldom found as a single antibody in patients who have received multiple transfusions, and it has proved very difficult to provoke the formation of anti-C by injecting suitable blood into D-positive recipients (see below). It is now thought that this is because C is always accompanied by the more antigenic factor G so that in G-negative

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subjects anti-G rather than anti-C is formed. Since G always accompanies D, anti-G behaves like anti-C plus anti-D (Allen and Tippett, 1958).

Jones, Diamond and Allen (1954) tried to produce anti-C and anti-E in suitable D-positive recipients by first giving frequent intravenous injections (about every 3 weeks). Amongst a total of thirty-two volunteers who were injected over a period of 1 to 1½ years, not one produced the desired antibody.

Van Loghem *et al.* (1948) gave twice-weekly injections of 0.5 ml. of C^w blood to three volunteers. After twenty injections given over a period of 3½ weeks one of these recipients produced anti-C^w.

In an attempt to produce anti-c, Wiener (1949b) gave repeated injections of Rh-negative (*cde/cde*) blood to nineteen R_1R_1 (*CDe/CDe*) individuals; not one produced antibody. However, Jones, Diamond and Allen (1954) succeeded in producing anti-c in two out of nine volunteers who were given repeated injections of c-positive blood over a period of 10 months.

By contrast, the patient of Callender and Race (1946) who was so adept at forming antibodies, produced anti-c after her second blood transfusion. The details were as follows: first transfusion in February, 1944 (patient *CDe/CDe*, donor *CDe/cde*); second transfusion in August, 1944 (donor *cde/cde*); 8 days later anti-c was found in the patient's serum.

Jones, Diamond and Allen (1954) gave repeated injections of e-positive blood to a volunteer of the probable genotype *cDE/cDE*. After 6 years anti-e was found for the first time. Van Loghem, Harkink and van der Hart (1953) gave injections of e-positive blood to three *cDE/cDE* recipients. Eight injections of 0.5 ml. of *cde/cde* blood were given at weekly intervals; after a 2 months' rest, weekly injections were begun again. After the third injection immune antibodies were found in one of the three recipients; seven more injections were given in an attempt to increase the strength of the antibody. The serum was now found to contain not only anti-e (titre 8) but also anti-K (titre 4) and anti-Fy^a (titre 2). Although anti-e is rarely formed as a result of transfusion, it is not uncommonly found in the serum of patients with acquired haemolytic anaemia; such a serum, after suitable absorption, may be used as an anti-e reagent in blood grouping.

It has been shown that most anti-e and anti-c sera also contain anti-f (Rosenfield *et al.*, 1953; Jones, Steinberg *et al.*, 1954). The original anti-f was found unmixed with other Rh antibodies in a patient of the probable genotype *CDe/cDE*, who had received many transfusions. Anti-f is now usually regarded as anti-ce (see above).

The Rh antibody, anti-V (now regarded as anti-ce^s (see above)) was

first described by DeNatale *et al.* (1955). It reacted well, using the indirect antiglobulin test, with red cells from a few white people and with those from approximately 30 per cent of negroes. It reacts with a product of the chromosomes cde^s and cDe^s. At least ten examples of anti-V have been encountered, mostly in Rh-negative subjects; some of these examples may have been anti-e^s, which reacts with all V-positive cells (Dr Eloise R. Giblett, personal communication).

It should be added that the existence of *d* as something other than the mere absence of *D* is not yet established. No generally accepted example of anti-d has yet been found.

In sera from immunized patients who have formed Rh antibodies other than anti-D, the antibodies most commonly found are anti-c and anti-E. Thus in one series of 136 sera containing single atypical antibodies other than anti-D there were forty-three examples of anti-c and twenty-four of anti-E (Levine, Robinson, Stroup *et al.*, 1956). In the same series there were nineteen sera containing multiple antibodies. In six of these cases anti-c and anti-E were found together, and two sera contained anti-C plus anti-e.

In a series of samples which have caused difficulty in cross-matching, anti-c may appear to be commoner than anti-E but this is because the c antigen is more common than E. Thus if the serum of a patient is matched against the red cells of only a small number of donors, anti-c is likely to be detected but the presence of anti-E may well go unrecognized. When the serum of potential recipients is routinely tested with E-positive as well as c-positive red cells, anti-E is seen to be distinctly commoner than anti-c. Thus Kellner (1955) reported that out of seventy-six sera found to contain antibodies during cross-matching (including twenty-seven sera with anti-D), six contained anti-E and three anti-c. Dr Eloise Giblett (personal communication), in screening the sera of prospective recipients, found 50 examples of anti-E and 24 of anti-c; on the other hand, in patients immunized by pregnancy, anti-c was far commoner than anti-E.

Immunization by Pregnancy

Passage of foetal red cells across the placenta. Rh antigens cannot be demonstrated in any tissues except red cells, and it is therefore believed that immunization to Rh during pregnancy occurs only when foetal red cells enter the mother's circulation. It is now known that some leakage of foetal red cells across the placenta is quite common in pregnancy. This has been demonstrated in four ways: first by a serological method capable of detecting very small amounts of foreign red cells (Richardson Jones and Silver, 1958; see also Chapter V); second, by a serological method capable

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of detecting very small amounts of foetal haemoglobin (Hosoi, 1958); third by a chemical method which elutes adult haemoglobin but not foetal haemoglobin from red cells in a smear (Kleihauer *et al.*, 1957; Zipursky *et al.*, 1959); and fourth, by the use of fluorescein-labelled antisera (Cohen *et al.*, 1960).

Hosoi (1958) using a serological method of detecting foetal haemoglobin, investigated a series in which the infant's red cells were ABO compatible with the mother's serum and obtained evidence of the presence of foetal cells in the mother's circulation in eighteen out of forty-seven cases.

The method of Kleihauer *et al.* (1957) was used by Zipursky *et al.* (1959) who obtained positive results in nine out of forty-two cases and by Finn *et al.* (1961), who, using stricter criteria for a positive result, found foetal red cells in the mother's circulation in thirty out of 256 cases; Finn *et al.* estimated that the amount of foetal blood present in the mother's circulation at the time of the test was rarely more than 1 or 2 ml.

Only a few results with the use of the fluorescein-labelled antibody method have so far been published; in one case the equivalent of 0.2 ml. of foetal blood could be detected in the mother's circulation (Cohen *et al.*, 1960).

It seems rather likely that leakage of red cells into the mother's circulation commonly occurs during parturition rather than during pregnancy itself. Thus not uncommonly Rh antibody may be absent from the mother's circulation during pregnancy but be present about 2 weeks later (Boorman *et al.*, 1947). Similarly, although Rh antibody is only rarely found in a first pregnancy it is sometimes detected if the mother's serum is tested after delivery (Weiner *et al.*, 1952). At the same time, this view cannot be taken as proven, since a considerable increase in anti-A titre is often observed during the first few days following delivery in a group O woman who has given birth to a group A infant (Dienst, 1905). In this case it is thought that immunization occurs by the passage of soluble A substance into the mother's circulation, since immunization is commoner when the infant is a secretor (Smith, 1945). Although it is possible to understand that foetal red cells might get into the mother's circulation more easily during parturition, it might be expected that water-soluble substances would pass freely during pregnancy. It may be that the rise in antibody titre following parturition is due rather to the removal of the foetus which has previously been 'mopping up' antibody, or is possibly due to a lowering of the level of corticoid hormones which have previously been tending to suppress antibody formation. In any case, in

trying to obtain potent anti-Rh sera for test purposes it is worth remembering that the titre of anti-Rh normally reaches a peak 1–3 weeks after delivery (Boorman, Dodd and Mollison, 1945).

Possible prophylaxis against immunization. Finn *et al.* (1961) obtained evidence that the number of Rh-positive red cells entering the mother's circulation might influence her chance of forming anti-Rh and they suggested a way of diminishing the risk. They 'followed-up' Rh-negative women by examining their serum for antibody 2 to 3 months after the delivery of an Rh-positive infant. Of seventy-five women in whom no foetal red cells had been detected immediately after delivery, only one had formed anti-Rh; of seven in whom small numbers of foetal red cells (equivalent to a total foetal bleed of about 1 to 4 ml.) had been detected, none had formed antibody but of three women in whom relatively large numbers of foetal red cells had been detected (equivalent to 5 ml. or more), two had formed anti-Rh.

Finn *et al.* argued that if the cases in which relatively large numbers of foetal red cells entered the maternal circulation could be detected, and if steps were then taken to remove the cells rapidly from the blood stream, that immunization might be prevented. The method they proposed was to inject serum containing anti-Rh, and they carried out some tests to demonstrate its efficacy. Six Rh-negative males were injected with 5 ml. of Rh-positive blood and in three cases this was followed 30 minutes later by an injection of 10 ml. of serum containing anti-Rh (titre in saline and in albumin 64). Two days later, the number of surviving cells in the subjects who had received anti-Rh was 20–50 per cent, compared with almost 100 per cent in those who had not. (For a discussion of the effects of injecting plasma containing 'incompatible' antibodies, see Chapter XIII.)

Effect of previous transfusion of Rh-positive blood on iso-immunization in pregnancy. It appears that if an Rh-negative woman receives a transfusion of Rh-positive blood and subsequently becomes pregnant with an Rh-positive infant she almost always produces Rh antibody. In a series collected by Nevanlinna (1953), out of eighteen Rh-negative women, previously transfused with Rh-positive blood, who later gave birth to one or more Rh-positive children, fourteen had anti-Rh in their serum. Nevanlinna compared this incidence of immunization with that in fifty-one Rh-negative women who had been transfused with Rh-positive blood but had not had any subsequent pregnancies with an Rh-positive infant; twenty-one of these were found to have anti-Rh.

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Thus, among women who had been transfused with Rh-positive blood but had not been pregnant with Rh-positive infants, about half were immunized, that is, had demonstrable anti-Rh in their serum, but nearly all could be regarded as 'sensitized' ('sensibilized'); that is, they could be expected to produce anti-Rh during a subsequent pregnancy with an Rh-positive infant.

In the United Kingdom the practice of determining the Rh group of patients before transfusion, and avoiding the transfusion of Rh-positive blood to Rh-negative recipients became common only in about 1947. Since then the number of Rh-immunized pregnant women giving a history of a previous transfusion has fallen steadily. Table XXVIII shows the experience at Hammersmith Hospital, London. The table shows that

TABLE XXVIII

TO SHOW THE PROGRESSIVE DECREASE IN THE NUMBER OF Rh-IMMUNIZED WOMEN GIVING A HISTORY OF A PREVIOUS BLOOD TRANSFUSION*

Total number of Rh-immunized pregnant women		*Number transfused previously and years in which transfusions given*					*Number previously transfused*	
		Pre-1940	1940-45	1946-47	1947-49	*1950 onwards*	*Total*	*Per cent*
1947-48	87	1	16	9	1	—	27	31
1949-50	130	1	9	8	4	0	22	17
1951-52	106	2	7	2	4	0	15	14
1953-55	92	0	5	1	1	0	7	8
1956-58	65	0	2	1	0	0	3	5
Total:	480	4	39	21	10	0	74	

* Of known Rh-positive blood or of blood not known to be Rh-negative.

most of the immunizing transfusions were given in 1940-45, which were, of course, years in which transfusion was used very enthusiastically. In this series, no immunizing transfusions were given after 1949, and this suggests that it should soon be rare to see a woman who has been immunized by the accidental transfusion of Rh-positive blood. It is logical now to consider whether one ought also to try to avoid immunizing women to c or E, and this is discussed in the section on compatibility tests (Chapter XII).

Frequency of immunization to anti-Rh (D). In about one out of every ten pregnancies in matings of Caucasian stock the mother is Rh-negative and the foetus Rh-positive. The calculation is as follows: 17 per cent of women are Rh-negative; of these, 17 per cent have Rh-negative husbands

and 83 per cent Rh-positive husbands. Of these eighty-three husbands, thirty-five are homozygous (*RR*) and forty-eight are heterozygous (*Rr*). All children born to the homozygous fathers will be Rh-positive but only half those born to the heterozygous fathers will be Rh-positive. Thus, out of every 100 pregnancies in the population, the number in which the mother is Rh-negative and the foetus Rh-positive will be $17\left(\frac{35}{100}+\frac{0.5\times 48}{100}\right)=10$. An alternative way of calculating this is to say that an Rh-negative woman can produce an Rh-positive infant only if her husband transmits the Rh-positive gene, so that the frequency of the combination Rh— mother with Rh+ infant is given by: frequency of the phenotype Rh— × frequency of the gene *R*, i.e. $0.17\times 0.59 = 0.10$ or 10 per cent.

In calculating the chance that an Rh-negative woman will carry two Rh-positive infants in succession it must be remembered that if her husband is heterozygous (*Rr*) the chance that he will pass on *R* twice in succession is 1 in 4. Thus, out of every hundred second pregnancies, the number in which an Rh-negative woman is carrying her second Rh-positive infant is given by: $17\left(\frac{35}{100}+\frac{0.25\times 48}{100}\right)=8$. These figures for the maximum frequency with which haemolytic disease could occur if an Rh-negative mother were always immunized by an Rh-positive foetus, may be compared with the frequency with which iso-immunization is actually detected.

In first pregnancies it is unusual to find Rh antibodies in the mother's serum and when one does find them, it is usually in a woman who is known to have received a previous transfusion or injection of Rh-positive blood. In one series of women all of whom were thought to be pregnant for the first time but none of whom was known to have received a previous injection of Rh-positive blood, Rh antibodies were found in twenty-three out of 30,000 corresponding to approximately five per 1000 Rh-negative women (Hartman and Brendemoen, 1953). This is higher than the estimate of Nevanlinna and Vainio (1960) who found that only about 1 per 1000 Rh-negative women produced Rh antibodies in their first pregnancy. The actual number of Rh-negative women in whose serum antibody was found during the first pregnancy was ten out of 4153 but six of these women had been immunized by a previous transfusion of Rh-positive blood.

In Rh-negative women pregnant for the second time the percentage

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with Rh antibody in their serum rises sharply; some estimates are: 6.6 per cent (Clemens and Walsh, 1954); 4.1 per cent (deduced from the estimate given by Mollison (1951) page 183); and 2.7 per cent (Nevanlinna and Vainio, 1960).

In succeeding pregnancies the number of Rh-negative women immunized to Rh rises steadily. Thus Nevanlinna and Vainio found the incidence to be: in third pregnancies 5.8 per cent; in fourth 6.3 per cent; in fifth 7.8 per cent; and in sixth or later pregnancies 10.2 per cent.

Nevanlinna (1953) drew attention to the fact that the risk of becoming immunized to Rh is greater in the second pregnancy than in a subsequent pregnancy. That is, the proportion of women who form anti-Rh for the first time while carrying their second Rh-positive infant is higher than the

TABLE XXIX

PERCENTAGE OF INDIVIDUALS WHO HAVING FAILED TO PRODUCE Rh ANTIBODY AFTER A GIVEN NUMBER OF PREGNANCIES WITH Rh-POSITIVE FOETUSES OR INJECTIONS OF Rh-POSITIVE BLOOD RESPOND TO THE NEXT STIMULUS

	Pregnancies (Nevanlinna and Vainio, 1960)	*Injections* (Wiener, 1949b)
Responding to second, having failed to respond to first	4.5	38
Responding to third, having failed to respond to second	2.8	26
Responding to fourth, having failed to respond to third	1.3	20
Responding to fifth, having failed to respond to fourth	approx. zero	10

proportion who form antibodies for the first time while carrying their third Rh-positive infant. Nevanlinna and Vainio (1960) calculated the probability of immunization in each succeeding pregnancy and their figures are shown in Table XXIX.

The findings in male volunteers deliberately injected with Rh-positive red cells reveal a similar phenomenon, that is the proportion who respond to two stimuli is greater than those who, having failed to respond to two stimuli, respond to the third stimulus.

These findings show that the injection of Rh-positive blood is a very much better stimulus than pregnancy with an Rh-positive infant; this may be because the number of Rh-positive red cells entering the circulation is greater following deliberate intravenous injections than when red cells 'leak' from the foetus.

Influence of ABO incompatibility on Rh immunization in pregnancy. The fact that immunization to Rh during pregnancy is less common when the father is 'ABO incompatible' with the mother was noted by Levine (1943). Thus, in a series of matings between Rh-positive fathers and Rh-negative mothers who had given birth to infants affected with haemolytic disease of the newborn, 24.7 per cent were ABO incompatible (father's red cells versus mother's serum) compared with 35 per cent of ABO incompatible matings expected in a random series. (For a later review, see Levine, 1958.)

This point was investigated further by Nevanlinna and Vainio (1956) who found that in families in which the mother was immunized to Rh but the father was ABO incompatible, the last healthy child (that is, the one immediately preceding the first affected child) was almost four times more commonly ABO compatible than incompatible. They concluded that an ABO compatible infant was much more likely to immunize its mother than one which was not. They also found that affected children were equally commonly ABO compatible and incompatible; that is to say, once the mother was immunized to Rh, ABO incompatibility of the infant had no protective effect.

The main conclusions of this work have been confirmed by Dr R. Finn (personal communication). In a series of families in which haemolytic disease of the newborn occurred, and the father was ABO incompatible with the mother, the 'immunizing foetus' could be identified with a high degree of certainty in twelve cases; in eleven it was ABO compatible. The conclusion that the protection given by ABO incompatibility is not complete agrees with that of Nevanlinna and Vainio, who found ABO incompatibility lacking in one-fifth of their cases, i.e. the mother was immunized even though all her previous infants were ABO incompatible.

It can be shown that in a Caucasian population 80 per cent of infants are ABO compatible with their mothers and that of all women having two infants, 73 per cent have two which are ABO compatible. Therefore ABO incompatibility between mother and infant does not by itself explain the fact that pregnancy with an Rh-positive infant is so much less effective in sensitizing the mother to Rh than is an injection of Rh-positive blood.

The reason why ABO incompatible Rh-positive red cells fail to immunize Rh-negative subjects has been discussed earlier in this chapter.

Effect of Rh group of grandmother. If Rh antigens passed from the mother to the foetus during pregnancy it might be expected that the Rh-negative

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foetus of an Rh-positive mother would become at least partly tolerant to the Rh antigen. It would then be expected that the maternal grandmother of infants with haemolytic disease of the newborn would more frequently be Rh-negative than Rh-positive. Evidence that this is the case is not very impressive.

Booth *et al.* (1953) determined the Rh groups of maternal grandmothers of 113 infants with haemolytic disease of the newborn and found that the frequency of Rh-negatives was exactly that expected in a random sample of the mothers of Rh-negative women.

Owen, Wood *et al.* (1954) found that women whose mothers were Rh-positive were just as liable to have an infant affected with haemolytic disease of the newborn in their first three pregnancies as were women with Rh-negative mothers, although there was some evidence that women with Rh-negative mothers formed Rh antibodies more frequently.

Ward *et al.* (1957) found a slight increase in the incidence of Rh-negatives amongst maternal grandmothers of affected infants. On the other hand the Rh antibody titre was slightly higher in women whose mothers were Rh-positive. The severity of the disease was not correlated with the Rh group of the maternal grandmother.

Relative Frequency of Formation of Different Antibodies in Pregnancy

In cases of haemolytic disease of the newborn due to Rh antibodies, anti-D is involved in the great majority of cases. Thus, van Loghem *et al.* (1957) found that in 99 per cent of a series of 2274 mothers whose infants were affected with haemolytic disease of the newborn, anti-D was present (with or without anti-C or anti-E). Of the remaining twenty-four sera, ten contained anti-E alone, eight contained anti-c, three anti-c plus anti-E, two anti-C^w and one anti-C.

Such figures as these may not give the true incidence of the formation of anti-c in pregnancy because Rh-positive mothers are usually tested only if they give birth to an infant which is clinically affected with haemolytic disease of the newborn. Mild cases, with only a positive direct Coombs test with little or no jaundice, constitute about a third of the total, and may well be overlooked. Nevertheless it seems probable that haemolytic disease of the newborn due to anti-c is about 100 times less common than haemolytic disease of the newborn due to anti-D. The opportunities for immunization during pregnancy by anti-c are about the same as those for immunization to D. Thus, supposing one counts only homozygous husbands, then matings likely to lead to the formation of anti-D may be taken as the frequency of *dd* × frequency of *DD*, i.e. 0.17 × 0.34 or 0.58; for anti-c the calculations are: *CC* × *cc*, i.e. 0.18 × 0.33 or 0.59. The conclusion is, then, that the opportunities are the same and that c must be roughly 100 times less antigenic than *D*.

This estimate may be compared with that of Giblett (1961), see Table XIV. The relative potency of c was calculated to be approximately 0.02; that is to say, a single transfusion of c-positive blood is estimated to stimulate the formation of anti-c in 2 per cent of c-negative subjects. If the corresponding frequency for D is taken to be 50–100 per cent, then D is estimated to be twenty-five to fifty times as potent an antigen as c.

Anti-E is found rather less commonly than anti-c in women immunized by pregnancy although opportunities for the formation of anti-E are slightly commoner.

Thus, if one multiplies the proportion of women lacking the relevant antigen by the incidence of the gene in the population, the figures are:

For E $0.72 \times 0.16 = 0.11$

For c $0.17 \times 0.57 = 0.10$

In a series of 7940 Rh-positive pregnant women whose serum was 'screened' for antibodies by Dr Eloise Giblett (personal communication), fifty-seven antibodies were found. Most of these were anti-Le^a but there were seven examples of anti-c, six of anti-E and three of anti-c plus anti-E.

The relative importance of pregnancy as a stimulus to the formation of blood group antibodies is also shown by some further figures from Dr Giblett. In the course of 4 years the blood of 60,000 patients was tested for antibodies, usually as a preliminary to transfusion. Among 35,000 female patients, antibodies were detected in 378 cases (1.1 per cent); in the 25,000 male patients 138 antibodies were detected – an incidence of 0.55 per cent. This survey also showed that Rh antibodies were much commoner in female than in male subjects. Thus anti-D was found in 283 women (0.81 per cent) but only in 95 men (0.38 per cent); and anti-c or anti-E or both in fifty-nine women but only in sixteen men. The difference between the two series was not due exclusively to the fact that the women had undergone pregnancies since the number of women receiving transfusions was greater than the number of men. The average male patient received a slightly larger amount of blood than the average female patient, due apparently to the higher incidence of gastro-intestinal bleeding, mainly from peptic ulcer, in males.

Persistence of Rh Antibodies

Once formed, Rh antibodies usually persist in the circulation for a very long period. For example, in a case mentioned by Stratton (1955b) anti-Rh was found in a woman 38 years after her last pregnancy.

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Ward (1957) drew attention to the fact that, whereas incomplete antibodies may persist for very long periods, complete agglutinins disappear comparatively rapidly. Thus, in women found to have saline agglutinins shortly after their last pregnancy, the titre was found to decline very rapidly during the following 12 months, so that at the end of this time only one-third of the women had a saline agglutinin titre of 1 in 8 or more, and after 4 years less than a tenth of the women had a saline agglutinin titre of 1 in 8 or more. In women whose serum contained incomplete anti-Rh the rate of decline was much slower: 6 years after the last pregnancy incomplete antibody could still be demonstrated in 460 out of 478 cases. The sera were tested for their ability to agglutinate Rh-positive red cells suspended in polyvinyl pyrrolidone, and it is possible that if other methods, such as the indirect antiglobulin test, or a test against enzyme-treated cells, had been used, the percentage of women with antibody demonstrable after 6 years would have been even higher.

Production in Animals of Antibodies resembling Anti-Rh

Landsteiner and Wiener (1940) injected the red cells of *Rhesus* monkeys into guinea-pigs and obtained sera which, after absorption with the red cells of certain human subjects (later shown to be Rh-negative) would still react with the red cells of other subjects (Rh-positive). The serum agglutinated the red cells of approximately 85 per cent of white subjects and it has generally been considered to be identical in specificity with anti-D.

Murray and Clark (1952) showed that anti-Rh could be produced by injecting into guinea-pigs material obtained from heated Rh-positive red cells. It had previously been shown by Calvin *et al.* (1946) and by Lubinski and Portnuff (1947) that heating Rh-positive red cells to 56° C. even for 5 minutes rendered the cells inagglutinable by anti-Rh (although such cells will still absorb anti-Rh, as shown by Brading, 1954), and Hubinont (1948) had shown that Rh substance could be detected in the supernatant of the heated red cell suspension. Murray and Clark (1952) showed that if the supernatant was spun at 10,000 rev./min., it lost its ability to inhibit anti-Rh. The deposit could be washed and concentrated and very small amounts would produce anti-Rh when injected into guinea-pigs. Murray and Clark tried injecting material obtained from heated Rh-negative red cells and very surprisingly found that 50 per cent of guinea-pigs injected with this material produced 'anti-D'.

These findings were confirmed by Levine *et al.* (1961). Heat extracts prepared from Rh-negative and Rh-positive red cells were centrifuged at 30,000 g. The washed sediment, after injection into rabbits, induced the

formation of an antibody resembling anti-D in specificity. The fact that this antibody was not identical with anti-D was shown by the fact that it would bring about the agglutination of human D-positive red cells 'blocked' with human anti-D. An antibody behaving in the same way was found in one out of twenty uninjected rabbits.

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CHAPTER TEN

BLOOD GROUP SYSTEMS OTHER THAN ABO, Le AND Rh

THE MNSs SYSTEM

The antigens M and N were first discovered by injecting human red cells into rabbits, absorbing the resulting immune serum with one sample of human cells and showing that it would still react with other samples (Landsteiner and Levine, 1927, 1928).

The frequencies of the different MN groups in Europe are shown in Table XXX.

S and *s* are allelic genes whose inheritance is closely associated with that of *M* and *N*. Thus, the antigen S is found significantly more frequently in blood which contains M than in blood which contains N (Sanger *et al.*, 1948; see also Table XXXI). 55 per cent of English people are S-positive and 45 per cent S-negative.

TABLE XXX

THE FREQUENCY OF THE MN GROUPS IN ENGLAND

Genotype	*Phenotype*	*Reaction with anti-M*	*Reaction with anti-N*	*Frequency per cent*
MM	M	+	−	28
MN	MN	+	+	50
NN	N	−	+	22

Other antigens of the MNSs system. M_1 is an antigen found more commonly in negroes than whites, which is thought to be an alternative form of the common M (Jack *et al.*, 1960). M^g is a rare allele of M and N, having an incidence in whites of less than 1 in 2500 (Allen, Corcoran *et al.*, 1958). Anti-M^g is, however, relatively common (see below).

Vw, Mi^a and Vr are antigens whose inheritance is determined by genes which may be regarded as part of the MNSs system. For example in whites the gene *Vr* has been found to accompany *Ms* (van der Hart *et al.*, 1958). Similarly, *Vw* usually accompanies *Ns* (Mohn *et al.*, 1958).

Mi^a was discovered by Levine, Stock *et al.* (1951), and Vw by van der Hart *et al.* (1954). When Levine, Robinson, Stroup *et al.* (1956) found that all the Dutch Vw(+) samples were Mi(a+), it seemed that the two antigens were identical.

However, Wallace *et al.* (1957) then showed that the explanation lay in the almost invariable presence of anti-Vw in anti-Mi^a sera and that only 50 per cent of Mi(a+) samples were Vw(+). No Mi(a−), Vw(+) sample was found in several thousand samples from whites. Vw has been shown by Simmons *et al.* (1959) to be identical with the Gr factor described by Graydon (1946).

The antigens Hu and He, present respectively in about 20 per cent and 3 per cent of West African negroes but very rare in Europeans, are also part of the MNSs system (for references, see Race and Sanger, 1958, p. 86).

Some individuals have red cells which are not agglutinated by anti-S or anti-s; they are thought to be homozygous for a gene S^u, allelic to *S* and *s*. No white people of this type (S^uS^u) have been found and the incidence in negroes is less than 1 per cent. S^uS^u individuals may form anti-U, otherwise known as anti-Ss (see below) following transfusion or pregnancy.

Antibodies of the MNSs System

Anti-M

Anti-M is a relatively rare antibody. For example, Dahr (1941) failed to find a single instance in the course of a deliberate search in 4000 blood samples. When anti-M does occur, it is usually as a cold agglutinin, acting best at 4° C. and weakly or not at all at 37° C. (Dahr, 1941; Paterson *et al.*, 1942; Unger *et al.*, 1946). In a case described by Crowley *et al.* (1957) an apparently naturally-occurring antibody had a titre of 64 at 4° C. and 25° C. and a titre of 8 at 37° C. Several examples of anti-M have been described in infants within the first year of life. Friedenreich's (1937) subject was only 2½ months old.

TABLE XXXI

SHOWING THAT THE ANTIGEN S IS FOUND MORE FREQUENTLY IN BLOODS OF GROUP M (DATA FROM RACE AND SANGER, 1954)

	S-positive per cent	*S-negative per cent*
M	73.40	26.60
MN	54.10	45.90
N	32.30	67.70
All samples	54.69	45.31

Occasionally anti-M seems to be formed as an immune antibody. Thus in the case reported by Broman (1944) eight blood transfusions had been

RCPE0002068_0176

given to a patient (type N) over a period of 9 months; a ninth blood transfusion was given and produced a severe haemolytic reaction. The patient's serum was found to contain an anti-M agglutinin with a titre of 64; the antibody reacted just as strongly at 37° C. as at refrigerator temperature. Van Loghem, Heier *et al.* (1953) described a case in which after transfusion of M-positive blood the titre of anti-M in the patient's serum rose from 2 to 128 and the antibody became active at 37° C.

Wiener (1950) described a case in which anti-M developed in an Rh-negative volunteer who had received repeated injections of Rh-positive blood with the intention of inducing the formation of anti-Rh. Over a period of 3 years the patient received injections at 4-6 monthly intervals. Nine days after the last of these injections the patient was bled and given in return a transfusion of 500 ml. of Rh-negative blood. She experienced a severe haemolytic reaction, which was shown to be due to the fact that she had developed anti-M.

In a case described by Strahl *et al.* (1955) an 11-month-old infant developed haemoglobinuria following the transfusion of M-positive blood. The infant's serum agglutinated M-positive cells only very weakly at 37° C. but at this temperature it sensitized the cells strongly to an antiglobulin serum.

In a case described by Freiesleben and Knudsen (1957) the patient's serum contained an anti-M which gave a strong indirect antiglobulin reaction; 20 days after the transfusion of incompatible blood the indirect antiglobulin titre had risen to 1000.

In a case described by Cutbush and Mollison (1958), an anti-M serum which, *in vitro*, agglutinated *MM* cells up to a temperature of 34° C. (but not higher) was shown to be capable of destroying *MM* cells very rapidly *in vivo*. The serum of this patient reacted distinctly more weakly with *MN* cells. Thus, it failed to agglutinate them at 34° C., although it did agglutinate them at 31° C. When *MN* cells were injected into the patient they were removed from the circulation very much more slowly than *MM* cells.

The fact that anti-M serum reacts far more strongly with *MM* than with *MN* cells has been reported by many authors. For example, Speiser (1956b) reported a case in which an anti-M had a titre of 32 with *MM* cells and only 2 with *MN* cells (indirect antiglobulin tests at 37° C.).

Haemolytic disease of the newborn due to anti-M is extremely rare. In a case described by Stone and Marsh (1959) a woman who had previously had one miscarriage and one normal (group N infant) gave birth to twins; one was born dead with hydrops and the other was living but

deeply jaundiced. The living infant (group MN) had a weakly positive direct Coombs test although the cells when incubated at 37° C. with an equal volume of 30 per cent albumin for 30 minutes were strongly agglutinated. The infant's serum contained free anti-M. The osmotic fragility of the red cells was greatly increased. The infant was treated by exchange transfusion with group N blood and recovered. The mother's serum contained anti-M with a titre at 37° C. of 64 in saline and 1000 in serum albumin.

In other cases in which anti-M develops during pregnancy, apparently as an immune antibody, it may fail to affect the foetus, as in the case described by Bowley and Dunsford (1949).

Anti-M_1. It appears that about one in three human anti-M sera also contain anti-M_1 (see Jack *et al.*, 1960).

Other sources of anti-M. Anti-M is found as a weak naturally-occurring agglutinin in horse serum; by the injection of human group O, M+red cells reasonably potent antisera can be produced, giving a definitely stronger reaction with *MM* cells than with *MN* cells (Levine, Celano, Lange and Berliner, 1957). Anti-M lectin prepared from the seeds of *Iberis amora* (Candytuft), appears to be quite a useful reagent (Nancy Allen, 1959).

The best anti-M sera are those produced by injecting M(+) red cells into rabbits; for a method see p. 140.

Anti-N

Anti-N agglutinins are encountered in human sera even less frequently than anti-M. Again there are cases in which the agglutinin has been found in subjects who have never been pregnant or received a transfusion. (e.g. Phansomboon *et al.*, 1949); and in subjects who have been pregnant but never transfused (Allott and Holman, 1947; de Kromme and van der Spek, 1948). In these cases the agglutinin was most active at 4° C. and was inactive at 37° C.

Only one really convincing instance of the production of immune anti-N seems to have been described (Callender and Race, 1946):

> The subject (type M) had received five transfusions of MN blood and one of N blood over a period of a year; then after an interval of 3 months five injections of MN blood and, 2 months later, a series of injections of N blood, and finally, 5 months later, seven more injections of type N blood. Thirteen days after the beginning of this last course of injections, anti-N was found in her serum, and within about 2 weeks its titre rose to 64 or more. This patient was particularly good at making antibodies, but she took a comparatively long time to make anti-N.

RCPE0002068_0177

Anti-N has twice been found as an agglutinin in the serum of subjects of the rare genotype S^uS^u (see Stern, Ellis and Masaitis, 1957).

Anti-N is present in the seeds of *Vicia graminea* (Ottensooser and Silberschmidt, 1953), and also in the more readily available seeds of *Bauhinea purpurea, var. alba* (Boyd *et al.*, 1958). Of the two, the lectin of *Vicia graminea* is the more potent but that of *Bauhinea* is apparently quite satisfactory. For example, Fletcher (1959) prepared an anti-N extract with a titre of about 64 by soaking the seeds of *Bauhinea varieagata* for 4 hours in M/15 phosphate buffer pH7, 0.4 ml. for each gramme of seed. They were then homogenized and left overnight and the supernatant separated and stored frozen. The extract weakly agglutinated MM cells but after the addition of 0.1 M glucose only NN and MN cells were agglutinated.

Hirsch *et al.* (1957) have shown that at low temperatures (23° C. or lower) anti-N will agglutinate M cells, although anti-M will not react with N cells. Similarly, anti-N will agglutinate enzyme-treated M cells.

It seems that anti-N does not react with *MM* S^uS^u cells (Allen *et al.*, 1960) and the strength of reaction between anti-N and M+ red cells is influenced by the Ss group of the cells (Dr R. R. Race and Dr Ruth Sanger, personal communication).

Anti-S

Anti-S has occasionally been found as a naturally-occurring antibody (e.g. Constantoulis *et al.*, 1955), but is more commonly found as an immune antibody in patients who have received many transfusions; for example, in the case described by Cutbush and Mollison (1949) the patient received a series of transfusions and developed increasingly severe haemolytic reactions with haemoglobinuria, and was then found to have developed anti-S, detectable only by the indirect antiglobulin test. After the last transfusion of S-positive blood the antibody showed a transient increase in titre, but then decreased steadily, and 12 months later was scarcely detectable.

A case in which anti-S seems to have been responsible for a fatal transfusion reaction is mentioned by Coombs *et al.* (1951).

Anti-S is also known to have caused haemolytic disease of the newborn. In a case referred to in the first edition of this book anti-S was found to be responsible for a positive direct Coombs test in a newborn infant without producing clinical evidence of haemolytic disease of the newborn. Levine, Ferraro and Koch (1952) subsequently reviewed this and eleven other cases and reported one fatal case of haemolytic disease of the newborn due to anti-S. The mother had previously been transfused. The anti-S reacted slightly more strongly at 37° C. than at 18° C.; although the serum agglutinated red cells suspended in saline the titre was higher when the cells were suspended in albumin or treated with trypsin, or when the indirect antiglobulin test was used.

Anti-s

The antibody anti-s is extremely rare; the first recognized example caused severe haemolytic disease of the newborn (Levine, Kuhmichel *et al.*, 1951). In the case described by O'Riordan and Cann (1959) the antibody was found as an agglutinin in the serum of a pregnant woman; the infant was s-positive but was unaffected. Treatment of the patient's serum with ethane-thiol destroyed the activity of this antibody, which may therefore have been composed of 19*S* globulin.

Anti-U (Wiener, Unger and Gordon, 1953), which may be regarded as anti-Ss (Sanger, Race *et al.*, 1955; Hackel, 1958), is rare; Dr E. R. Giblett (personal communication) knows of eight cases recognized in the U.S.A., some of which were associated with haemolytic disease of the newborn.

Anti-Vw, Anti-Mi^a, Anti-M^g

All these antibodies are comparatively common but are of small clinical importance because of the rarity of the corresponding antigens. Darnborough (1957) found nine examples of anti Vw in testing 402 sera; at least five, and probably all, were naturally occurring. All agglutinated cells more strongly in saline than in albumin and better at room temperature than at 37° C.; all were detectable by the indirect antiglobulin test. Zeitlin *et al.* (1958) found eight examples of anti-Vw in 703 normal sera. As explained above, anti-Vw is now known to be the same as anti-Gr.

According to Mohn *et al.* (1958), all anti-Mi^a sera contain anti-Vw as well as anti-Mi^a. Both anti-Mi^a and anti-Vw are known to have caused haemolytic disease of the newborn (Levine, Stock *et al.*, 1951; van der Hart *et al.*, 1954).

Anti-M^g occurs fairly commonly as an agglutinin. Allen *et al.* (1958) found four examples in testing 500 normal sera, and Zeitlin *et al.* (1958) found twenty-three examples in testing 703 normal sera.

THE P SYSTEM

The P blood group system was discovered by Landsteiner and Levine (1927) by testing immune sera prepared by injecting human red cells into rabbits.

RCPE0002068_0178

About 74 per cent of English people are P-positive. There is considerable variation in the strength of the P antigen, and this variation is inherited. P-positives can be classified as P-strong, P-medium and P-weak (Henningsen, 1952).

Sanger (1955) showed that all people who had formed the very rare antibody anti-Tj^a were P-negative, and suggested that the terminology of the P system should be modified so as to subdivide P-negatives into those (*pp*) whose red cells were not agglutinated by anti-Tj^a and those (P_2p) or (P_2P_2) whose red cells were agglutinated by anti-Tj^a but were not agglutinated by anti-P. Her suggested amendments are shown in Table XXXII.

As the table shows, anti-Tj^a is regarded as being a mixture of two anti-

TABLE XXXII

P BLOOD GROUP SYSTEM; RELATIONSHIP OF Tj^a AND P ANTIGENS

P group		*Genotype* (Sanger, 1955)	*Tj^a group*	*Antibodies in serum*	
Old terminology	*New terminology*			*Old terminology*	*New terminology*
P negative	P negative	*pp*	Tj(a—)	anti-Tj^a	anti-P+-P_1
	P_2	P_2p, P_2P_2	Tj(a+)	anti-P	anti-P_1
P positive	P_1	P_1P_1, P_1P_2, P_1p	Tj(a+)	—	—

This table ignores the existence of the very rare allele p^k and the corresponding antibody anti-p^k (see Matson, Swanson, Noades *et al.*, 1959).

bodies, anti-P which reacts with all samples except *pp*, i.e. Tj(a—) and anti-P_1 corresponding to the antibody previously called anti-P. Absorption of anti-P+ anti-P_1 with P_2 red cells yields a pure anti-P_1 reagent.

A fourth allele at the P locus, called P^k, is apparently very rare. The antigen P^k can be recognized only in individuals of the genotype pP^k or P^kP^k. Antisera previously thought to contain only anti-P+P_1 are now known to contain anti-P^k as well (Matson, Swanson, Noades *et al.*, 1959).

Anti-P_1

Henningsen (1949b) found that anti-P agglutinins (now called anti-P_1) were present in the sera of approximately two out of three unselected P-negative individuals; the agglutinins were usually very weak and were

active at low temperatures only. In pregnant P-negative women the incidence of anti-P_1 was almost 90 per cent, but no evidence was obtained that this was connected with iso-immunization in pregnancy. Moreover, some of the most potent anti-P_1 sera came from men who had never had injections or transfusions of blood. Even with the strongest anti-P_1 sera, Henningsen could obtain no reaction at 37° C but there is no doubt that anti-P_1 sera active at 37° C. do exist. Wiener and Unger (1944) described a case in which anti-P_1 was found to be weakly active at 37° C.; the patient had been transfused but there was no evidence that there had been a haemolytic reaction. In a case described by Moureau (1945), a patient with acquired haemolytic anaemia became increasingly jaundiced after the transfusion of P-positive blood and was found to have an anti-P_1 agglutinin with a titre of 32 at 37° C. *In vitro* the addition of guinea-pig serum resulted in the lysis of P-positive cells.

Following the injection of small volumes of P-positive red cells to patients whose serum contains anti-P_1, weakly active at 37° C. *in vitro*, the author has not found any increase in the potency of the antibody in samples taken during the following 3 weeks.

The fact that anti-P_1 is occasionally capable of causing red cell destruction *in vivo* has been demonstrated by injecting isotope-labelled P-positive red cells into a patient whose serum contained a potent anti-P_1 (Mollison and Cutbush, 1955). It was shown that there was rapid destruction of a proportion of P-positive cells but no destruction of P-negative cells. When tested *in vitro* at 37° C. the patient's serum only very weakly agglutinated red cells but it caused the adsorption of complement, as could be shown by tests with an appropriate antiglobulin serum. The patient's serum also partially lysed ficin-treated P-positive cells at 37° C. (Cutbush and Mollison, 1958). The serum was tested after 4 years' storage and was found to lyse untreated P-positive cells very weakly if rabbit complement was added (Mollison and Thomas, 1959).

Patients whose serum contains anti-P_1 which only doubtfully agglutinates P-positive cells at 37° C. may fail to bind complement, or do so only very weakly (Cutbush and Mollison, 1958). This contrasts with the behaviour of sera containing Lewis antibodies, which may fail to agglutinate red cells above a temperature of about 25° C. but readily fix complement at 37° C. (see for example the anti-Le^b described by Mollison and Thomas, 1959).

Sera containing anti-P_1 commonly agglutinate P-negative as well as P-positive red cells if tests are carried out at about 20° C. or lower, and this frequently complicates the problem of identifying the antibody.

RCPE0002068_0179

Echinococcus cyst fluid. Cameron and Staveley (1957) found that the live scolices of hydatid disease inhibited anti-P_1 and stated that they and Merritt and Hardy (1955) had detected potent anti-P_1 in patients with hydatid disease.

Levine, Celano and Staveley (1958) have produced anti-P_1 in rabbits by injecting P-negative cells (or rabbit cells) tanned and coated with Echinococcus cyst fluid; the injection of cyst fluid alone failed to elicit the formation of antibodies.

Echinococcus cyst fluid may be lyophilized and stored, and may conveniently be used to establish the specificity of a suspected anti-P_1 agglutinin. Sera which are inhibited by a 1 in 100 dilution of cyst fluid may be presumed to contain anti-P_1; see Chapter XII.

Anti-P+-P_1 (anti-Tj^a)

Anti-Tj^a, called anti-P+-P_1 in Sanger's terminology, is a very rare antibody, only thirteen examples having been encountered up to about 1954 (Levine and Koch, 1954). Several of the first examples of anti-Tj^a were found in women who had had miscarriages, and Levine and Koch (1954) suggested that the antibody might be the cause of these accidents. This no longer seems likely, since more than one woman with anti-Tj^a in her serum is known to have given birth to a live normal Tj(a+) child. One such case was described by McNeil, Trentelman and Thomas (1957); another was observed in the author's hospital, although in this case the happy outcome may have been due to the fact that the father was P-negative (old terminology).

Mrs E., whose serum contained anti-P+-P_1 (anti-Tj^a), gave birth to her first infant at term. The infant appeared healthy, and it never became jaundiced. A sample of cord blood had a haemoglobin concentration of 17.4 g./100 ml.; the red cells gave a negative direct antiglobulin test. A further blood sample taken some hours after birth was examined more fully. The osmotic fragility of the red cells was found to be strictly normal, and the reticulocyte count to be 2.6 per cent. The serum was examined by Dr R. R. Race, who found that the baby was P-negative but Tj(a+), i.e. P_2p. Its serum contained a fairly strong anti-P_1 and weak anti-P. In this case the baby presumably escaped trouble by being P-negative (or more strictly, P_1-negative).

Anti-Tj^a, as might be expected, is capable of causing rapid red cell destruction *in vivo*. In the first subject (Mrs 'Jay') in whom anti-Tj^a was found, a test injection of 25 ml. of Tj(a+) blood was given. Following the injection there was an immediate severe reaction with haemoglobinaemia. Subsequently the titre of anti-Tj^a rose from 8 to 512 (Levine, Bobbitt *et al.*, 1951).

THE LUTHERAN (Lu) SYSTEM

About 8 per cent of the English population are Lu(a+). Almost all of these have the genotype Lu^aLu^b, the overall frequency of the genotype Lu^aLu^a in the English population being only 0.15 per cent.

Anti-Lu^a

The first example of anti-Lu^a was found by Callender and Race (1946) in a patient who had had two blood transfusions. It was not possible to say with certainty whether the antibody had played any part in the haemolytic transfusion reaction, since the patient had also developed anti-c. It seemed that the antibody was immune, because its strength diminished rapidly during the subsequent weeks. About 2 months after the transfusion of Lu(a+) blood the antibody had practically disappeared. Callender and Race reported that the type of agglutination produced by anti-Lu^a was characteristic: 'The agglutinates were large, but many unagglutinated cells were constantly present.'

Eleven months after the patient had formed anti-Lu^a an attempt was made to re-stimulate the antibody by giving small intravenous injections of Lu(a+) blood. Six injections were given during 4 weeks; then, after a rest of 5 weeks, seven more injections were given over a further period of 4 weeks. No anti-Lu^a was found, though the patient did, for the first time, produce anti-N.

A further example of anti-Lu^a was reported by Mainwaring and Pickles (1948) in a patient who had received multiple transfusions. There was no evidence that it had been the cause of rapid blood destruction. The antibody, when first found, had a titre of 16, but then diminished in strength progressively. It was possible to distinguish strongly and weakly reacting Lu(a+) blood. An attempt was made to assess the immunizing capacity of the Lu^a antigen by using Lu(a+) blood for transfusion to eight Lu(a−) patients. After a single transfusion two out of eight patients developed anti-Lu^a of the agglutinating type. Both these patients had received previous transfusions, but in one case the previous donors were traced and found to be Lu(a−). In two patients who developed anti-Lu^a, the agglutinin was found 15 and 28 days after transfusion respectively, but was then detectable in the serum for a short time only. It was not stated whether, in these two particular cases, the antibody was more active at

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37° C. than at 20° C., and the survival of the Lu(a+) cells was not measured.

From the experiments of Mainwaring and Pickles it might appear that anti-Lu^a is formed quite readily by Lu(a−) subjects receiving more than one transfusion of Lu(a+) blood. This evidence is difficult to reconcile with the experience of Race and Sanger (1954, p. 208), who gave four injections of Lu(a+) blood at 3-monthly intervals to each of twelve Lu (a−) subjects, but did not observe the formation of anti-Lu^a in a single case.

Even if the Lu^a antigen readily provoked the formation of anti-Lu^a, the antibody would not often be formed, since the antigen occurs in only 8 per cent of the population, and thus the chance of receiving blood from two Lu(a+) donors is small, except in patients receiving a large number of transfusions.

The best estimate of the frequency of anti-Lu^a is provided by the survey of Greenwalt and Sasaki (1957), who examined the serum of 18,600 normal blood donors and found three examples of anti-Lu^a. All these antibodies reacted better at 12° C. than at higher temperatures and showed some dosage effect.

Although anti-Lu^a has not yet been clearly incriminated as the cause of a haemolytic transfusion reaction, there can be no doubt that a potent example of the antibody would cause red cell destruction *in vivo*. Since most examples of anti-Lu^a appear to be 'naturally-occurring' and are therefore presumably 19*S* gamma globulins, haemolytic disease of the newborn due to anti-Lu^a is expected to be extremely rare.

Anti-Lu^b

The first example of this antibody was found in a woman who had never been transfused but had had three previous pregnancies. The antibody reacted well with saline suspensions of red cells; the reactions were stronger at room temperature than at 37° C. The indirect Coombs reaction was weakly positive, but only with *Lu^{b}Lu^b* cells. The identity of the antibody was first suspected when it was found that the patient's own cells reacted very strongly with an anti-Lu^a serum and that the patient's serum gave weaker reactions with Lu(a+) than with Lu(a−) cells (Cutbush and Chanarin, 1956). Initially the antibody appeared to react with all samples of red cells, a fact which is not surprising in view of the very low incidence of *Lu^{a}Lu^a* subjects in the English population — 0.15 per cent, according to Race and Sanger (1954, p. 215). The patient's sister had the genotype *Lu^{a}Lu^a*; her serum did not contain anti-Lu^b.

This example of anti-Lu^b was found to bring about the destruction of

Lu(b+) red cells *in vivo*. *Lu^{b}Lu^b* red cells underwent considerably greater destruction than *Lu^{a}Lu^b* red cells (Cutbush and Mollison, 1958). In the case described by Greenwalt and Sasaki (1957) a patient whose serum contained anti-Lu^b developed jaundice 10 days after the transfusion of Lu(b+) blood, and her serum was found to contain methaemalbumin.

A case in which anti-Lu^b may have caused very mild haemolytic disease of the newborn was described by Kissmeyer-Nielsen (1960) but the possibility that anti-B was responsible could not be completely excluded.

THE KELL (K) SYSTEM

Nine per cent of the English population are K+, having the genotype *KK* or *Kk*; only 0.2 per cent belong to the genotype *KK*.

There are other antigens within the K blood group system called Kp^a and Kp^{b}f but since the corresponding antibodies are extremely rare they will not be discussed here. The relationship of the genes *Kp^a* and *Kp^b* to *K* and *k* has not yet been clearly demonstrated. Some references are as follows: Allen and Lewis (1957); Chown *et al.* (1957); Allen, Lewis and Fudenberg (1958); Dunsford *et al.* (1959).

Anti-K

No example of a naturally-occurring anti-K has yet been described, but anti-K is a relatively common immune blood group antibody.

A thorough investigation into the frequency with which K-negative subjects form anti-K following the transfusion of K-positive blood was published by Kornstad and Heistö (1957). One hundred and thirty K-negative subjects who had received a transfusion which included at least one bottle of K-positive blood were examined; of fifty-three tested at 4-12 months, five had anti-K; of seventy-seven tested at 13-37 months, only one had anti-K. The authors concluded that the probability that a K-negative subject transfused with a bottle of K-positive blood would develop anti-K was about 1 in 10.

The conclusion that K is a less effective antigen than Rh(D) is supported by the experiment of Wiener, Samwick *et al.* (1955). Sixteen subjects, at least ten of whom were K-negative, received three injections of pooled red cells from eight donors, some of whom were K-positive. There was an interval of 4 months between the first and second injections, and of 13 months between the second and third injections. None of the recipients formed anti-K.

Nevertheless, in any large series of cases a good many examples of anti-K will be found. Thus, Levine (1955) reported that in examining the

RCPE0002068_0181

sera of 7500 patients who received a total of 15,000 blood transfusions, fourteen examples of anti-K were found; and Dr Eloise R. Giblett (personal communication) in the course of 'screening' samples of serum from 21,000 patients found thirty examples of anti-K.

Opportunities for iso-immunization to K in pregnancy are relatively common although the formation of anti-K by pregnant women who have not been previously transfused with K+ blood is rare. If two pregnancies with a K-positive (or Rh-positive) foetus are sufficient to immunize a woman, anti-K would be found during pregnancy one-third as commonly as anti-Rh (Wiener, Brancato and Waltman, 1953); that is to say in one-third of six per 1000, or two per 1000.

It seems that this figure should really be one-fourth as common, not one-third. Thus, if 17 per cent of women are Rh-negative, 35 per cent of their husbands homozygous (*DD*) and 48 per cent heterozygous (*Dd*), the number who will have two Rh-positive infants in succession is $0.17 \times \left(0.35 + \frac{0.48}{4}\right)$, or 0.08, or 8 per cent. If 91 per cent of women are K-negative, 0.2 per cent of their husbands homozygous (*KK*) and 8.8 per cent heterozygous (*Kk*), the number who will have two K-positive infants in succession is $0.91 \times \left(0.002 + \frac{0.09}{4}\right)$, or 0.02, or 2 per cent. In these calculations the reason for dividing the frequency of heterozygous husbands by four is that there is only a one in four chance that the husband will pass on the relevant gene twice in succession.

Among 13,000 pregnant women tested by Dr Eloise R. Giblett (personal communication) anti-K was found in thirteen — an incidence of one in 1000, but this does not imply that pregnancy is a frequent stimulus, for most of the women whose sera contained anti-K gave a history of a previous transfusion.

Since most K-positive subjects are heterozygous (*Kk*), and thus are unlikely to have a large number of K-positive children, it is not surprising that when a K-negative woman is found to have formed anti-K during pregnancy it often turns out that she has been transfused previously with K-positive blood. For example, in the case in which anti-K was first discovered (Coombs *et al.*, 1946) the woman whose infant suffered from haemolytic disease of the newborn had received a transfusion of blood from her husband (history elicited in a later interview with the patient). The same thing had happened in a case described by Chown (1949), who emphasized the moral, 'Never transfuse a woman with her husband's blood'.

Many severe haemolytic reactions due to anti-K have been described; see for example a case described by Wiener and Sonn-Gordon (1947b) in which the patient's antibody was at first given the name anti-Si, but was later recognized to be identical with anti-K. In the case referred to by Young (1954) the patient developed haemoglobinuria as a result of a transfusion of K-positive blood. Ottensooser *et al.* (1953) described a haemolytic reaction due to anti-K in a woman who had received the blood of two K-positive donors 23 years previously. Anti-K can cause severe haemolytic disease of the newborn (Van Loghem, de Raad and van Hattem, 1953).

Serological Characteristics of Anti-K Sera

Sera containing anti-K usually fail to agglutinate red cells suspended in saline; they give better agglutination in a medium of AB serum than in concentrated albumin (Grove-Rasmussen *et al.*, 1954). Simmons (1956) found that agglutinating activity could best be demonstrated by mixing 1 drop of serum with 1 drop of a 10 per cent cell suspension in citrate-glucose on a slide, and reading the results after the slide had remained in a moist chamber for 30-60 minutes; the optimal temperature was 20° C. rather than 37° C. in all of eight cases.

The most reliable test for anti-K is the indirect antiglobulin test. Some anti-K sera bind complement, and are then best detected when fresh serum is used to sensitize K-positive cells, or fresh normal serum is added as a source of complement (and the cells are then tested with a serum containing an adequate amount of anti-β_1 (complement) globulin).

Sera containing complement-binding anti-K may lyse K-positive cells if rabbit serum is added as a source of complement (Mollison and Thomas, 1959).

Anti-k is a rare antibody; the first case to be described was found in the serum of a recently delivered woman whose infant was mildly affected with haemolytic disease of the newborn (Levine, Backer *et al.*, 1949). The rarity of the antibody can be partly accounted for by the fact that only two in 1000 white subjects are k-negative (i.e. *KK*).

THE DUFFY (Fy) SYSTEM

The red cells of about 66 per cent of the English population are Fy(a+), belonging to the genotype Fy^aFy^a or Fy^aFy^b (Cutbush *et al.*, 1950). Fy^aFy^a samples react more strongly than Fy^aFy^b samples with some anti-Fy^a sera; the distinction between the two types of blood can be made directly by testing with an anti-Fy^b serum (see Table XXXIII).

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In negroes, 68 per cent of subjects have the phenotype Fy(a—b—); this type has not yet been found in Europeans (Sanger, Race and Jack, 1955).

Anti-Fy^a

Most examples of anti-Fy^a are 'immune' and are found in the serum of patients who have received several transfusions. Only one example has been described in which the antibody may have been naturally-occurring. In this case a man developed a mild haemolytic reaction following his first transfusion. The pre-transfusion sample of serum was found to contain anti-Fy^a, though the reactions were weak; 4 days after the transfusion the serum reacted very strongly with Fy(a+) cells (Rosenfield *et al.*, 1950).

Many examples of anti-Fy^a can be detected only by the indirect anti-

TABLE XXXIII

THE FREQUENCY OF THE DUFFY GENOTYPES AND PHENOTYPES IN ENGLAND

Genotype	*Phenotype*	*Reaction with* anti-Fy^a	anti-Fy^b	*Frequency per cent*
Fy^aFy^a	Fy(a+b—)	+	—	17
Fy^aFy^b	Fy(a+b+)	+	+	49
Fy^bFy^b	Fy(a—b+)	—	+	34

globulin test. Quite often the antibody binds complement; therefore in making tests for anti-Fy^a, if the sample of serum is not fresh, normal complement should be supplied (see Chapter XII).

The Fy^a antigen is destroyed by many enzyme preparations. Morton (1957) found that Fy(a+) cells treated with impure preparations of trypsin failed to react with anti-Fy^a serum whereas cells treated with crystalline trypsin were either agglutinated by the anti-Fy^a serum or gave a positive indirect antiglobulin test. Fy(a+) cells treated with ficin absorb very little anti-Fy^a (Haber and Rosenfield, 1957).

Occasionally anti-Fy^a sera agglutinate Fy(a+) red cells suspended in saline. One such serum was used in extensive studies by Race, Sanger and Lehane (1953), who found that the red cells of homozygotes (Fy^aFy^a) were agglutinated more strongly than those of heterozygotes (Fy^aFy^b). Sometimes an anti-Fy^a which can originally be detected only by the indirect antiglobulin test becomes weakly active in saline after antigenic

stimulation. Two examples were described in the second edition of this book (Mollison, 1956, p. 242).

Anti-Fy^a can pass the placenta. Thus, in a case described previously (Mollison, 1956, p. 344) a woman whose serum contained an antibody later shown to be anti-Fy^a was transfused with a large amount of incompatible blood. No free antibody remained in her circulation, but serum obtained from her newborn infant (whose red cells were Fy(a—)) was shown to contain anti-Fy^a. This was actually used for cross-matching blood for the mother.

In a paper published in 1959 Greenwalt *et al.* gave details of eleven cases of haemolytic disease of the newborn due to anti-Fy^a which had come to their notice. The first published case was that of Baker *et al.* (1956); in this case, as in five other of the eleven cases, the mother had been immunized by an earlier transfusion of Fy(a+) blood. Two of the eleven infants died from haemolytic disease of the newborn.

Anti-Fy^b is a much rarer antibody than anti-Fy^a. Race and Sanger (1958, p. 223) recommend the antiglobulin test or the trypsin antiglobulin test for its detection; one example of a potent agglutinating anti-Fy^b has since been found (Dr I. Buchanan, personal communication).

THE KIDD (Jk) SYSTEM

About 75 per cent of English people possess the Jk^a antigen; 25 per cent have the genotype Jk^aJk^a (phenotype Jk(a+b—) and 50 per cent have the genotype Jk^aJk^b (phenotype Jk(a+b+)). The remaining 25 per cent of the population have the genotype Jk^bJk^b (phenotype Jk(a—b+)).

The type Jk(a—b—) has been recognized in a Filipino (Pinkerton *et al.*, 1959).

Anti-Jk^a and Anti-Jk^b

The first example of anti-Jk^a was discovered in the serum of a woman who had given birth to an infant with haemolytic disease of the newborn (Allen *et al.*, 1951). The patient had never been transfused and had previously given birth to five normal infants. The mother's serum also contained anit-K, and since the baby was typed as K-positive it seemed uncertain whether the anti-Jk^a had played any part. Later the infant was found to be really K-negative (personal communication from Dr F. H. Allen, cited by Greenwalt *et al.*, 1956). A case of haemolytic disease of the newborn due to anti-Jk^a and severe enough to cause kernicterus was described by Matson, Swanson and Tobin (1959).

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Anti-Jk^a occasionally causes severe haemolytic transfusion reactions, with haemoglobinuria (e.g. Kronenberg *et al.*, 1958).

Anti-Jk^b is a rarer antibody than anti-Jk^a, and has usually been found in sera which also contain other immune blood group antibodies (e.g. Plaut *et al.*, 1953). The antibody anti-Jk^{a}Jk^b (i.e. inseparable) was found, apparently occurring naturally, in the serum of the Jk(a—b—) patient described by Pinkerton *et al.* (1959).

Anti-Jk^a and anti-Jk^b are best detected by the indirect antiglobulin test; they are complement-binding antibodies, and thus, if the serum being tested is not fresh, fresh normal serum should be added during the stage of incubation with test cells. According to Stratton (1956a) better results can be obtained by using concentrated fresh serum, prepared by freezing the serum and removing the watery layer. Anti-Jk^a may lyse ficin-treated red cells, and characteristically shows a striking dosage effect, so that lysis of *Jk^{a}Jk^a* cells may be 100 per cent but that of *Jk^{a}Jk^b* cells only 25 per cent (Haber and Rosenfield, 1957).

Similarly, anti-Jk^a may lyse untreated Jk(a+) cells if rabbit complement rather than human complement is used (Mollison and Thomas, 1959), and may sensitize red cells to an immuno-conglutinin serum (Mollison and Polley, 1960).

In the detection of very weak Kidd antibodies the tests may be made more sensitive by first treating the test cells with ficin or trypsin and then carrying out the indirect antiglobulin test. In identifying anti-Jk^a it must be remembered that dosage effect is common with all tests, so that positive results may be obtained only with *Jk^{a}Jk^a* cells (van der Hart and van Loghem, 1953).

Occasionally anti-Jk^a sera agglutinate red cells suspended in saline; an example discovered by Dr M. Metaxas of Zürich and sent to the author's laboratory gave good reactions at room temperature and at 37° C.

Anti-Jk^a and anti-Jk^b have the reputation of 'going off' on storage, but this is because complement, rather than antibody, deteriorates on storage. The antibodies cannot be so very unstable for one case has been published in which serum kept in the deep freeze for 9 years was shown to contain anti-Jk^b (Kornstad and Halvorsen, 1958).

THE DIEGO (Di) SYSTEM

The antibody revealing the existence of the antigen Di^a was found in a woman whose infant was affected with haemolytic disease of the newborn (Layrisse *et al.*, 1955); the family was of mixed native Venezuelan and white race. It was subsequently found that all of 1000 samples from white donors were Di(a—) whereas about 36 per cent of samples from certain South American Indians were Di(a+) (Levine, Robinson, Layrisse *et al.*, 1956; Layrisse *et al.*, 1955). The antigen is found in 5–15 per cent of Japanese and Chinese people (Layrisse and Arends, 1956).

The Di^a antigen has already been shown to be inherited independently of most of the other blood-group antigens and seems almost certain to belong to an independent blood-group system (Layrisse *et al.*, 1959).

THE SUTTER (Js) SYSTEM

The antibody revealing the existence of the eleventh main blood-group system in man was found in a young white male adult. This patient had received a transfusion 10 months previously of blood from two donors, one of whom was a negro. This point turned out to be very relevant because the antigen now named Js^a was found to be present in the blood of 20 per cent of negroes but was not present in any of 500 samples from white donors (Giblett and Chase, 1959). The system of terminology follows that of the Lu, Le, Fy, Jk and Di systems.

ANTIGENS WITH A LOW INCIDENCE

Some antigens are very rare and may thus at first appear to be confined to the family in which they are first discovered. Hence the name 'private' antigens, originally suggested by Levine (1953). Some low incidence antigens which originally seemed to be independent of the nine main blood group systems described above have subsequently been shown to be a part of them (e.g. Vw(Gr), Mi^a). Others have been recognized from the start to be part of one of the main blood group systems (e.g. C^x, described by Stratton and Renton, 1954).

A few low incidence antigens remain which have up to the present not been shown to be related to any of the nine blood group systems. For example:

Levay. This, the very first 'private' antigen to be discovered, was named after the first donor in whose red cells it was present (Callender and Race, 1946). The blood of this donor was given to a patient who formed several previously unknown blood group antibodies in addition to anti-Levay. Subsequently, less than 1 per cent of blood samples were found to be Levay positive.

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Wr^a. This antigen is present in about one in 1000 blood samples in England (Dunsford, 1954; Cleghorn, 1960). The corresponding antibody, anti-Wr^a, was first found in the serum of a woman who had given birth to two infants affected with haemolytic disease of the newborn; the antibody could be detected only by the indirect antiglobulin test (Holman, 1953). According to Dunsford (1954) the antibody is commoner than the antigen and can be detected in approximately one in 100 blood samples. It is sometimes found in males who have never been transfused. For some reason which has not yet been explained, anti-Wr^a is often found in the serum of patients who have formed other blood group antibodies; an association with anti-E was noted by Dunsford (1954).

If the incidence of the antigen is only one in 1000, but that of the antibody one in 100, incompatibility from Wr^a would be encountered only once in 100,000 transfusions, even if no direct matching tests were applied. It is not surprising that only one haemolytic reaction from anti-Wr^a has been reported (see van Loghem, van der Hart *et al.*, 1955).

Cutbush and Mollison (1958) injected a very small volume of Wr(a+) red cells into a patient whose serum contained anti-Wr^a and found that the cells were removed with a half-time of the order of 80 minutes. This should not be taken to indicate that anti-Wr^a is unlikely to cause serious red cell destruction, because in the patient tested the antibody was present as an agglutinin acting only weakly at 37° C.

Be^a. Anti-Be^a was first discovered in the serum of a woman who had been transfused with her husband's blood after her first (normal) pregnancy. She subsequently gave birth to an infant mildly affected with haemolytic disease of the newborn, and she later had two stillbirths (Davidsohn *et al.*, 1953). In two volunteers transfused with 200-400 ml. of Be(a+) blood, anti-Be^a was detected 3 to 8 months later (Stern *et al.*, 1958).

Sw^a. This antigen is found in only one in 6000 of English donors. Its special interest lies in the fact that the corresponding antibody was found in the serum of a patient with the cold antibody type of acquired haemolytic anaemia and in the serum of several other patients with auto-immune haemolytic disease, whereas no example of the antibody was found in testing serum from more than 1200 normal subjects. In every case in which anti-Sw^a was found it was accompanied by anti-Wr^a and sometimes also by anti-Vw and anti-Mi^a (Cleghorn, 1959).

By. Anti-By was discovered by Simmons and Were (1955) in the serum

of a woman who had very recently given birth to an infant with very mild haemolytic disease of the newborn.

Antibodies to the rare antigens Wr^a, Sw^a and By, as well as C^x, Mi^a and Vw are not uncommonly found in the serum of patients with 'symptomatic' acquired haemolytic anaemia, for example haemolytic anaemia associated with chronic lymphatic leukaemia (Cleghorn, 1960).

HIGH INCIDENCE ANTIGENS

When an antibody is found which appears to react with all blood samples, it may be one of the following (Rosenfield, Haber, Kissmeyer-Nielsen *et al.*, 1960):

(1) Known to belong to one of the main blood group systems: anti-Ss (anti-U), anti-P+-P$_1$ (anti-Tj^a), anti-H (O), anti-k, anti-Kp^b, anti-Lu^b or anti-Jk^{a}Jk^b.
(2) Thought to be independent of the main blood group systems: anti-Vel, anti-Yt^a, anti-I or anti-Ge.

Incidence of positive reactions. Anti-Lu^b, anti-k and anti-Yt^a react with about 99.8 per cent of all blood samples; anti-Ss and anti-Vel give negative reactions with less than one in 1000 and anti-H (O), anti-P+-P$_1$, anti-I and anti-Ge react with almost all blood samples.

The antibodies reacting with an antigen belonging to one of the main blood group systems have already been described and it remains to give an account of anti-Vel, anti-Yt^a, anti-I and anti-Ge.

Anti-Vel, Anti-Yt^a and Anti-Ge

Of the first two examples of anti-Vel described, one was an agglutinating antibody and the other haemolytic (Sussman and Miller, 1952; Levine, Robinson, Herrington and Sussman, 1955). An example described by Levine *et al.* (1961) was also haemolytic and had apparently been formed as an immune response to transfusion. Six relatives of the propositus were also Vel negative, or Ve(a—), but none of them had anti-Vel (anti-Ve^a) in the serum.

The only example of anti-Yt^a described so far was an antibody demonstrable only by the indirect antiglobulin test and apparently not diminishing the survival of incompatible red cells *in vivo* (Eaton *et al.*, 1956).

The first three examples of anti-Ge, all of which came from pregnant or recently delivered women were described by Rosenfield, Haber, Kissmeyer-Nielsen *et al.* (1960). All were incomplete antibodies and could

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be revealed only by the indirect antiglobulin test. The infants born to all three women had a positive direct antiglobulin test but no evidence of haemolytic disease.

Anti-I

Cold auto-agglutinins, occurring as weak antibodies in the serum of normal subjects, or in much higher titre in the serum of certain patients with acquired haemolytic anaemia, were until recently thought to have no blood group specificity. Although it was recognized that there were some samples of red cells which consistently reacted more strongly with such antibodies, no one had found any blood samples which failed to react. In 1956, Wiener, Unger *et al.* described investigations on the serum of two patients with the cold antibody type of acquired haemolytic anaemia. The serum of the patients agglutinated almost all blood samples, but in the course of testing 22,000 samples, five were found which failed to react. It was concluded that the antibody had blood group specificity, and it was named 'anti-I'. This work was confirmed and extended by Jenkins *et al.* (1960) and Tippett *et al.* (1960). The latter authors described the occurrence of anti-I in three circumstances: (*a*) as a powerful cold antibody in the serum of certain patients with acquired haemolytic anaemia; (*b*) as a weak cold auto-agglutinin in the serum of subjects without haemolytic anaemia; (*c*) in very occasional normal subjects whose red cells had an abnormally weak I antigen.

Categories (*a*) and (*b*) will be discussed in Chapter XI, which deals with auto-antibodies. Category (*c*) will be discussed here briefly.

The first example of a normal subject with a potent anti-I in his serum was discovered by Jenkins *et al.* (1960); five further examples were described by Tippett *et al.* (1960). Red cells from three of these subjects were not agglutinated at all by serum from all the other subjects; the red cells from the remaining three subjects (three brothers) were very weakly agglutinated. All the sera reacted more strongly at 6° C. than at 20° C., and reacted more strongly with ficin-treated red cells than with untreated cells. There was a strong suggestion of some relationship of I to the ABO system, for the red cells of two of the donors gave outstandingly weak reactions with anti-H and anti-O, and group O red cells were slightly more strongly agglutinated by anti-I than were cells of other groups. Anti-I was sometimes accompanied by 'pseudo anti-A_1', that is, the serum reacted more strongly with A_1 cells than with those of other ABO groups but did not react with A_1i cells. Nevertheless the inheritance of I was shown to be independent of that of the ABO groups. Anti-I can readily

be distinguished from anti-H by the fact that it reacts with 'Bombay', i.e. H-negative cells.

There was also a suggestion of some similarity between the I and P_1 antigens. Thus, hydatid cyst fluid, which inhibits anti-P_1, also inhibits some examples of anti-I, although a greater concentration is required to inhibit anti-I than anti-P_1.

Anti-i was described by Jenkins and Marsh (1960). The serum weakly agglutinated the patient's own red cells and the red cells of 500 normal adults (average titre 16 at 12° C.) but reacted extremely strongly with I-negative red cells (titre 250,000). Red cells from cord blood, which react weakly with anti-I, were shown to react very strongly with anti-i. During the first 18 months of life the red cells gradually come to react strongly with anti-I and weakly with anti-i. Jenkins and Marsh concluded that *I* and *i* are not allelomorphic genes but rather that *i* is a precursor of I and that the proper development of I is genetically determined.

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CHAPTER ELEVEN

AUTO-ANTIBODIES AND OTHER CAUSES OF INTERACTION BETWEEN RED CELLS AND THEIR OWN SERUM OR PLASMA

The production of antibodies by an individual capable of damaging that individual's own cells was for a long time considered an impossibility (see Ehrlich and Morgenroth, 1900), but this view has now been modified. As mentioned in Chapter VII it is now believed that there are several different ways in which the normal 'tolerance' of the body to its own antigens may be broken down. So far as red cell auto-antibodies are concerned it is thought that the breakdown is due to some change in the antibody-forming tissues rather than in any alteration in the red cell antigens.

In the general field of immunology the term 'auto-antibody' is normally restricted to an antibody which produces pathological effects; for example antibodies against thyroglobulin produce changes in the thyroid gland and signs of hypothyroidism. As described later in this chapter, comparable auto-antibodies against red cells occur occasionally in human sera but in addition to these, human sera regularly contain so-called normal auto-antibodies. These are active *in vitro* only at temperatures near 0° C., and are assumed to be inactive in the body. Their origin, like that of the naturally-occurring iso-agglutinins, is uncertain. Some people consider that they are hetero-agglutinins, whose formation is stimulated by the ingestion of antigenic material in the tissues of animals, plants or bacteria (e.g. Wiener, 1951b). Others believe that they are normal globulins with configurations which by chance correspond with those of antigens on the red cells.

Naturally-Occurring Cold Auto-antibodies

Cold Auto-Agglutinins

Landsteiner (1903) observed that if the serum of an animal was mixed with its own red cells at a temperature near 0° C. agglutination occurred. He later showed that the serum from most human subjects would agglutinate red cells from the same subject at 0° C. (Landsteiner and Levine, 1926).

The titre of normal auto-agglutinins at 0-2° C. does not usually exceed 64 using a tube technique with a 2 per cent cell suspension and reading the

results microscopically (Dacie, 1954b, p. 190) and is found to be less than 10 if the end-point is read macroscopically (Finland *et al.*, 1945).

Normal cold auto-agglutination can be reversed by warming the agglutinated cells to 20° C.; it seems to be characteristic of cold agglutination that the temperature required to reverse it is higher than the temperature at which agglutination occurs.

Normal cold auto-agglutinins appear to have the same specificity as pathological cold auto-agglutinins, that is to say, anti-I (see Chapter X). Thus, the only red cells with which they fail to react are those from the very rare donors whose red cells lack, or almost lack, the I antigen.

In contrast to the normal incomplete cold antibody (see below), cold auto-agglutinins are not inhibited by secretor saliva.

Milgrom *et al.* (1957) were led to believe that all subjects form auto-agglutinins active up to body temperature, but that these are normally adsorbed by the subject's own red cells. The experimental basis for this belief was the observation that if blisters were raised in the palms of normal subjects, a fluid was obtained with a relatively high protein concentration (3 per cent), containing an auto-agglutinin acting preferentially on group O red cells but not inhibited by secretor saliva. The agglutinin was weakly active at 37° C. and more strongly active at lower temperatures.

Cold agglutinins can usually be demonstrated in cord blood, but only if they are present in the mother's blood. Using group O cells from adults, it was found that the titre in the infant's serum was usually a little lower than that in the mother's serum (Crawford and Mollison, unpublished observations).

A phenomenon which deserves further investigation was reported by Parish and Macfarlane (1941). The serum of a patient was found to agglutinate his own cells and other cells only when they were suspended in citrate and not when they were suspended in saline. A similar phenomenon was described by Jakobowicz and Bryce (1949); in their case the serum appeared to contain anti-O(H) but it did not agglutinate cells suspended in saline. Agglutination occurred only if ionized calcium was absent and sodium was present.

In some states of disease, powerful cold agglutinins are found in the serum; for example, in some cases of acquired haemolytic anaemia and following virus pneumonia. It is not known whether these cold agglutinins simply represent an increased amount of the normal cold agglutinin or whether they differ qualitatively. Their characteristics will be discussed below in a separate section.

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Normal Incomplete Cold Auto-Antibody

Dacie (1950b) showed that if red cells are left in contact with normal human serum at 0–4° C. for 2 hours or more and then washed in warm saline (to wash off 'complete' antibodies) they give a positive reaction with antiglobulin serum. He showed that this incomplete cold antibody binds firmly on to red cells only in the presence of factors present in fresh serum; the optimum pH for binding is 8.0 (Dacie, 1954b, p. 263). Serum which has been heated to 56° C. does not sensitize red cells; its ability to do so can be partly restored by the addition of a small amount of absorbed fresh serum. Heparin, oxalate or citrate added to serum also inhibit the sensitization (Dacie, 1950b).

Dacie (1951) also found that red cells sensitized with incomplete cold antibody were agglutinated by an antiglobulin serum to which gamma globulin had been added, and later showed that it was complement, and not antibody, which was detected by the antiglobulin serum under these circumstances (Dacie *et al.*, 1957).

Normal incomplete cold antibody, like other cold antibodies, is capable of binding complement and will, under appropriate conditions, lyse red cells or sensitize them to an immuno-conglutinin serum. Thus, if fresh normal serum and PNH red cells are used (Dacie *et al.*, 1956), or if normal red cells and rabbit complement are used (Mollison and Thomas, 1959), and the mixtures are put first at 0° C. and then at 37° C., lysis occurs. Similarly, if normal red cells are sensitized at 0° C. with fresh normal serum they will be agglutinated by an immuno-conglutinin serum (Mollison and Polley, 1959).

Since the normal incomplete cold antibody is bound to red cells only when complement is present (Dacie, 1950b) it is difficult to demonstrate the existence of the antibody as something apart from complement. However this can be done by using inactivated human serum as a source of incomplete cold antibody, fresh horse serum as a source of complement and normal human red cells. If these are left together at 0° C., the red cells become agglutinable by an immuno-conglutinin serum, but negative reactions are obtained (*a*) if the horse serum is inactivated by heating or (*b*) if the cells are first exposed to heated human serum, then washed and exposed to fresh horse serum; that is, complement and antibody must be present at the same time for sensitization to occur (Polley and Mollison, 1961).

The normal incomplete cold antibody has a specificity closely related to, or identical with, anti-H. Thus, it reacts far more strongly with O cells than with A_1B cells, and the reactions are inhibited by the saliva of

secretors but not by the saliva of non-secretors. Subjects whose cells react weakly with anti-H have slightly more incomplete anti-H in their serum than subjects whose cells react strongly with anti-H (Crawford *et al.*, 1953b). Normal incomplete cold antibody does not react with 'Bombay' red cells (unpublished observations).

As a rule red cells can be sensitized by the normal incomplete cold antibody only at temperatures up to about 12° C. (unpublished observations) although occasionally cells are sensitized at room temperature. In one case investigated in the author's laboratory, blood was taken from the subject (an apparently healthy blood donor) and examined the same day; the direct antiglobulin reaction of her red cells was moderately strongly positive. When the test was repeated on blood taken straight from a syringe into warm saline, the direct antiglobulin reaction was negative. It was shown that the patient's serum contained an unusually potent incomplete cold antibody which would 'coat' normal red cells at room temperature. The survival of the subject's own red cells in her own circulation was measured by labelling them with ^{51}Cr and, as expected, found to be normal.

The titre of incomplete cold antibody appears to be quite unrelated to the titre of normal cold agglutinin in the same sample (Schubothe, 1954).

When citrated blood is stored at 4° C. the red cells may adsorb some incomplete cold antibody despite the presence of citrate which, by virtue of its anti-complementary activity, interferes with the binding of the antibody. Thus, when the indirect antiglobulin test is used for direct matching, stored red cells may appear to be incompatible. That is to say, if the cells are incubated with the recipient's serum and then tested with antiglobulin serum, they will be weakly agglutinated and this may be ascribed to the presence of a specific antibody in the patient's serum. The correct interpretation of the results will be reached if the cells are also tested directly with antiglobulin serum, that is, without first being incubated with the recipient's serum.

Incomplete cold antibody was demonstrated in twenty-nine out of thirty-three samples of cord blood that were tested (Crawford and Mollison, unpublished observations). The cells of newborn infants contain very little H substance (Watkins, 1952); thus the presence of anti-H in their serum is unlikely to produce a 'false' positive direct antiglobulin reaction.

Nevertheless Holländer (1952) has reported one case in which the red cells of a newborn infant gave a weak positive direct Coombs reaction with undiluted

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antiglobulin serum. This was due apparently to the transfer from the mother of incomplete cold antibody which was inactive at 37° C. but had a titre of 8 at 22° C. and of 128 at 4° C.; the antibody appeared to be a 'normal' incomplete cold antibody since the mother's serum contained only low titre cold agglutinins. A fresh sample from the infant gave a negative direct antiglobulin reaction, but after storage at 4° C. for 48 hours the reaction became moderately strong.

Pathological Auto-Antibodies

Cold Antibodies Associated with Acquired Haemolytic Anaemia

In some patients with acquired haemolytic anaemia, the serum contains extremely potent cold auto-agglutinins with titres usually lying in the range 500 to 100,000 at 2° C. If, in titrating the serum, the dilutions are made in normal inactivated serum (heated to 56° C. for 30 minutes) instead of in saline, the reaction may be greatly enhanced, giving much higher titration values (Rossi, 1958).

According to Dacie (1959) cold antibodies rather than warm ones are found in about 20 per cent of cases of 'idiopathic' acquired haemolytic anaemia, in about 30 per cent of cases secondary to some other condition, such as chronic lymphatic leukaemia, and in all cases following virus pneumonia.

The auto-agglutinin appears at first sight to be non-specific. Thus, Crookston *et al.* (1956) tested fourteen sera containing potent cold agglutinins and found that, although there were striking differences in their reactivity with red cells from various donors, these differences did not seem to be related to the ABO groups of the donors or to any other blood groups. On the other hand, the samples of red cells could be arranged in a definite order of reactivity, so that cells which reacted strongly with one serum reacted strongly with all, and so on.

Wiener, Unger *et al.* (1956) produced evidence that this kind of antibody might have blood group specificity. The serum they tested came from a patient with acquired haemolytic anaemia, and contained a high titre cold agglutinin. The serum was tested against 22,000 samples of red cells, and five of these were not agglutinated. Wiener *et al.* suggested that the name 'anti-I' should be given to the agglutinin. They concluded that patients who formed anti-I must be I-negative but Wiener *et al.* (1960) showed that these patients, with the cold antibody type of acquired haemolytic anaemia were in fact I-positive. Further details about the I antigen and antibody have been given in Chapter X.

Agglutination of normal red cells by pathological cold agglutinins is reversed by warming to between 25 and 32° C. (Dacie, 1954a). In most cases the patient's own whole blood is visibly agglutinated, even at 30° C.,

and often this auto-agglutination can be noted in a syringe containing blood taken only a few minutes earlier.

A simple screening test for high-titre cold auto-agglutinins was devised by Garrow (1958). 0.2 ml. of whole blood obtained by skin prick is mixed with 0.2 ml. of citrate in a precipitin tube (60×7 mm.) which is corked. The tube is chilled by holding it by the cork and rubbing it on the cold tray of a refrigerator. Auto-agglutination is reversed by warming the tube in the hand. Garrow found that the test was usually positive when the cold agglutinin titre was 64 or more and usually negative when the titre was 32 or less.

The serum of these patients displays other characteristic features; the serum will often haemolyse normal red cells at room temperature, particularly if the pH is adjusted to approximately 6.5-7.0. It can be shown that, *in vitro*, the binding of antibody occurs at temperatures up to about 30° C. or even 35° C. but not at 37° C. It is convenient to carry out the test at 20° C. because the haemolytic antibody binds readily at this temperature (Dacie, 1954a). The haemolysins are thermostable, but a labile component of fresh serum (presumably complement) is necessary during both the cold and warm phases of the reaction (Dacie, 1950a). The relationship between the 'cold haemolysin' of acquired haemolytic anaemia and the Donath-Landsteiner antibody is discussed below.

There is good evidence that the high titre cold agglutinin is a globulin with a relatively high molecular weight, sedimenting in the ultra-centrifuge with a sedimentation constant of 18*S* or 19*S* and having a mobility on electrophoresis of a fast gamma globulin (Christenson *et al.*, 1957; Fudenberg and Kunkel, 1957; Mehrotra and Charlwood, 1960). If the serum of patients with high-titre cold agglutinins is examined electrophoretically there may be an abnormal peak in the gamma$_1$ region (Christenson and Dacie, 1957; Fudenberg and Kunkel, 1957). As expected, treatment of the serum with mercapto-ethanol abolishes or greatly reduces the serological activity of the cold antibody (Mehrotra and Charlwood, 1960). Finally, after elution from red cells the antibody can be shown, by immuno-electrophoresis, to be a β-2M globulin (Mehrotra, 1960).

The serum of patients with haemolytic anaemia of the cold antibody type almost always gives a strongly positive indirect antiglobulin reaction with normal red cells at 20° C. In many cases binding of incomplete antibody also occurs at 37° C., although less strongly and usually only if the serum is first slightly acidified (Dacie, 1954a, 1954b, p. 194). Heated serum, or plasma, will not sensitize red cells to an antiglobulin serum

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apparently because complement is needed for firm binding of antibody (Dacie, 1954b, p. 259).

The red cells of patients whose serum contains potent cold auto-antibodies give a positive direct antiglobulin reaction. Characteristically when the red cells are tested with serial dilutions of antiglobulin serum, no 'prozone' is observed (Van Loghem, Stallman and van der Hart, 1951). The reaction with antiglobulin serum is due to the adsorption of complement on to the red cells, and it is therefore not inhibited by the addition of gamma globulin to the antiglobulin serum (Dacie, 1951; Dacie *et al.*, 1957).

Cold Antibodies Associated with Paroxysmal Haemoglobinuria

In the syndrome of paroxysmal cold haemoglobinuria the patient's serum contains a haemolysin which can be demonstrated by first bringing cells and serum together in the cold, to allow adsorption of the antibody, and then warming the cells to allow the activity of complement to produce haemolysis. This is known as the Donath-Landsteiner reaction, after its discoverers. The optimum pH for haemolysis is in the region of 7.0-8.0. The antibody responsible differs from the cold haemolysin described above in being active over a wider range of pH.

The following method (Dacie, 1954b, p. 493) is a rapid and convenient means of demonstrating the presence of the Donath-Landsteiner antibody:

> Samples of the patient's blood are delivered directly into two tubes or 'bijou' bottles previously warmed in a 37° C. water-bath. One sample is left to clot at 37° C.; the other is placed immediately in crushed ice at 0° C. and left undisturbed for 30 minutes. The tube or bottle is then replaced in the water-bath at 37° C. without disturbing the clot. The samples are finally re-examined when the clots have retracted. In paroxysmal cold haemoglobinuria the serum expressed by the clot which was chilled before it was warmed should be tinged deeply red with haemoglobin; the serum of the sample kept at 37° C. should be entirely free from haemoglobin.

Sera which give a positive Donath-Landsteiner reaction usually have only a normal titre of cold agglutinins. The direct antiglobulin reaction of the cells is usually positive; it may become negative when the patient is symptom-free (Dacie, 1954b, p. 282).

Paroxysmal cold haemoglobinuria was at one time considered to be invariably associated with syphilis. However, according to Dacie (1954b, p. 286), in some cases there is no evidence of syphilitic infection; in some cases the serological reactions for syphilis are positive during the attack but become negative when the patient recovers, suggesting that the reactions for syphilis were 'false positives'. Nevertheless the association of many cases of paroxysmal cold haemoglobinuria with syphilis is undoubted and as syphilis has become less common so has the syndrome of cold haemoglobinuria.

Warm Auto-Antibodies

In the so-called warm antibody type of acquired haemolytic anaemia the red cells are 'coated' with an antibody which reacts best at 37° C. and there are no abnormal cold agglutinins in the serum.

The warm antibody can be eluted from the red cells and will then sensitize other red cells to an antiglobulin serum. The patient's serum only sometimes gives a positive indirect antiglobulin test — a finding associated with a poor prognosis according to Dausset and Colombani (1959). When the serum does give a positive indirect antiglobulin test, the reaction is enhanced by acidification of the serum (Gardner, 1949).

Almost invariably the serum will agglutinate normal red cells treated with enzymes such as trypsin (Dacie and de Gruchy, 1951). Occasionally the serum will haemolyse trypsinized red cells or red cells from patients with paroxysmal nocturnal haemoglobinuria (Dacie and de Gruchy, 1951): (normal serum, unless it is slightly acidified, causes little or no lysis of PNH red cells). Very occasionally the patient's serum agglutinates red cells suspended in saline.

In most cases the patient's red cells appear to be coated only with gamma globulin because they fail to react with an antiglobulin serum to which gamma globulin has been added. In other cases the red cells do react with antiglobulin serum to which gamma globulin has been added showing that the red cells have adsorbed some 'non-gamma' globulin; it is not known whether this material is complement. In rare cases the red cells seem to react as well with anti-non-gamma globulin as with anti-gamma globulin (Dacie, 1959).

Warm auto-antibodies may display blood-group specificity. Thus, in a case described by Weiner *et al.* (1953), a patient with haemolytic anaemia with the apparent genotype R_1R_1 (*CDe/CDe*) had in his serum an antibody with the specificity anti-e; the same antibody could be eluted from the patient's cells. Many similar cases have been described since. Thus Dacie and Cutbush (1954) investigated one patient of the apparent genotype R_1r (*CDe/cde*) whose serum contained a mixture of anti-C and anti-e; the serum of this patient did not react at all with cells of the genotype R_2R_2 (*cDE/cDE*). In eight other patients the serological findings were less clear-cut; the common finding was that the serum — and the eluate from

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the patient's cells — reacted more strongly with e-positive cells than with e-negative cells but did react to some extent with the latter; it was suggested that this might be due to the presence of both non-specific and specific components.

Several other investigators have found that when the auto-antibody displays blood-group specificity it is usually within the Rh system. For example, Kissmeyer-Nielsen (1957) reported that in twelve adequately investigated cases there was a definite specificity in five, namely, anti-C plus anti-D, anti-D plus anti-E, anti-D (two cases) and anti-c. In a case reported by Gold, Ikin and Mourant (1958), a patient with the Rh phenotype CcDee had an auto-antibody behaving as a mixture of anti-C, anti-D and anti-e, that is, the patient formed three out of the four possible Rh auto-antibodies that he could make.

Dacie (1959) summarized his experience to date by saying that of twenty-nine cases in which he had investigated the specificity of warm auto-antibodies, the antibody had been completely specific in only one case. In eight more it had behaved as if it were a mixture of anti-e (or in some cases of anti-e+anti-c or anti-e+anti-D) together with a non-specific component, and in the remaining twenty cases it exhibited no specificity at all.

There have been a few reports of auto-antibodies with a specificity outside the Rh system. For example, van Loghem and van der Hart (1954 a and b) found that out of ten selected cases which they investigated, seven had auto-antibodies exhibiting specificity within the Rh system (anti-D, three cases; anti-c, one case; anti-c plus anti-e, three cases); in three other cases the antibody appeared to be anti-O, anti-B and anti-Jk^a respectively.

Positive Direct Antiglobulin Test in apparently Healthy Subjects

Occasionally the red cells of an apparently healthy person are found to be 'sensitized' with incomplete antibody of the warm type. In one such case it was shown by labelling with ^{51}Cr that the life-span of the red cells was strictly normal (Mollison, 1956, pp. 165 and 258; see also Chapter VI); in this case the reaction of the red cells with antiglobulin serum was inhibited by gamma globulin.

In a case reported by Darnborough (1958) the red cells of a normal donor were found to give a positive direct antiglobulin test; the donor's red cells were Rh-negative and an eluate yielded a specific anti-c. Stratton and Tovey (1959) reported the finding of a positive direct antiglobulin test in eight blood donors; two had a history of previous virus pneumonia but the remaining six appeared to be normal.

In most of these cases the donor was discovered to have a positive direct antiglobulin test as a result of finding apparent incompatibility when cross-matching the cells against a patient's serum.

Auto-Agglutinin Acting only in Albumin

A few cases have been described in which a patient's serum has been found to agglutinate his own red cells (and all other red cells) suspended in albumin but not when they are suspended in saline. In these cases the serum does not sensitize red cells to an antiglobulin serum and the patient's red cells are not themselves agglutinated by antiglobulin serum. The auto-agglutinin was rather more active at 37° C. than at lower temperatures, in the cases described by Weiner, Tovey *et al.* (1956), but was equally strong at 4° C., 20° C. and 37° C. in the case described by Moore, Linins and McIntyre (1959). In one case the agglutinin was shown to pass the placenta (Weiner and Hallum, 1957).

INTERACTIONS BETWEEN RED CELLS AND THEIR OWN SERUM NOT DUE TO BACTERIA

Rouleaux Formation

If red cells are allowed to sediment in their own plasma, they tend to adhere together in a characteristic way, 'like a pile of coins'. The rate of sedimentation of the red cells depends upon the degree of this tendency to aggregate. Thus, rapid, intense rouleaux formation and a high sedimentation rate go hand in hand.

Fibrinogen is the protein that has the greatest influence on rouleaux formation, and the addition of fibrinogen to blood has been used as a method for the rapid separation of the red cells from the white cells and plasma (Emerson, 1949). Gray and Mitchell (1942) tested the effect of purified protein fractions on the sedimentation rate and found that gamma globulin had one-sixth the effect of fibrinogen. There is a relation between the shape and size of molecules and their tendency to induce rouleaux formation, large asymmetrical molecules being the most active. Thus, lipoprotein, though very large (molecular weight 1,300,000) is symmetrical, i.e. more or less spherical, and has little tendency to cause rouleaux (Oncley, 1949). Fibrinogen molecules are much smaller (molecular weight 400,000) but are elongated. Dextran molecules are also elongated but produce rouleaux formation only when they exceed a certain size (Bull *et al.*, 1949).

In blood grouping work serum, rather than plasma, should be used as a

RCPE0002068_0191

routine because of its lack of fibrinogen. Occasional samples of serum cause strong rouleaux formation due to a raised globulin concentration; for example, in one patient with a serum globulin concentration of 7 g./100 ml. the serum caused strong rouleaux formation even when diluted with an equal volume of saline. Most samples of human serum will not cause rouleaux when diluted with an equal volume of saline, and this fact is of great value in distinguishing rouleaux from agglutination.

It is often thought that rouleaux formation can be distinguished from true agglutination by simple microscopic examination, but in fact the distinction is often difficult to make. In large rouleaux the cells do not all adhere together in neat piles but tend instead to form large clumps which may easily be mistaken for agglutinates. Conversely, weak agglutination in colloid media can closely resemble rouleaux formation; this may be observed for example in the titration of partially neutralized anti-A immune sera against A cells in a medium of serum.

The rouleaux formation caused by dextran may interfere seriously with blood-grouping tests; there is no serious difficulty in testing the red cells because these can be washed free from dextran, but agglutination tests with sera containing dextran may be impossible to interpret because of heavy rouleaux formation. In such cases the indirect antiglobulin test must be used to detect antibodies.

Various substances have some inhibitory effect on rouleaux formation but none of them are of much practical value. The author has tested glycine (Koop and Bullitt, 1945) and sodium salicylate (Inokuchi, 1950).

Serum to which dextran with a molecular weight of approximately 200,000 had been added in a concentration of 3.25 g./100 ml. was mixed with an equal volume of a 2 per cent suspension of cells in saline, to which was added various concentrations of glycine or sodium salicylate. The control suspension showed heavy rouleaux formation. Glycine had only a slight inhibitory effect; for example, cells mixed with 24 per cent glycine (final concentration 12 per cent) still exhibited slight rouleaux; this concentration of glycine was slightly lytic. Sodium salicylate was more effective; cells mixed with 6 per cent sodium salicylate (final concentration 3 per cent) showed no rouleaux formation. However the cells also showed considerable sphering and specific agglutination was interfered with. When the final concentration of sodium salicylate was 1.5 per cent there was some rouleaux formation; agglutination by anti-Rh and anti-A was diminished. It must be concluded that sodium salicylate is of no practical value under these circumstances (unpublished observations with Miss H. Crawford).

Other Factors Causing Non-Specific Clumping of Red Cells

Effect of Colloidal Silica on Red Cells

Renton and Hancock (1957) reviewed earlier work showing that colloidal silica is adsorbed by red cells and that it may agglutinate them or, if complement is present, lyse them. Colloidal silica may also coat red cells without agglutinating them, and such red cells may agglutinate if suspended in serum.

Harley and Margolis (1961) showed that silica particles of an appropriate size would bring about the haemolysis of red cells in the absence of complement. Thus a solution containing 1 μg. silica per ml. of particles with an average size of 30 mμ would produce 50 per cent lysis of a 2 per cent suspension of three-times washed red cells after 30 minutes at 37° C. Silica particles with a diameter of about 4 mμ were found to produce negligible lysis although they produced powerful agglutination. Exposure of red cells to particles less than 3 mμ in diameter was found to protect against exposure to large particles. One in 200 plasma completely protected red cells against the effects of colloidal silica.

When solutions are autoclaved or stored in glass bottles, the solution may become contaminated by colloidal silica; this is particularly likely to happen when the solution is alkaline (e.g. trisodium citrate). Solutions containing traces of colloidal silica bring about non-specific clumping of red cells, although this does not occur if serum is present. In practice trouble arises only when dealing with suspensions of red cells in citrate or saline. Thus when preparing solutions of citrate or saline for washing red cells it is safest not to autoclave the solutions in glass bottles but to prepare them freshly on the day on which they are to be used or, alternatively, if they are to be stored, to Seitz-filter them (Stratton and Renton, 1955).

When washed red cell suspensions are stirred on a glass slide with a glass rod (or with another glass slide) the cells may agglutinate, presumably due to the presence of colloidal silica. This may cause trouble when the antiglobulin test is carred out on glass slides, using diluted antiglobulin serum, but it is unlikely to interfere with blood grouping tests on slides since there is then sufficient serum to inhibit the agglutination caused by silica. In any case trouble can be avoided by using a polythene rod as a stirrer (Stratton and Renton, 1955).

There are many other substances which cause red cells to agglutinate non-specifically; for example, multivalent metallic ions such as Cr^{+++}; tannic acid; Wharton's jelly (containing hyaluronic acid); and various enzymes. The treatment of red cells with chromic chloride has been used in experiments on the removal of agglutinated red cells from the circulation (Jandl and Simmons, 1957); treatment with tannic acid has been used

RCPE0002068_0192

to make red cells adsorb soluble antigens (Boyden, 1951); contamination with Wharton's jelly is considered below; red cells treated with certain enzymes produced by bacteria became polyagglutinable (see below) although when treated with some enzymes such as trypsin, ficin or papain they react specifically (see Chapter XII).

Contamination with Wharton's jelly

Samples of blood contaminated with Wharton's jelly may agglutinate spontaneously (Wiener, 1943, p. 49). This phenomenon is likely to cause trouble only when cord blood is collected by cutting the cord and allowing the blood to drain into a tube. For many reasons, this is a very unsatisfactory way of obtaining a sample.

Flanagan and Mitoma (1958) found that contamination of cord blood with a one in 1000 dilution of Wharton's jelly in saline would cause the red cells to clump. They also observed that the clumps tended to form more slowly than true agglutinates and were smaller, more free cells being present. When the clumps were dispersed they tended to form 'strings'. On standing, the clumps disappeared spontaneously but re-appeared when the sample was agitated. Clumping produced by contamination with Wharton's jelly is dispersed by adding hyaluronidase; for example, one drop of a solution containing 1 mg. in 10 ml. saline (Killpack, 1950). This was confirmed by Flanagan and Mitoma (1958) who found that whereas hyaluronic acid alone would not produce false agglutination, the hyaluronic acid (sodium salt) incubated with 20 per cent human albumin would do so. The resulting false agglutination was dispersed within 15 seconds by 0.05 ml. of a solution containing 150 USP units of hyaluronidase.

If a suspension of red cells is contaminated with Wharton's jelly, the cells can be freed from it by washing three times although occasionally five washes may be necessary (Flanagan and Mitoma). A far more satisfactory approach is to take blood sample with a syringe and needle from the umbilical vein rather than by simply allowing the cord to drain into a tube.

Antibodies against Red Cells which have Adsorbed Penicillin

Ley *et al.* (1958) described a serum which agglutinated any red cells to which penicillin had been added. They searched for other similar sera using red cells prepared in the following way: 4 ml. of blood was mixed with 4 ml. of Alsever's solution (see Chapter I), added to 200,000 units of powdered 'Penicillin G' and incubated at 37° C. for 1 hour. The red cells were then washed three times.

Of 2000 sera tested, twenty-five reacted with penicillin-treated red cells; some sera agglutinated the cells; others gave only a positive indirect antiglobulin test.

Fudenberg and German (1960) investigated five patients whose sera contained an antibody against penicillin-treated red cells. In four cases the patient's serum sensitized the red cells to an antiglobulin serum and the activity was confined to the 7*S* fraction of serum. In two of the cases the serum also agglutinated treated cells and this activity was associated with the 19*S* globulin fraction. In the fifth case the agglutinating activity was in the 7*S* fraction; in this case the patient's own red cells were treated *in vitro* with penicillin after being labelled with ^{51}Cr. The red cells had a grossly shortened survival in the patient's circulation ($T_{\frac{1}{2}}Cr$ 8 days) but a normal survival in the circulation of a normal subject.

An Agglutinin Active against Stored or Enzyme-Treated Red Cells

A cold agglutinin which would react only with stored red cells was described by Brendemoen (1952). Red cells became agglutinable after 4–7 days' storage at room temperature or 2 days at 37° C. or 30 minutes at 56° C. The agglutinin could be absorbed by agglutinable cells. The person whose serum contained the cold agglutinin had a positive Wassermann reaction; her serum did not contain the 'Donath-Landsteiner' antibody; Brendemoen examined the sera of 300 normal subjects and sixteen subjects with a positive Wassermann reaction but failed to find another serum containing this special kind of cold agglutinin.

Jenkins and Marsh (1961) found three examples (all in women with severe haemolytic anaemia) of sera containing antibodies against stored red cells. Two were agglutinating antibodies and the third, which was very powerful, could be detected only by the indirect antiglobulin test. All three antibodies were active at 37° C. as well as at lower temperatures. (By contrast, the antibody described by Brendemoen was not active above a temperature of about 25° C.) Two of the three sera gave a positive Wassermann reaction and this appeared to be a 'false positive' reaction because in one case the reaction became negative when the agglutinin against stored red cells disappeared.

Jenkins and Marsh confirmed Brendemoen's observation that heating fresh red cells to 56° C. for 30 minutes activated the antigen, which they called H_T. The antigen was also activated by heating red cells to 37° C. for 60 minutes or by exposing the red cells for very short periods to enzymes; for example, the antigen was fully activated by exposure to ficin for 60 seconds. Two thousand samples of fresh red cells all failed to

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react with anti-H_T but 600 of these, after brief enzyme treatment, were agglutinated. Similarly fifteen samples of red cells from cord blood, one sample of i red cells and one of Bombay red cells, after enzyme treatment, were agglutinated by anti-H_T.

In a case described by Stratton *et al.* (1960) the serum of a patient weakly agglutinated all samples of fresh red cells, including her own cells, and reacted more strongly with stored samples of her own and other red cells. The agglutinin was active at 37° C. As in the case described by Jenkins and Marsh, cells heated to 56° C. were more strongly agglutinated; heating to 56° C. for 30 minutes was found to be equivalent to storage at +4° C. for 14 days. Treatment of saline suspensions of red cells with alumina, which is known to result in the removal of a considerable amount of lipids (see Lovelock, 1954) did not alter the reaction of the red cells. Stratton *et al.* noted that only some of the cells were agglutinated and they suggested that these were the older cells but the evidence in support of this view seems a little thin.

An Agglutinin Active against Freshly-washed Red Cells

Freiesleben (1959) described three examples of a panagglutinin which would act on the subject's own red cells or on other red cells only if they had been freshly washed. The panagglutinin could be removed by absorption and recovered by elution: it reacted best at 4° C., less well at room temperature and not at all at 37° C. Stored cells, provided that they had been freshly washed, reacted as well as fresh cells.

These reactions have not yet been explained, although it is tempting to suppose that they are related to the removal of some component of the red cell membrane. For example, Lovelock (1954) found that when red cells were suspended in normal saline there was a rapid diffusion of lipid and lipoproteins. Similarly, he found that if red cells were washed repeatedly in saline there was a progressive loss of lipids.

PHENOMENA DUE TO INFECTION OF RED CELLS OR SERUM

Bacteria or their products may cause red cells to become polyagglutinable or panagglutinable, that is to say, agglutinable respectively by many or all samples of normal serum. The term polyagglutinable is often restricted to one particular phenomenon, namely, the activation *in vivo* of the T-receptor in red cells, causing the cells to be agglutinated by any sample of serum containing a sufficiently potent anti-T agglutinin. T-activation can readily be produced *in vitro* and this will be described first.

Activation of T-Receptor *in vitro*

All human red cells contain a latent receptor, T, which can be activated ('changed') by various strains of bacteria or by enzymes derived from them. Red cells which have been 'changed' in this way are agglutinated by normal human serum, since the agglutinin anti-T is normally present.

The antigen T has been shown to be different from such very common antigens as H, U and Yt^a. Similarly, anti-T is different from anti-P+-P_1, anti-I, etc. (Sanger and Race, 1958).

The titre of anti-T varies considerably in samples of serum from different adults; anti-T agglutinins are usually not present in the serum of newborn infants and appear only at the age of 6 months. They are distinct from normal cold agglutinins, although they also act more strongly at low temperatures than at 37° C. (Lind and McArthur, 1947). Anti-T can be removed from human serum by absorption with 'changed' cells.

Knowledge of this phenomenon was first gained by observations on red cell suspensions infected *in vitro* (Hübener, 1925; Thomsen, 1927; Friedenreich, 1930); it was observed that suspensions of red cells might become agglutinable by ABO-compatible serum after standing for many hours at room temperature and that this was associated with infection of the suspension with certain enzyme-producing bacteria, for example, corynebacteria. This phenomenon came to be known as the Thomsen phenomenon or the Hübener-Thomsen-Friedenreich phenomenon. Chu (1948) found that a wide range of organisms including pneumococci, streptococci and *Staphylococcus albus, aureus* and *citreus*, were capable of 'changing' cells *in vitro*.

Burnet and Anderson (1947) prepared anti-T in animals by injecting an eluate containing T antigen. Red cells were treated at 4° C. with a filtrate of a culture of *V. cholerae*; this filtrate contains an enzyme which destroys virus receptors and also, apparently, partially destroys the receptors for cold agglutinins, perhaps converting them to active T-receptors. The red cells were then warmed to 37° C. to elute the enzyme from the red cells. In this process, T-receptor was evidently removed from the red cells, for the eluate, when injected into animals, stimulated the formation of specific anti-T agglutinins. (The receptor-destroying enzyme is now known to be a sialidase, or neuraminidase, and thus produces some of the same effects as trypsin; see Chapter VII.)

Polyagglutinability (Activation of the T-Receptor *in vivo*)

Red cells may become 'changed' *in vivo*, that is to say, agglutinable by anti-T. Usually, this polyagglutinability occurs as a transient phenomenon, disappearing within a few weeks or months of the time when it is first

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observed. The phenomenon is not very common. Thus, Stratton and Renton (1958) reported that they had observed only ten cases in 12 years. Most of the cases are detected by finding discrepancies between the results of testing red cells and sera in the course of ABO grouping. It is therefore to be expected that the phenomenon will be detected most often in group O subjects and will not be detected in subjects of group AB. Out of sixteen cases of polyagglutinability described in the years between 1938 and 1955, eleven were in group O subjects, four in group A and one in group B.

In many cases the patient has an obvious bacterial infection, but the phenomenon has also been observed in apparently healthy subjects; for a review of some of the earlier reported cases, see Henningsen (1949a). In a case reported by Reepmaker (1952) an organism that was shown to be capable of inducing T-transformation was isolated from the patient's urine. Chorpenning and Hayes (1959) have made the point that T-transformation is not the only kind of polyagglutinability induced by bacterial infection, and that in many reported cases of polyagglutinability it has simply been assumed that the change was T-transformation.

Activation of a receptor T_N 'in vivo'. In one very fully investigated case, polyagglutinability *in vivo* not due to T-transformation was discovered (Dausset *et al.*, 1959). In this case the patient belonged to group B and his red cells were agglutinated by all normal adult sera. This agglutination was shown to be due to a 'new' agglutinin, anti-T_N and not to anti-T. Thus, when anti-T was removed from normal serum by absorption with red cells 'changed' by exposure to a filtrate of *Vibrio cholerae*, the serum still agglutinated the patient's red cells. Conversely, absorption of normal serum with the patient's red cells (having the agglutinogen T_N) failed to remove anti-T. Anti-T_N was found in all normal adult sera tested, but it was not present, or was present in only low titre, in sera from newborn infants (cord blood).

The patient's serum apparently contained an immune form of anti-T_N and it was thought that this was responsible for the haemolytic anaemia from which the patient had suffered for 12 years.

Reactions of Polyagglutinable Cells

Polyagglutinable cells are agglutinated by the sera of a proportion of adults but fail to be agglutinated by most sera from newborn infants. The reaction is strongest at room temperature and may be very weak or absent at 37° C. Although the agglutinates may be large, there are many free cells present.

In the first case of polyagglutinability *in vivo* to be described, the cells were agglutinated by only 15 per cent of adult sera (Levine and Katzin, 1938). Stratton (1954) described a case in which the cells were agglutinated by 9 per cent (ten out of 116) of adult sera; he stressed the importance of excluding polyagglutinability before concluding that rare blood-group antigens have been detected.

Polyagglutinable cells react most strongly with fresh serum and sometimes fail to react with serum which has been stored frozen (Stratton, 1954; Hendry and Simmons, 1955; Chorpenning and Hayes (1959)).

Because anti-T appears to 'go off' on storage it is usually possible to determine the correct ABO group of polyagglutinable cells using stored blood-grouping sera (personal observations).

Polyagglutinable cells fail to agglutinate in their own serum and give a negative direct antiglobulin reaction. At 37° C. they are not sensitized to an antiglobulin serum by human sera which are capable of causing their agglutination at room temperature.

It has been reported (Stratton, 1954) that polyagglutinable cells stored in glycerol at −22° C. lose the property of polyagglutination; however, this is not always the case (personal observations). *In vivo* the phenomenon may disappear after as short a time as 4 days but usually persists for several months.

A typical case. Polyagglutinable cells are usually recognized when discrepancies are found during ABO grouping as in the following case:

Mrs S. was admitted to hospital with a septic abortion; mixed coliform and non-haemolytic streptococci were grown from a vaginal swab. Her red cells were strongly agglutinated by anti-A serum and partially agglutinated by anti-B; her serum agglutinated and lysed B cells but failed to agglutinate A cells, suggesting that she really belonged to group A. On further testing, her red cells were found to be agglutinated by four adult AB sera, the reactions being strongest at 4° C. and weakest at 37° C; the cells were not agglutinated by several samples of cord blood serum from group A infants.

The patient made a good recovery and 1 week after admission her cells were only very weakly polyagglutinable.

Clinical Significance of Polyagglutinability

As a rule, polyagglutinability does not cause any obvious increase in red cell destruction although in at least one reported case it has been suspected of being the cause of haemolytic anaemia (see above).

Van Loghem, van der Hart and Land (1955) described a severe haemoly-

RCPE0002068_0195

tic reaction in an infant, due apparently to anti-T in the plasma of the donor's blood. It was shown that the donor's serum reacted at 37° C. with the infant's red cells.

Polyagglutinability has been produced experimentally *in vivo* by injecting pneumococcus cultures (Type 19) into guinea-pigs (Ejby-Poulsen, 1954a). In one animal injected subcutaneously, the red cells became polyagglutinable 48 hours after the injection. In a further paper, Ejby-Poulsen (1954b) demonstrated the production of haemolytic anaemia in guinea-pigs by injecting concentrated enzymes from pneumococcus, Type 19. Animals with a high titre of anti-T were chosen for the experiment. The animals developed haemoglobinuria for 48 hours and their haemoglobin concentration fell to 50 per cent of the original level.

Brading *et al.* (1959) injected a preparation of R.D.E. (receptor destroying enzyme) intravenously into animals and found there was a fall in haemoglobin and a later rise in reticulocyte count and a temporary disappearance of anti-T from the circulation.

A haemolytic anaemia in rabbits associated with 'polyagglutinable cells', using the term in the wide sense, has been described by Evans *et al.*, 1959; the rabbits were suffering from mucoid enteritis.

Agglutination of Red Cells which have adsorbed Bacteria or their Thermostable Products

Keogh *et al.* (1948) showed that if polysaccharide was extracted (without denaturing it) from various organisms including *Salmonella typhi*, meningococci or staphylococci, it would adhere to red cells and cause them to be agglutinated by the appropriate bacterial antibody. Similarly Jochem (1958a) showed that most streptococci (*Strep. pyogenes* and *S. viridans*) produce a substance which can be separated from the bacteria by centrifugation or Seitz-filtration and can 'transform' red cells so that they are agglutinable by normal serum. The transforming substance is not an enzyme and is not destroyed by being heated to 100° C. for 6 hours or more. The substance therefore does not destroy the virus receptor on red cells, nor does it activate the T-receptor. It is produced not only by streptococci but also by *B. anthracis*, *B. cereus*, some staphylococci and some pneumococci. The agglutination of 'transformed red cells' by human serum can be inhibited by adding the bacterial product to the serum; the inhibition is specific, e.g. the addition of *B. anthracis* filtrate to the serum will prevent the agglutination of red cells transformed with *B. anthracis* filtrate but not that of red cells transformed with other organisms (Jochem, 1958a; 1958b).

In a case observed in the author's laboratory, contamination of a sample of serum with *B. cereus* was found to lead to positive indirect antiglobulin tests. The serum was being tested by the two-stage antiglobulin test (see Chapter XII). In the first stage, red cells exposed to patient's serum in order that they should take up any blood group antibody that was present, in fact adsorbed the bacteria; in the second stage the cells were exposed to fresh serum as a source of complement, and they adsorbed an antibody against *B. cereus*-treated red cells (present in most normal sera), and also adsorbed complement. When tested against an antiglobulin serum these cells therefore gave a strongly positive reaction. It was shown that red cells treated with a filtrate of a broth culture of the organism would take up antibody and complement from normal serum but relatively large amounts of the filtrate had to be used. By contrast, red cells could be sensitized after treatment with a relatively small amount of unfiltered broth culture (Weeden *et al.*, 1960).

Panagglutination by Infected Serum

Davidsohn and Toharsky (1940) found that if sera were inoculated with a gram-positive organism, *Corynebacterium H.* and left for a few days at room temperature, 69 per cent of sera developed an agglutinin, which they named anti-h. These sera would agglutinate all normal red cells, which were therefore considered to contain an antigen 'H' (presumably nothing to do with H of the ABO system). If a filtrate of *Corynebacterium H.* was added to normal serum, anti-h developed within 24 hours; plasma was found to be more easily 'transformed' than sera; plasma or serum from cord blood was as easily transformed as plasma or serum from adult blood.

(For a fuller review of bacterial agglutination and haemolysis, see Neter, 1956.)

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CHAPTER TWELVE

BLOOD GROUPING TECHNIQUES

Introduction

Blood grouping tests consist either of determining the antigen content of red cells, using sera of known antibody content, or of determining the antibody content of sera, using red cells of known antigen content. The determination of the antigen content of red cells is the more straightforward, because it is comparatively easy to prepare sera containing a single blood group antibody. To determine the antibody content of a serum it may be necessary to make tests with a large panel of red cells, because all red cells contain many antigens and serum often contains more than one antibody.

Thus, to determine whether a sample of red cells contains the antigen E it suffices to make a test with an anti-E serum. On the other hand, to discover whether a serum contains anti-E alone needs more elaborate tests; the mere demonstration that the serum (of a D-positive person) reacts, for example, with cells of the probable genotype *cDE/cDE* is not enough, because the positive reaction might be due either to anti-E or to anti-c, or to both, or might be due to an antibody of another blood group system. Tests with a panel of red cells of known blood groups are needed to solve such a problem.

In all blood grouping work the use of controls is essential, to show that the standard cells or standard sera are giving the reactions expected of them and to show that the 'unknown' cells or sera are not giving false positive results. For example, suppose a sample of red cells is being tested for the presence or absence of the antigen E. The following tests would be needed as a minimum:

(i) Control E+ cells versus standard anti-E serum.
(ii) Control E− cells versus standard anti-E serum.
(iii) 'Unknown' cells versus standard anti-E serum.
(iv) 'Unknown' cells versus subject's own serum, or versus normal compatible human serum.

In detecting red cell antigens specially selected sera are used. The main requirements are that the serum should contain only a single antibody; in most cases this implies that other antibodies must be removed by absorption or neutralization. To ensure that the serum contains only one blood group antibody it must be tested against an appropriate panel of red cells.

There are several other requirements for test sera. They must be sufficiently potent to detect all grades of the relevant antigen and to detect antigens present in single dose (e.g. Jk^a in Jk^aJk^b subjects); they must not contain 'non-specific' cold agglutinins, nor must they tend to form rouleaux; ideally, they should be tested to see that they do not have the property of agglutinating all stored red cells or all freshly washed red cells (see Chapter XI).

Test sera capable of agglutinating red cells suspended in saline are the most convenient. The next most convenient are those which agglutinate enzyme-treated red cells and the least convenient are those whose reactions can be detected only when the indirect antiglobulin test is used.

Blood group antibodies are usually detected in the course of routine procedures such as compatibility tests or antenatal screening tests. Their identification is discussed later in this chapter. Meanwhile the various ways of detecting the interaction of red cell antigens and antibodies will be described. As a preliminary the effect of storage upon the reactions of blood group antigens and antibodies will be discussed.

Storage of Red Cells before Testing

If tests are to be made within a few hours, blood can be obtained by skin prick, collected in a small tube containing dried heparin, 3 per cent sodium citrate solution or any other convenient anticoagulant.

If the red cells are to be used the following day or later a venous sample should be obtained, mixed with one-fourth of its volume of acid-citrate-dextrose solution (see Chapter I) and kept at 4° C. Red cells stored under these conditions for 2 or 3 weeks react almost as well as fresh red cells. Provided that reasonably potent antisera are used, there is no difficulty in correctly determining the various blood groups, although if very weak antisera are used, some antigens, for example P, may not be detected (Crawford *et al.*, 1954). Linins *et al.* (1959) found that after 3 weeks' storage in ACD, the antigen Fy^a was scarcely affected, K was 20 or 30 per cent weaker, c was 40 per cent weaker and P about 50 per cent weaker than in fresh cells.

Provided that potent antisera are used, red cells stored at 4° C. even for several months can be correctly grouped. Thus, Simmons *et al.* (1945) reported that Rh subgroups could be correctly determined in samples stored for as long as 5 months. They used a slightly modified Rous-

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Turner solution, that is to say, a rather larger volume of citrate-glucose in relation to the volume of blood, but there is no reason to suppose that the results would have been appreciably different if acid-citrate-dextrose had been used.

When blood is taken into a sterile container and stored at 4° C. in the form of a clot, the red cells are as well preserved after 1 week as red cells stored in ACD from the point of view of reactions with blood grouping sera. After 2 weeks red cells stored as clotted blood are definitely less well preserved; for example, Crawford *et al.* (1954) found that the reactions with anti-K were almost as good as those of fresh cells in the case of blood stored with ACD but far weaker in the case of clotted blood. Chaplin, Wallace and Chang (1956) found that the agglutination reactions of A cells in blood stored as a clot for 1 week were weaker than those of fresh blood, whereas even after 3 weeks cells from blood stored with ACD reacted as well as fresh cells.

A disadvantage of storing blood at 4° C. without anticoagulant, i.e. as clotted blood, even for short periods, is that a good opportunity is afforded for the binding of normal incomplete cold antibody to the cells. Thus, red cells obtained from clotted blood which has been standing in the refrigerator occasionally give a positive direct antiglobulin test. Red cells stored as citrated blood at 4° C. may take up some incomplete cold antibody but they do so to a much smaller extent.

If red cells are stored as a weak suspension in ACD, for example 5 volumes of cells to 100 volumes of ACD, the cells rapidly lose their reactivity. Thus after 2 weeks' storage (at 4° C.) the reactions with anti-Rh sera may be almost completely lost (Kooptzoff, 1954), although the reactions with anti-A are not lost to the same extent.

If blood mixed with ACD is stored at room temperature for periods up to 3 days, no significant deterioration in reactivity occurs (Crawford *et al.*, 1954). If blood samples are to be stored at room temperature (or sent by post), or if they are to be kept at 4° C. and opened occasionally for the withdrawal of samples, antibiotics should be added to discourage the growth of bacteria. Streptomycin or tetracycline in a final concentration of 25 microgrammes per millilitre are effective against most organisms. When antibiotics are added to blood samples to discourage infection, it is as well to remember that if very large amounts are added, unwanted effects may be produced. Thus red cells incubated with very large amounts of penicillin react with a small proportion of normal sera (see Chapter XI) and if concentrated solutions of streptomycin are added to serum some antibodies may be inhibited (Kout and Kubickova, 1959).

Storage of Red Cells in the Frozen State

If red cells are to be kept for blood grouping tests for really long periods they must be stored in the frozen state. Red cells can be frozen and thawed without haemolysis if they are first mixed with glycerol (Polge, Smith and Parkes, 1949). If the red cells are to be kept at −20° C. a final glycerol concentration of approximately 3.0 M is adequate to prevent serious haemolysis, provided that the mixture contains citrate rather than saline (Chaplin and Mollison, 1953), although with this concentration there will be slight progressive haemolysis, usually not exceeding 10 per cent after 1 year's storage (Hughes Jones, Mollison and Robinson, 1957).

Red cells stored at −20° C. deteriorate progressively so that post-transfusion survival is about only 40 per cent after 2 years' storage. At −45° C. or −50° C., red cells are better preserved, although in order to prevent haemolysis when cells are frozen to this temperature and subsequently thawed, the concentration of glycerol must be increased. If a final concentration of approximately 4.5 M is achieved, the cells remain unhaemolysed even after 1 or 2 years' storage (Hughes Jones *et al.*, 1957).

Although storage at −50° C. with a final glycerol concentration of 4.5 M gives excellent preservation for an indefinite period (certainly many years) the use of the higher glycerol concentration means that the cells have to be given extra washes, as described below, to remove all the glycerol before the cells can be used for blood grouping tests. For this reason, storage at −20° C. with a final glycerol concentration of 3.0 M is more convenient and is recommended for storage periods not exceeding a few months.

In describing glycerol concentrations, confusion arises unless it is stated whether the concentration is in terms of volume or weight, because glycerol has a high specific gravity (1.24). Thus a molar solution of glycerol is one which contains 9.2 g. per 100 ml., i.e. 9.2 per cent (w/v) or 7.4 ml. per 100 ml., i.e. 7.4 per cent. v/v. In describing the composition of glycerol solutions for laboratory use, amounts will be given in terms of volume since it is more convenient to measure out volumes in making up solutions.

A further source of confusion in describing glycerol concentrations in blood mixtures is the fact that glycerol diffuses into red cells but mixes only with the water-phase of cells, which is generally taken to be about 65 per cent of the total volume of packed red cells.

Thus, if one volume of a 30 per cent (v/v) glycerol solution is added to an equal volume of tightly packed cells, the final glycerol concentration in the fluid phase of the mixture will be $\frac{30 \times 1.24}{1+0.65} = 22.5$ per cent w/v or 2.45 M.

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In the present book, unless otherwise stated, glycerol concentrations refer to the final concentration in the water-phase of the mixture after allowing the glycerol to equilibrate with the red cell contents.

A suitable solution for the storage of red cells at −20° C. may be prepared as follows:

Tripotassium citrate ($K_3C_6H_5O_7.H_2O$)	19.4 g.
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	3.1 g.
Disodium hydrogen phosphate (Na_2HPO_4)	2.8 g.

Dissolve in 600 ml. of distilled water and add 400 ml. of glycerol.

When a sample of red cells is to be stored, blood is taken and mixed with one-quarter of its volume of ACD. After centrifugation, the supernatant is discarded and then one volume of the above citrate-phosphate-glycerol solution is added to an equal volume of packed red cells. When the glycerol solution is added slowly to packed red cells only slight haemolysis (approximately 1 per cent) occurs if mixing is efficient. It is possible to avoid haemolysis completely by adding glycerol in two stages. Thus instead of adding to 1 volume of packed red cells an equal volume of a solution containing 40 per cent glycerol (v/v), one can add first a half volume of a solution containing 20 per cent glycerol (v/v), then a half volume of a solution containing 60 per cent glycerol (v/v). In each case the concentration of citrate and phosphate is the same as in the solution described above.

Since glycerol diffuses into human red cells within a few minutes at room temperature, the mixtures of red cells and glycerol can be placed in the refrigerator straight away.

As described above, if red cells are to be stored at about −45° C. they should be mixed with sufficient glycerol to give a final concentration of 4.5 M in the fluid phase of the mixture. This can be achieved as follows:

To 1 volume of packed red cells is added about 4 volumes of a solution containing 0.06 M potassium citrate, 0.02 M NaH_2PO_4, 0.02 M Na_2HPO_4 and 30 per cent v/v glycerol. After centrifuging the mixture and discarding the supernatant, 1 volume of a solution containing the same concentration of citrate and phosphate, but with a glycerol concentration of 40 per cent v/v, is added. The final concentration in the fluid phase of the mixture should be about 4.5 M.

Removal of Glycerol from Red Cells

Removal by successive washes. When the frozen red cells are wanted for grouping tests a tube is thawed in warm water at 37° C. and the red cells are freed from glycerol by washing them in successively more dilute solutions of glycerol. Red cells stored in a final concentration of approximately 3.0 M glycerol should be washed successively in 16 per cent, 8 per cent, 4 per cent and 2 per cent glycerol (v/v) in 3 per cent trisodium citrate. If the cells have been stored at −20° C. for more than 6 months, it may be necessary to reduce the glycerol concentration even more gradually if haemolysis is to be avoided during washing, e.g. the cells can be washed in 12 per cent glycerol in sodium citrate between the 16 per cent and 8 per cent washes. The cells are finally washed twice in 1 per cent saline.

Red cells stored in a final glycerol concentration of 4.5 M must be washed first in 30 per cent (v/v) glycerol in 3 per cent citrate, then in 25 per cent, then in 20 per cent, and then in the series of solutions described above.

More rapid methods of washing. A more rapid method of freeing the cells from glycerol is to mix them with hypertonic citrate and then wash them in saline (Lovelock, 1952). For example, Moore, McIntyre and Bruce (1959) recommend adding to the thawed suspension of red cells in glycerol an equal volume of warm 0.67 M potassium citrate, leaving the tube at 37° C. for 5 minutes and then washing the cells in saline until no more haemolysis occurs.

Another method is that described by MacDonald and Marsh (1958) and is also meant to be used with red cells stored with a final concentration of approximately 30 per cent (w/v) glycerol. The sample is thawed and centrifuged and the supernatant discarded. The red cells are washed first in 12 per cent (v/v) glycerol in 5 per cent trisodium citrate then in 5 per cent (v/v) glycerol in 5 per cent trisodium citrate and finally in saline. The method gives a recovery of the order of 75 to 98 per cent depending upon the period for which the red cells have been stored.

The above rapid methods give too much lysis if the red cells have been stored with glycerol concentrations of the order of 4.5 M. For such cells the following method has been used successfully in the author's laboratory:

The red cells are first washed in 10 per cent (v/v) glycerol in 10 per cent trisodium citrate, then in 10 per cent (v/v) glycerol in 5 per cent trisodium citrate, then in 1.6 per cent (v/v) glycerol in 5 per cent trisodium citrate and finally in saline. There should be very little lysis at any stage, provided that the solutions used are buffered to about pH 7.

Removal of glycerol by dialysis. A convenient method is to place a few millilitres of the thawed glycerolized red cells in a length of dialysis

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tubing and then place the tubing in saline for 30 to 60 minutes (Weiner, 1961). In the author's laboratory it has been found that the amount of lysis can be minimized by dialysis against a citrate-phosphate solution (the one described on p. 67 is convenient), rather than saline and after dialysis giving the cells one wash in citrate-phosphate before washing them in saline. Provided that dialysis is allowed to continue for 60 minutes and that the concentration of glycerol in the original red cell mixture does not exceed 30 per cent (w/v) there should be very little lysis.

Reactions of Red Cells Stored in the Frozen State

Red cells stored at −20° C. in glycerol for 1 year react almost as well as fresh red cells; this conclusion appears to apply to the reactions of all blood group antigens (Crawford *et al.*, 1954).

Occasional samples of red cells stored at −20° C. in glass containers give 'false positive' results when used in the indirect antiglobulin test. It is therefore advisable to incubate a sample of the thawed cells with AB serum and to see that these cells do not react with the antiglobulin serum before interpreting any positive results obtained with the cells.

In the author's laboratory red cells stored in polythene containers at −50° C. with a final glycerol concentration of 4.5 M have given very few false positive results. It is not yet known whether this is due solely to the use of plastic containers, thus avoiding the action of colloidal silica (see Chapter XI), or partly to the better preservation of red cells stored at −50° C.

Very Rapid Freezing of Blood Mixed with a Sugar

Small amounts of red cells can also be stored in liquid nitrogen. As described by Meryman (1956) if blood is sprayed into liquid nitrogen, the droplets freeze very rapidly; after storage in liquid nitrogen the droplets can be thawed by transferring them to warm saline and, provided that the blood has originally been mixed with a suitable sugar, most of the red cells can be recovered after thawing. Huntsman *et al.* (1960) described a simple method in which blood mixed with half its volume of 40 per cent sucrose was dropped into tubes containing liquid nitrogen. Recovered red cells gave normal reactions in blood grouping tests.

Storage of Blood Grouping Sera

Blood grouping sera may retain their potency for long periods when stored at 4° C., provided that infection is prevented. According to one report, the 'half-life' of antibodies in gamma globulin solution stored at

4° C. is not less than 20 years (Gerlough, 1958). If sera are to be stored at 4° C., it is advisable to add some bacteriostatic agent; sodium azide in a final concentration of 0.1 per cent seems to be satisfactory. In this concentration it has been shown not to affect the titre of Rh antibody (Batson *et al.*, 1950), and not to alter the electrophoretic pattern of serum, although a concentration of more than 0.3 per cent may produce such changes (Saravis, 1959). Merthiolate is less satisfactory; in a concentration of more than one in 5000 it inhibits the action of anti-Rh sera and tends to cause haemolysis (Simmons and Woods, 1946).

Most laboratories store their sera in the frozen state at −20° C.; it is then unnecessary to add any bacteriostatic agent. To avoid frequent thawing and refreezing of a particular sample the serum should be stored in small amounts.

Deterioration of Complement on Storage

If serum is kept at 37° C. the preservation of complement is much affected by the presence of red cells. Thus Fischer *et al.* (1958) obtained sera from clotted blood, 60 minutes after obtaining the blood, and then incubated the serum at 37° C. for 24 hours with and without washed fresh red cells. In sera stored alone the complement titre fell to about 60 per cent of the original value but when kept in contact with red cells it fell almost to zero. Contact with cells was found to result in a considerable fall in C'_3 and C'_2; the fall in C'_4 was similar but C'_1 was not affected at all; properdin disappeared from the serum kept in contact with cells. These findings emphasize the need to separate serum promptly from clotted blood. Defibrination seems to be the best method for obtaining separated serum as quickly as possible.

The following method of defibrination has been found to be very satisfactory: about six glass beads and a similar number of short (1 cm.) pieces of stick, conveniently prepared by breaking up a wooden applicator stick, are placed in a 'Universal container', that is, a screw-capped glass pot with a capacity of about 28 ml. Approximately 15 ml. of blood is taken into the pot and, after replacing the cap, the pot is repeatedly inverted by hand, or clipped on to a rotating turntable, so that the beads slide in turn from one end of the pot to the other until defibrination occurs. After 10 minutes the pot is centrifuged and the serum taken off.

At 4° C. complement deteriorates relatively quickly. Crosby and Scarborough (1942) found that in whole citrated blood the amount of complement fell to 50 per cent in 2-4 days and 20 per cent in 12 days, although Chaplin, Wallace and Chang (1956) found that in serum stored

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for 3 weeks there was still enough complement to bring about haemolysis of A cells by anti-A.

At −20° C. complement deteriorates much more slowly; for example, serum stored at −20° C. for a few weeks is usually almost as effective as fresh serum as a source of complement in the antiglobulin test (Polley and Mollison, 1961). Similarly Osler *et al.* (1952) found that the haemolytic property of guinea-pig serum for sensitized sheep red cells was virtually unimpaired after 4-6 weeks' storage at −18° C. At −50° C. complement does not deteriorate appreciably during a period of some months.

Sera stored at −20° C. may become strongly anti-complementary and then the presence in them of antibodies, such as anti-Le^a, may be overlooked if an ordinary 'one-stage' indirect antiglobulin test is used (Polley and Mollison, 1961); methods of dealing with this problem are described in a later section.

AGGLUTINATION TESTS

In principle, agglutination takes place in two steps. Firstly, antibody must be adsorbed on to the red cells, and secondly, red cells which have adsorbed antibody must come together. As far as the adsorption of antibody is concerned, in most cases there is a definite thermal optimum, that is to say, a temperature at which adsorption occurs most rapidly. Thus, most Rh antibodies have a thermal optimum of 37° C., whereas most anti-A and anti-B agglutinins have an optimum of about 0° C.

So far as the second phase of the agglutination reaction is concerned, any factor which increases the number of cell contacts will accelerate agglutination. Thus, if red cells are simply allowed to sediment in a tube, agglutination will not be maximal until sedimentation is complete. Agglutination can be greatly accelerated by centrifugation since it immediately brings together the cells at the bottom of the tube.

Strength of Red Cell Suspension

It is important to standardize the strength of cell suspension; most workers use a 2 per cent suspension (see below).

When weak agglutinins are being detected the test can be made more sensitive by using dilute suspensions of cells, because this will increase the amount of antibody relative to the red cells. Thus, Lund (1941) found that a final cell concentration of 0.06 per cent was eight times as sensitive as 0.5-1.0 per cent. If such weak cell suspensions as this (0.06 per cent) are used, the mixture must be centrifuged after it has stood for 1 to 2 hours. Then the supernatant fluid is removed and the cells resuspended in a smaller volume of fluid. The suspension, now much less dilute, is then examined microscopically. The use of very weak cell suspensions is never necessary in routine clinical work.

Need to Wash Red Cells

Red cell suspensions used in blood grouping tests should be washed free of their own plasma. If this is not done clots will form when the red cells are mixed with grouping serum. If whole blood is mixed with serum on a slide, the flakes of fibrin which form may be mistaken for agglutinates.

A further reason for washing red cells is that plasma tends to cause rouleaux-formation, and this interferes with the interpretation of agglutination tests. Moreover plasma contains anticoagulants which are anti-complementary and may thus interfere with the detection of complement-binding antibodies.

Finally, in the ABO and Lewis systems the serum contains blood group substances corresponding to those on the red cells and these substances may inhibit the antibody in the grouping serum. A case in which this happened was reported by Barber and Dunsford (1959).

A patient of group A was thought at first to belong to a weak sub-group because her red cells were not agglutinated by group B (anti-A) serum and were only very weakly agglutinated by group O serum. Subsequent tests showed that the absence of a reaction with anti-A serum was due to using an unwashed suspension of red cells. The patient's serum was particularly rich in A substance and thus had inhibited the anti-A. A similar case was reported by Moullec *et al.* (1960)—see Chapter VIII.

Tests with Sera which Agglutinate Red Cells Suspended in Saline

Tests in Tubes

A 2 per cent suspension of red cells is prepared after first washing the red cells twice in saline. For routine use it is sufficient to gauge the strength of the cell suspension by eye, that is to say, by colour. A standard can be prepared from time to time, containing four volumes of whole blood (approximately two volumes of red cells) in 100 volumes of saline, as a check.

One volume of cell suspension is added to an equal volume of the test serum in a small tube; precipitin tubes, 7 mm. in diameter and 50 mm. high, are convenient. Much time is saved by delivering each volume as a drop from a fine-bored Pasteur pipette, rather than by delivering from a mark on the stem of the pipette.

The mixture of cells and serum is left for 2 hours at an appropriate

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temperature. For ABO grouping room temperature (18° C. to 28° C.) is satisfactory; for Rh antibodies 37° C. is the optimum; a few antisera, such as anti-P_1, react distinctly more strongly at 10° C. than at room temperature. In practice the temperature at which each sample of blood grouping serum gives the best reactions must be discovered by trial.

At the end of the incubation period red cell sediments are examined. If there is strong agglutination the sediment will have an irregular edge and when the tube is tapped the cells may adhere together in one large clump or in a few large clumps. When agglutination is weak or absent the sediment forms a smooth compact deposit. In such cases the tubes should not be tapped, but some of the deposit should be drawn up into the stem of a Pasteur pipette, delivered on to a glass microscope slide, gently spread out with the stem of the pipette and examined under a low-powered microscope. The spreading should be performed in such a way that the cells are actually moving when they are examined. This makes it easier to distinguish between small clumps due to cells lying on top of one another and clumps in which the red cells adhere together even though moving.

When the result of a test is required in a hurry the tubes can be centrifuged after only a brief period of incubation; for example, when using known potent sera the tubes can be centrifuged after only 5 minutes' incubation.

Slide Tests (with Suspensions of Red Cells in Saline)

If a drop of cell suspension and agglutinating serum are mixed together on a slide, agglutination will occur more rapidly than when they are mixed in a tube, because the distance through which the cells have to sediment is much shorter and they will therefore come into contact with one another more rapidly. The chief disadvantage of slide tests is that drying occurs rapidly, so that only a limited time is available for reading the reaction. This disadvantage can be overcome by leaving the mixtures in a moist atmosphere; for example, they can be placed in a covered container in which the atmosphere is kept damp with wet blotting paper, as in the technique described by Simmons *et al.* (1943). If slide tests are used for the *rapid* determination of blood groups, it is essential to use very potent antisera capable of producing visible agglutination within 1 minute.

Routine ABO Grouping

The determination of the ABO groups differs from the determination of other blood groups in two ways. Firstly, the ABO system is by far the most important blood group system in transfusion practice; secondly, the ABO system is the only one in which the antigen content of the red cells can usually be deduced from a knowledge of the agglutinin content of the serum.

The standard method of determining the ABO group of a subject is therefore to test the red cells against anti-A and anti-B serum and to test the serum against group A and group B red cells (see Table XXXIV).

As the table shows, 'standard practice', for example that recommended in M.R.C. Memo. No. 36, includes testing the red cells against group O serum. The main object of this is to detect rare group A samples which are not agglutinated by anti-A from a group B donor, but are agglutinated by group O serum. The practical importance of detecting these very weak group A blood samples is not great, particularly when the subject is a potential recipient of a transfusion. Therefore, in the writer's opinion, the testing of group A samples with group O serum, though desirable, is not essential for safe transfusion practice.

TABLE XXXIV

DETERMINATION OF ABO GROUPS OF RECIPIENT; (IN THE EXAMPLE THE PATIENT IS GROUP A)

Red cells	*Serum*			
	Recipient	*Anti-A*	*Anti-B*	*Anti(-A + -B)* † (group O)
Recipient	—	+	—	+
Control Group A*	—	+	—	+
Control Group B	+	—	+	+

* Preferably subgroup A_1 to make the test for anti-A more sensitive; when the recipient's serum contains anti-A_1 a further test will have to be made with A_2 cells.

† Not essential.

It is usual to test the serum against A_1 rather than A_2 cells; A_1 red cells, apart from having an extra receptor A_1, are richer than A_2 cells in A substance and thus serve better to detect anti-A. In some subjects of subgroups A_2 and A_2B, the serum contains anti-A_1 and in these the serum has to be re-tested to demonstrate that it will not react with cells of subgroup A_2.

Determination of Subgroups of A

Three methods may be used. The classical method is to use a group B serum which has been absorbed with cells of subgroup A_2. This removes anti-A and leaves anti-A_1 alone. The following method is suggested:

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Titrate several anti-A sera with A_1 and A_2B cells; select one with which the A_2B cells react weakly and the A_1 cells react strongly. To one volume of this serum add an equal volume of well-washed packed red cells of subgroup A_2. Leave at 4° C. for 1 hour. Now centrifuge the mixture and take off the supernatant serum. Test the serum against A_1 and A_2 cells; it should react well with A_1 cells and fail to react with A_2 cells. It is advisable to test the effect of absorption with a lesser volume, for example one-tenth, as well as with an equal volume of A_2 cells since in some cases absorption with an equal volume considerably reduces the anti-A_1 activity of the serum.

In interpreting the results of tests with an anti-A_1 serum, some difficulty is caused by bloods which react weakly; such bloods are often regarded as intermediate in type between A_1 and A_2. Witebsky (1948) has shown that there is another and probably more satisfactory way of dividing group A bloods:

An immune anti-A serum is 'partially neutralized' with A substance. The serum now fails to agglutinate A_2 cells suspended in serum but will still agglutinate A_1 cells suspended in serum. Witebsky found that only 10 per cent of A bloods failed altogether to react when tested in this way, and these he considered to belong to subgroup A_2. Seventy-five per cent of bloods were A_1 and the remaining 15 per cent he considered as intermediate. If an immune anti-A serum is neutralized only to the point where agglutination of A_2 cells in saline is abolished (and A_1 cells are still agglutinated) a positive antiglobulin reaction is obtained with A_2 cells.

The third way of determining A subgroups is to test the samples against an extract of *Dolichos biflorus*; at a suitable dilution such an extract will fail to react with A_2 cells but will strongly agglutinate A_1 cells.

The method of preparing the extract is as follows: one volume of seeds is soaked overnight in three volumes of saline. The mixture is then ground in a mortar and finally centrifuged to obtain the extract in the supernatant.

Use of Reagents containing Anti-H

The most convenient source of anti-H is an extract of the seeds of *Ulex europaeus*. (For a method of preparing an extract, see previous paragraph.)

Another anti-H lectin can be obtained from the seeds of *Lotus tetragonolobus*.

Before the introduction of seed extracts the most generally used source of anti-H was eel serum.

Approximately two out of five eels (*Anguilla anguilla*) have a sufficiently potent anti-H to give good reactions with group O cells when the serum is diluted one in

100 to one in 500. The animals may be anaesthetized either by immersing them in water containing barbiturate or by packing them in ice, and may then be bled from the ventral aorta using a Pasteur pipette. Up to 8 ml. of blood may be obtained from a large eel.

Determining Blood Groups of Agglutinated Red Cells

When red cells are agglutinated by a potent cold auto-agglutinin it may be possible to prepare a smooth suspension by washing the red cells in saline at 37° C. to elute the agglutinin, but when they are agglutinated by an antibody with a wider thermal range, heating even to 56° C. may fail to remove all the antibody and it in any case damages the cells. An alternative approach is then to add soluble antigen. This method was used by Booth *et al.* (1957) in investigating the blood of a human chimera. Thus to obtain a smooth suspension of A cells from a mixture of A and O they first agglutinated the A cells and separated them. Group A substance was then added and agglutination was reversed. It was easier to get good reversal of agglutination when an extract of *Dolichos biflorus* rather than human anti-A was used to agglutinate the cells.

Vos and Kirk (1958a) showed that A or B red cells which had been strongly agglutinated by human anti-A or anti-B would form a smooth suspension after treatment with A or B substance although the cells still gave a positive antiglobulin test. If more A and B substance was used, the direct antiglobulin test became negative.

Detection of Blood Group Substances in Saliva

The presence of ABO and Lewis substances in saliva (or serum) can be detected by a specific inhibition of agglutination.

Testing saliva. The subject should spit into a clean beaker until he has contributed about 5 ml. of saliva. The saliva is then poured into a hard glass test tube and put into a boiling water-bath for 10 minutes. The purpose of this is to destroy enzymes which would otherwise destroy the group-specific substances. The saliva is now centrifuged at high speed and the supernatant fluid placed in a series of small tubes. The saliva will keep well at −20° C.

In testing for the presence of a particular blood group substance, control salivas must be used; for example, in testing for H, the saliva of a non-secretor of H and of a secretor are needed as controls. The test is made most sensitive by diluting the serum containing antibody to a point at which, after mixture with saline, it still just gives a positive reaction. However, for routine use, this is rather too sensitive, and it is better to

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adjust conditions so that the serum, when mixed with saline, gives about a +++ reaction. The amount of blood group substance in the saliva can be estimated by making serial dilutions in saline (see Table XXXV); to each dilution of saliva an equal volume of diluted agglutinating serum is added. After 5 minutes (cf. Halvorsen, 1944) a volume of test cells is added. The mixtures are allowed to stand for 1 to 2 hours and are then examined microscopically for agglutination.

In making tests to decide if a person is a secretor of ABH, it is normally sufficient to test for H substance alone and the use of extracts of *Ulex europaeus* is convenient. If equivocal results are obtained and the subject

TABLE XXXV

DETECTION OF H SUBSTANCE IN SALIVA

Dilutions of the saliva to be tested are added to equal volumes of a suitable dilution of an extract of *Ulex europaeus* (anti-H), mixed with O cells and subsequently examined for agglutination.

'Secretor' status	*Dilutions of Saliva* 1/5	1/20	1/80	1/320	Control
Non-secretor	++	++	++	++	++
Weak secretor	—	—	+	++	++
Normal secretor	—	—	—	+	++

belongs to groups A, B or AB, an additional test can be made for the presence of A or B substance.

In testing for Le^a substance, saliva of an Le(a—b—) subject should be used as a negative control. The subject must be a selected one because a small proportion of subjects whose red cells have the phenotype Le(a—b—) secrete Le^a in their saliva (Ceppellini *et al.*, 1959); perhaps the safest negative control is a subject with anti-Le^a in his serum. As a positive control a subject of the phenotype Le(a+b—) or Le(a—b+) may be used, although the amount of Le^a will be much less in the saliva of the Le(a—b+) subject, see Table XXXVI.

In testing for Le^b substance, only a few anti-Le^b sera are suitable, the remainder being inhibited by H substance, presumably due to a cross-reaction, as discussed in Chapter VIII.

Tests in Capillary Tubes

Chown (1944) and Chown and Lewis (1946, 1951) described a method of Rh grouping in capillary tubes. This method has been found very useful

by some laboratories for routine work, its chief advantages being that only very small amounts (0.005 ml.) of serum are required, that the capillary tubes are discarded after use, and that the results can be read in 15 minutes. It must be emphasized that the method requires a serum which will agglutinate red cells suspended in saline. It is thus essentially a method for determining the Rh group of a sample using a selected reagent and it is not a suitable method for the routine detection of Rh antibodies. The method is of course equally suitable for use with saline-agglutinating sera other than anti-Rh and is particularly valuable when only very limited supplies of serum are available.

TABLE XXXVI

ESTIMATION OF LEWIS SUBSTANCES IN SALIVA

Dilutions of the saliva to be tested are added to an equal volume of a suitable dilution of an i-Le^a (in testing for Le^a substance) or anti-Le^b (in testing for Le^b substance), mixed with Le(a+) or Le(b+) cells and subsequently examined for agglutination

Lewis phenotype of red cells of donor of saliva	*Tests with anti-Le^a* — *Dilutions of saliva* 1/5	1/20	1/80	1/320	1/1280	*Tests with anti-Le^b*† — *Dilutions of saliva* 1/5	1/20	1/80	1/320
Le(a—b—)*	++	++	++	++	++	++	++	++	++
Le(a+b—)	—	—	—	—	+	—‡	±	++	++
						or ++	++	++	++
Le(a—b+)	—	—	—	+	++	—	—	—	(+)

* A selected subject: see text.

† Only a few anti-Le^b sera are suitable, others being inhibited by H substance (see Chapter VIII).

‡ Only some anti-Le^b sera — the anti-Le^bL of Ceppellini *et al.* (1959) — are inhibited by the saliva of Le(a+b—) individuals; the inhibition is thought to be due to a cross-reaction with Le^a substance.

The technique is as follows: the blood sample whose group is to be determined can be obtained by skin prick or venepuncture. The red cells are washed once in saline and a heavy suspension (20-40 per cent) of cells in saline is prepared.

The apparatus required consists of capillary tubes of about 0.4 mm. bore and 8 cm. length, some plasticine and a small rack. One end of a capillary tube is dipped into the agglutinating serum and a column of about 2 cm. is allowed to run in. (It is advisable to use a potent anti-Rh serum diluted 1 in 8 or 1 in 16 in saline, to avoid rouleaux formation). The same end is now dipped into the cell suspension and an equal volume allowed to enter. There must be no air bubble between the serum and the cell

RCPE0002068_0204

suspension. The tube is inverted and the other end pressed into a small piece of plasticine (or alternatively sealed by heat). The tube is then inclined at an angle of 45° and read at 5 minutes and again at 15 minutes, by inspecting the tube in a good light against a white background. An Rh-positive sample forms a beaded layer of red cells along the length of the tube, whilst an Rh-negative sample forms a long, smooth line.

Since this method is essentially one for the rapid determination of the Rh group of a prospective recipient of a transfusion, doubtful results must provisionally be considered as negative.

Tests in capillaries with enzyme-treated cells are described later in this chapter.

Agglutination of Red Cells Suspended in a Colloid Medium

Tube Tests

A satisfactory medium is a mixture of equal parts of AB serum and 30 per cent bovine albumin.[1] Red cells can be suspended in this medium and the test carried out in test tubes in the same way as with cells suspended in saline except that, because sedimentation occurs more slowly, the tubes should be left for 2 or 3 hours before being 'read'.

An alternative method is to use a saline suspension initially, and leave the mixtures of serum and saline suspension for 1½ hours, then remove the supernatant saline, replace it, without disturbing the cells, with an equal volume of serum-albumin mixture (20 per cent albumin in the original description of the method), leave the tubes for 30 minutes and then examine the deposits microscopically. This method, sometimes known as the 'Sheffield removal' technique, was found very reliable by Dodge (1952). A further modification consists in leaving the serum and saline suspension for 1½ hours, then, without removing the saline, running a drop of 30 per cent albumin down the side of the tube.

A shorter method which seems to be just as sensitive and has the advantage of giving strong positive results with sera which zone with standard methods has been described by Lewis and Chown (1957). Ten drops of 30 per cent bovine albumin are added to one drop of three times washed, packed red cells and mixed well. One drop of the mixture is incubated for 10 minutes at room temperature with one drop of the serum to be tested. The tube is spun at 1000 r.p.m. for 2 minutes and then 'read' macroscopically after gently rotating the tubes so as to free the cells from the bottom.

[1] Obtainable from Armour Laboratories.

A dextran medium for immune anti-A and anti-B. Munk-Andersen (1956a) found that the following medium greatly enhanced the reactions of immune anti-A and anti-B sera:

	Final concentration in the mixture
Dextran (Macrodex Pharmacia DRI 204)	5 per cent (w/v)
AB serum	25 per cent (v/v)
NaCl	1.3 per cent (w/v)

Slide Tests

Diamond and Abelson (1945) devised the following test, which is sometimes known as 'Diamond's rapid slide test'. An oval about one inch by two-thirds of an inch is marked on an ordinary glass slide using a glass-writing pencil. A drop of anti-Rh serum and two or three drops of oxalated blood are mixed within the oval and spread out so as to cover the area. The slide is now placed over a slightly warmed surface, illuminated from below, and rocked. If a really potent antiserum is used, massive agglutination occurs within 30 seconds. It is essential to have as controls (i) two or three drops of the patient's oxalated blood alone; (ii) anti-Rh serum versus known Rh-positive blood; (iii) anti-Rh serum versus known Rh-negative blood. Even then, a negative result does not exclude the possibility that the patient is a 'weak' Rh-positive. The main use of the test is simply to establish rapidly that a person is Rh-positive and can therefore safely be given Rh-positive blood. Provided that only unequivocal results are accepted, and the above controls included, the test is useful. One disadvantage is that, because serum is mixed with plasma, fibrin strands form and may be mistaken for agglutination.

Munk-Andersen (1956a) used the special dextran medium described above to demonstrate sensitization of the red cells of infants with haemolytic disease of the newborn due to ABO incompatibility. One volume of the medium was added to one volume of a 5 per cent suspension of red cells in saline on a slide. Positive results were obtained in haemolytic disease of the newborn due to ABO incompatibility but in cases due to anti-Rh the results were negative or only very weakly positive.

RCPE0002068_0205

'Stratton's Sandwich'

Diamond's rapid slide test is unsuitable for use with weak anti-Rh sera since by the time that the agglutination develops the preparation has partially dried and rouleaux formation interferes with the reading. To overcome this difficulty Stratton (1955b) suggested the following method, designed for the rapid typing of recipients prior to transfusion:

Mix one volume (about 0.03 ml.) of anti-Rh serum with an equal volume of 30 per cent albumin and one volume of once-washed packed red cells. Spread the mixture over two-thirds of the surface of an ordinary glass slide and cover immediately with a second slide. As a control, make a second sandwich in which AB serum replaces anti-Rh serum. Leave the 'sandwiches' at 37° C. for 10 minutes, then examine under a microscope; pressure on the top slide makes the cells move and facilitates reading.

Stratton states that even sera containing weak incomplete Rh antibodies can be used for this test and that in the course of testing 851 D-positive bloods only four (all D^u) were falsely typed as negative.

Blood Grouping on Cards

It has often been suggested that blood grouping should be carried out on cards, one advantage being that a permanent record of the test is thereby secured. Thalheimer and Myron (1942) used globulin fractions of anti-A and anti-B to obtain more potent reagents and identified them by adding aqueous eosin to one and methylene blue to the other. One drop of serum was mixed with one drop of blood on a card and stirred with a toothpick. When the cards had dried a permanent record of the subject's blood group was available. It was found that if the serum alone was placed on the card and allowed to dry, it lost its efficacy within a week.

Eldon (1955) has devised a method of grouping on cards which overcomes many of the shortcomings of previous methods. Small volumes of anti-A, anti-B and anti-Rh sera, mixed with dextran to enhance agglutination, are dried on separate marked panels on the card. Each card is enclosed in a moisture-proof, tin-foil envelope and it is claimed that even after 1 year's storage reliable results are obtained.

The idea of grouping on cards is certainly attractive but there are important objections to be considered. Firstly, the apparent extreme simplicity of determining the ABO and Rh group by this method may lead to its use in an emergency by inexperienced persons. Secondly, the only available control is another card and it is uncertain whether this could ever be regarded as identical. Thirdly, it has been found that when the cards have been used and allowed to dry they may no longer show the original result; that is, a result which was originally negative may become 'positive' during drying (Dr D. M. Parkin, personal communication).

A distinction should be made between tests on cards on which blood-grouping sera have been dried ready for use, as described above, and tests on cards using freshly added reagents. In this latter method, ordinary liquid grouping serum is mixed with red cell suspension on a card rather than a glass slide so that when the mixture has dried it can be filed for reference. Dr Eloise Giblett (personal communication) has used this method in about one-third of a million cases and has found no evidence that the results alter on storage.

Titration of Sera

In most laboratories the method is as follows: ten (or more) small tubes are set up in a rack. One volume of saline, delivered from a marked Pasteur pipette, is added to tubes 2 to 10; with the same pipette a volume of serum is added to tubes 1 and 2. Now the contents of the second tube are mixed and a volume of the mixture is withdrawn and added to the third tube. The contents of the third tube are now mixed and a volume of the mixture is withdrawn and added to the fourth tube, and so on, until the last tube is reached, when the volume withdrawn is discarded. In this technique it is customary not to attempt to clean the pipette after transferring a volume of mixture from one tube to another; therefore a minute amount of mixture from, say, tube 2, may reach tube 4, and so on. Because of this, a tube which is supposed to contain only one part in 500 of the original serum may in fact contain one in 400. If the titration is performed carelessly, greater errors may result, but with care the error is usually within one 'tube', e.g. serial dilution method, apparent titre 512; direct dilution method, titre 400. The extent of the error can be determined by making a precise dilution of the original serum. For example, if by the standard serial dilution technique a serum has a titre of 256, separate dilutions of the original serum of one in 100, one in 200, one in 300 can be made, using clean pipettes each time. The results can then be compared.

Method of describing the serum dilution. In describing the dilution of a serum some workers ignore the volume of cell suspension whilst others take it into account. Thus a mixture of one volume of undiluted serum with one volume of cell suspension is described by some as a 1 in 1 dilution and by others as a 1 in 2 dilution. Perhaps the description of 1 in 1 is simplest and most widely used.

RCPE0002068_0206

The end-point of agglutination. Very weak agglutination can be detected with certainty only by examination under the microscope. Thus, if the end-point of a titration is decided by examination under the microscope, higher values will be recorded. Very weak agglutination is easily dispersed; thus a technique which involves shaking up the mixtures before examining them will yield lower results than will a technique in which the cell sediments are examined after being very gently transferred to a slide under a microscope.

Results of Iso-Agglutinin Titrations

The titre of a serum is defined as the reciprocal of the highest dilution which will cause agglutination. As already explained, there are many variable factors which affect agglutination. In theory, all these factors can be standardized, but in practice there are many differences in technique between different laboratories. Thus, whereas in one laboratory an anti-A titre of 500 is considered exceptional (see Wiener and Moloney, 1943), in another a titre of 500 or more is found in 40 per cent of unselected group O subjects (Aubert, Boorman, Dodd and Loutit, 1942). As an example of the widely differing results obtained by using differing techniques the findings in Table XXXVII has been assembled. Where the authors have included the volume of cell suspension in calculating the dilution of the serum, their figures have been halved to make them comparable with the others.

Although at first sight the results in Table XXXVII are grossly discrepant, there is no reason to suppose that the various samples tested really differed appreciably from one another. The different results can be explained by considering the effect of the factors already discussed. For example, Wiener and Moloney not only used A_2 cells but also employed a very insensitive technique. Wiener has stated elsewhere that this particular technique gives titres about one-sixth as high as the values obtained in similar titrations set up in test tubes and read after standing for 2 hours at room temperature, or after centrifugation for 1 minute at low speed (Hurst *et al.*, 1946). Baxter's results demonstrate the considerable difference between end-points read macroscopically and microscopically.

In practice iso-agglutinin titrations are almost always needed simply to study changes, and any method which gives reproducible results will serve.

Titration in Colloid Media

The serum to be titrated can be diluted serially in a mixture of equal parts of AB serum and 30 per cent albumin, rather than in saline. The disadvantage of preparing dilutions in a mixture containing albumin is

that the albumin tends to froth when it is sucked up and down in a Pasteur pipette. It is therefore more convenient to prepare the dilutions of the serum in saline and to incubate each dilution with the test cells and then to replace the saline with albumin or a mixture of albumin and AB serum, as in the Sheffield removal technique (see above).

TABLE XXXVII

PROPORTION OF GROUP O SERA WITH AN ANTI-A TITRE OF 200-300, OR MORE, ACCORDING TO VARIOUS AUTHORS

Authors	*No. tested*	*Percentage of O sera with anti-A titre exceeding 200*	*Notes on technique*
Wiener and Moloney (1943)	approx. 65	0	Subgroup A_2 cells. Mixtures shaken for 10 minutes, in well-slides, then read (naked eye and low-power of microscope).
Ebert and Emerson (1946)	184	1	Pooled A cells; 2% suspensions. Mixture centrifuged, read microscopically.
Ervin, Christian and Young (1950)	1375	9	Pooled A cells; 2% suspension. Serum diluted to 1/200 in one step, i.e. by adding 0.05 ml. serum to 10 ml. saline. Mixtures centrifuged after 15 minutes. Examined macroscopically for grossly detectable agglutination.
Tisdall, Garland *et al.* (1946)	1650	15	Pooled A cells. Mixtures centrifuged after 15 minutes. Examined macroscopically.
Brewer (1937)	250	45	Mixtures allowed to stand overnight at room temperature. End-points read macroscopically.
Baxter (1949)	96	27	A_1B cells } macroscopic end-point
		35	A_1 cells }
		52	A_1B cells } microscopic end-point
		58	A_1 cells }
			In all cases serum diluted to 1/200 in one step. Mixtures centrifuged after ½ hour.
Aubert, Boorman, Dodd and Loutit (1942)	250	66	2% cell suspension. Mixtures allowed to stand for 2 hours. Read microscopically. (End-point: clumps of three to four cells.)

Titration of Partially Neutralized Anti-A and Anti-B Sera

As already described in Chapter VIII, Witebsky (1948), showed that the presence of incomplete anti-A could be revealed by titrating the 'partially

RCPE0002068_0207

neutralized' serum against A_1 cells in a medium of AB serum. Witebsky added two volumes of AB substance (prepared commercially[1] from hog and horse material) to one volume of the serum to be tested and then titrated the mixture against cells suspended in AB serum. The results of titrating an immune anti-A serum treated in this way are illustrated in Table XXI (see p. 269).

A medium of 20 per cent or 30 per cent albumin, which is optimal for agglutination by incomplete Rh antibodies, gives less satisfactory results than AB serum as a medium for agglutination by immune anti-A or anti-B; if albumin is used for anti-A or anti-B a concentration of 7 per cent gives the best results, but even so, is less satisfactory than AB serum (Dr J. F. Mohn, personal communication).

A more sensitive method of detecting small amounts of immune anti-A or anti-B in the serum is to add graded doses of saliva containing A (or B) substance, or of a preparation of animal A (or B) substance, to an immune serum, and then to carry out the indirect antiglobulin test on the partially neutralized samples incubated with test cells; details will be found on pp. 414-415.

'Mixed Agglutination'

Coombs and Bedford (1955) demonstrated A (and B) antigens in platelets in the following way:

Platelets from a group A person were shown to adhere to group A red cells which had adsorbed anti-A agglutinins, but not to adhere to B red cells which had adsorbed anti-B. The reaction was described as 'specific mixed agglutination'. This reaction was later used to demonstrate A and B antigens in human epidermal cells (Coombs *et al.*, 1956) and human leucocytes (Gurner and Coombs, 1958).

Mixed agglutination may also be non-specific. Thus Bakemeier and Swisher (1957) found that leucocytes and platelets would adhere to a subject's own red cells if these had been incubated with a complement-binding antibody in the presence of complement, and Richardson Jones and Kaneb (1959) found that if anti-A was added to a mixture of a very small number of A cells and a large number of O cells some of the O cells adhered to the A cells. Despite these findings, there seems to be no reason to doubt that mixed agglutination may be used to demonstrate the presence of an antigen possessed in common by two kinds of cell, provided that rigorous controls are used.

[1] Obtainable from Messrs. Sharp & Dohme.

THE USE OF ENZYME-TREATED CELLS TO DETECT INCOMPLETE ANTIBODIES

Morton and Pickles (1951) gave the following instructions for using trypsin in the detection of incomplete antibodies:

Prepare a 1 per cent stock solution of crystalline trypsin (Armour Laboratories) by dissolving 0.1 g. in 10 ml. N/20 HCl. This stock solution will keep for 4 months. Before use, mix one part of the stock solution with nine parts of M/10 phosphate buffer of pH 7.7.

A preparation of crystalline trypsin, sold in England (in 50 mg. amounts) as 'Trypure Novo' (Evans Medical Supplies, Ltd.) has been found to be satisfactory. A stock 0.1 per cent solution (not 1 per cent) is prepared in N/20 HCl; for use this is diluted 1 in 20 in M/10 phosphate buffer of pH 7.7. This buffer can be prepared by adding nine volumes of 0.1 M-Na_2HPO_4 (14.2 g./litre) to one volume of 0.1 M-KH_2PO_4 (13.6 g./litre).

Wash the test cells three times in saline, to remove the 'trypsin-inhibitor' present in normal serum. Add four volumes of dilute buffered trypsin solution to one volume of packed cells, mix and place in a water-bath at 37° C. for 30 minutes. Wash the cells once and make up to a final 5 per cent suspension in saline.

The cell suspension is now ready to add to the serum containing incomplete antibody, but the serum should first be left in the water-bath at 37° C. for 5 minutes, to avoid possible interference from cold antibodies. When the serum is warm, an equal volume of 'trypsinized' cells (which have meanwhile been kept warm) is added and left for one hour in the water-bath at 37° C. The mixtures are then examined macroscopically.

Morton and Pickles (1951) found that this method was more sensitive than the indirect Coombs test, or the 'albumin test', in detecting incomplete Rh antibodies; among the sera which reacted well with trypsin-treated cells were three which had given prozones with albumin and several which had given negative reactions with the Coombs test.

Morton and Pickles originally suggested that it was also satisfactory to use crude trypsin preparations for the detection of Rh antibodies. They later found that cells treated with some brands of trypsin failed to react with anti-M, anti-N and anti-S, and suggested that it might be desirable to use the more refined products (Morton and Pickles, 1951).

Morton (1957) found that Fy(a+) cells treated with impure preparations of trypsin might fail to react with anti-Fy^a, whereas Fy(a+) cells treated with crystalline preparations generally reacted, either being agglutinated by serum containing anti-Fy^a or giving a positive indirect

RCPE0002068_0208

antiglobulin reaction. The same author found that the S antigen was also destroyed by impure trypsin preparations, but was less sensitive than Fy^a. He concluded that chymotrypsin was probably one of the enzymes that destroys the Fy^a and S antigens. M and N receptors were found to be transiently enhanced by treatment with crystalline trypsin, but were inactivated by further treatment. M and N receptors are also destroyed by certain influenza viruses and by an extract of *Vib. cholerae* (Mäkelä and Cantell, 1958; Springer and Ansell, 1958).

The reactions of K-positive cells with anti-K are not enhanced by treating the red cells with trypsin (Unger and Katz, 1952) and may even be abolished by impure preparations (Morton, 1957).

Rosenfield and Vogel (1951) noted that the reactions of Lewis antisera (both anti-Le^a and anti-Le^b) were enhanced by treating the test cells with trypsin. Fresh antisera were found to haemolyse the treated cells. The reactions of anti-P were also enhanced by trypsin treatment. (Morton and Pickles (1951, Table IX) had previously found that a sample of incomplete anti-Le^a haemolysed Le(a+) cells which had been treated with trypsin.)

Reversible agglutination of trypsinized cells. If trypsinized red cells are mixed with normal human serum at 37° C. and centrifuged immediately they will usually be found to be agglutinated; this agglutination is completely reversed after 1 hour's incubation (Rosenthal and Schwartz, 1951). Rosenfield and Vogel (1951) reported that agglutination took place if the cells were centrifuged within 20 minutes of being mixed with serum and also found that the agglutination was reversed after 1 hour at 37° C. They found that 1 per cent of sera agglutinated the subject's own cells even after 1 hour at 37° C.

If normal serum is heated to 60° C. for 2 hours the agglutinin responsible for this phenomenon is destroyed; however another factor, 'reversor', is left, and treatment of cells with heated serum renders them non-agglutinable by normal serum (Spaet and Ostrom, 1952).

The agglutinin responsible for the agglutination of trypsinized cells is not related to normal cold agglutination and the activity is not enhanced by low temperatures (Spaet and Kinsell, 1953); it is no weaker in the serum of newborn infants than in adults (Spaet and Anderson, 1954).

The Use of Enzymes other than Trypsin

Papain has been preferred to trypsin by some authors; it is claimed to be more sensitive and it is cheaper. When papain is used with an activator, the 'reversal phenomenon' (see above) is inhibited. Goldsmith (1955) suggested the following method:

Prepare a stock solution by suspending 1 g. of papain in 100 ml. of 0.85 per cent NaCl. (Goldsmith found several brands of papain to be suitable; for example, that supplied by Biddle Sawyer & Co. Ltd, London.) The stock solution may be stored for several months at 4° C., although the bulk of the solution is best kept at −20° C. If desired, the suspension can be centrifuged after keeping for 24 hours at 4° C. with occasional agitation; the clear supernatant is slightly less active than the original suspension.

For use, one volume of stock solution is added to nine volumes of M/15 Sorensen phosphate buffer, pH 7.3.

This buffer can be prepared by adding three volumes of 0.067 M-Na_2HPO_4 (9.46 g./litre) to one volume of 0.067 M-KH_2PO_4 (9.07 g./litre).

Two volumes of buffered papain solution are added to 1 ml. of thrice-washed packed cells and incubated at 37° C. for 30 minutes. The cells are then washed three times, made up to a 5 per cent suspension in saline and warmed to 37° C. before being added to the serum to be tested. Goldsmith claims that this method is very sensitive and that it gives very few false positives (only eleven in 3370 cases).

Stratton (1953 a, b) described a slide method, which enabled Rh antibodies to be detected at room temperature; it was very sensitive and highly suitable as a screening test, for example, for the sera of pregnant women. The method gave few false positives; by using the patient's serum with the patient's own cells as a control these could be detected.

Details of Stratton's method will be found in the previous (2nd) edition of this book, p. 297; they are not repeated here because it has now been found more convenient to add the enzyme directly to the mixture of serum and cells (see below).

Stratton and Renton (1958, p. 42) recommended that when Rh antibodies were found by a slide screening test against papainized red cells at room temperature, the results should be confirmed in capillaries at 37° C. Previously papainized red cells are drawn into a capillary with serum and saline and left for 30 minutes on a warm viewing box.

Ficin. Wiener and Katz (1951) reported that ficin was a satisfactory enzyme for the detection of Rh antibodies, and Haber and Rosenfield (1957) gave a very full account of its use in detecting many different antibodies. The latter authors recommend the following method for using ficin:

Two hundred and fifty milligrammes are weighed out in 60 ml. stoppered vials. The powder should be handled with care, as in sensitive individuals it is capable of causing sloughing of the mucous membranes. The powder is stable indefinitely, and it is therefore kept dry in the vials until it is needed. When needed, each 250 mg. is dissolved in 25 ml. of

RCPE0002068_0209

Hendry's buffer (pH 7.4). This stock solution will keep for 1 week at 4° C. or for many months if stored at —20° C.

The buffered solutions described by Hendry (1948) are designed to be iso-osmotic with human red cells; Hendry's buffer pH 7.4 is prepared by adding nineteen volumes of a solution containing 2.34 g. $NaH_2PO_4.2H_2O$ per 100 ml. to 100 ml. of a solution containing 1.63 g. Na_2HPO_4 per 100 ml.

The treatment of red cells is carried out as follows:

One drop of the stock 1 per cent solution is added to nine drops of a 2 per cent suspension of red cells and incubated at 37° C. for 15 minutes. The red cells are then washed once in saline and resuspended in saline to give a 2 per cent concentration. Two drops of the suspension of ficinized red cells are mixed with two drops of the serum and incubated at 37° C. for 1 hour. Haber and Rosenfield (1957) found that the titre of antibodies against ficin-treated cells was as high as with cells treated with activated papain, and higher than with trypsinized cells. Results with Lewis and P antibodies were also found to be better with ficin-treated cells than with trypsin-treated cells. They found that the reactions of the antigens K and k were not enhanced by ficin treatment, and that reactions with cells containing the Fy^a antigen were almost completely abolished.

When enzyme-treated red cells are used in the antiglobulin test, a lower concentration of enzyme should be used (see p. 415).

Bromelin is apparently about as effective as ficin and is probably less noxious; a method of using it is described in the following section.

Direct Mixture of Enzyme, Red Cells and Serum

A highly convenient method is to mix a suitable enzyme preparation with the serum and cells to be tested, thus allowing the enzyme treatment of the cells and the reaction with antibody to occur in a single stage. The technique advocated by Löw (1955) was as follows:

Two grammes of papain (papayotin Merck 1 : 350) are ground in a mortar with 100 ml. of M/15 phosphate buffer, pH 5.4. After filtration, 10 ml. of 0.5 M cysteine is added and the solution is diluted with the buffer to 200 ml.; it is then incubated for 1 hour at 37° C. This activation with insteine is essential to overcome the inhibitory effect of serum. After cycubation the enzyme is ready for use. It can be stored at —20° C. for many months. For use it is diluted 1 in 10 in buffered saline, pH 7.3.

The M/15 phosphate buffer pH 5.4 referred to above is prepared by adding 0.35 volumes of M/15, i.e. 0.067 M, Na_2HPO_4 (9.46 g./litre) to 9.65 volumes of 0.067 M-KH_2PO_4 (9.07 g./litre).

Three volumes of diluted enzyme are mixed with one volume of serum. One volume of this mixture is placed in a test tube with one volume of a 3 per cent cell suspension, mixed and incubated at 37° C. for 2 hours. The tubes can be read macroscopically or microscopically. When using sera containing weak antibodies, it is advisable to use equal parts of serum and enzyme solution.

Lewis *et al.* (1958) found that a modification of Löw's method worked very well in capillaries with anti-K sera. They filled a capillary successively with 1 c.mm. of anti-K, suitably diluted with AB serum, 2 c.mm. of papain solution, prepared as described above, and 0.5 to 1 c.mm. of a 50 per cent suspension of washed red cells.

Hekker *et al.* (1957) have used a papain slide test for mass Rh typing. In the course of testing 125,000 sera they checked 10,000 positives and all negatives with a saline-albumin tube test and found no discrepancies. The details of their test are as follows:

Ten grammes of papain were dissolved in 250 ml. of phosphate buffer (pH 6.2). The mixture was centrifuged and the deposit discarded. 4.85 g. of cysteine hydrochloride and 25 ml. of phosphate buffer were now added, and the whole made up to 1000 ml. with phosphate buffer and then incubated at 37° C. for 1 hour. The mixture was now filtered through an asbestos filter (K 5) and was stored at —20° C. For use, one drop of the papain solution, one drop of anti-Rh and one drop of 5 per cent cell suspension were mixed and left at room temperature for 15 minutes before being read. The authors mention that it may be necessary to spin the papain solution before use to get rid of the deposit.

A simple tube test using papain without cysteine has been described by Stapleton and Moore (1959). One per cent papain is made up in M/15 phosphate buffer pH 5.3 and filtered. One volume of this solution is mixed with one volume of incomplete anti-D and one drop of the mixture is added to one drop of a 3–5 per cent suspension of Rh-positive cells and incubated for 1½ hours at 37° C.

Stapleton and Moore (1959) found that this method was more sensitive than the albumin replacement method in detecting D^u samples but they pointed out that the method was not very suitable for the typing of recipients before transfusion because 'sensitized' red cells, e.g. sensitized with incomplete cold antibody or with the warm or cold auto-antibodies of acquired haemolytic anaemia, are agglutinated on incubation with enzyme and so appear to be Rh-positive whatever their true Rh group.

Pirofsky and Mangum (1959) described the following method, using the enzyme bromelin (see also Pirofsky, 1959):

RCPE0002068_0210

A stock solution containing 0.5 per cent bromelin in nine parts saline and one part phosphate solution, pH 5.5, was prepared; when sodium-azide in a final concentration of 0.1 per cent was added and the stock solution was stored at 4° C. it kept its potency for 2 months.

One drop of the stock bromelin solution was mixed with 0.1 ml. of serum and one drop of a 4 per cent suspension of red cells and incubated at 37° C. for 15 minutes (or at 4° C. when the antibody concerned was known to be anti-A, anti-B, anti-Le^a or anti-P_1). The mixtures were then centrifuged at 1000 rev./min. for 1 minute and inspected for macroscopic agglutination.

This method, as expected, failed to detect anti-Fy^a and anti-S but appeared to be a sensitive way of detecting other blood group antibodies.

TESTS FOR HAEMOLYSIS

Strength of Red Cell Suspension

When the serum contains a weak haemolysin it may fail to produce any lysis of a 5 per cent suspension of red cells, but may lyse a 2 per cent suspension (Chaplin, Wallace and Chang, 1956). Some sera will produce complete lysis *in vitro* only when the ratio of serum to cells exceeds 500 to 1 (equivalent to one volume of serum mixed with one volume of 0.4 per cent suspension of cells (Jandl *et al.*, 1957)).

In practice the problem is not to detect very weak haemolysins but to detect strong ones, namely those in the serum of group O donors whose blood might be transfused to recipients of other groups. Accordingly a test which will detect only fairly potent haemolysins is appropriate, and it is common to use a 5 per cent cell suspension (see below).

Enzyme-treatment of cells. Some antibodies, e.g. anti-Le^a, anti-Le^b and anti-Jk^a, will bring about the lysis *in vitro* of enzyme-treated cells, although they fail to lyse untreated cells. The technique of looking for haemolysins with enzyme-treated cells is the same as that used for agglutinins, except that complement must be present. The supply of complement for haemolysin tests is discussed below.

Use of Human Serum as a Source of Complement

If serum from a freshly drawn blood sample is being examined for the presence of a haemolytic antibody, an adequate amount of complement will normally be present. If samples of serum which have been stored for 24 hours are being tested, fresh serum should be added to ensure that

sufficient complement is present. Alternatively, serum which has been separated from freshly drawn blood within an hour or two and then stored at −20° C. for periods of up to a few weeks, or at −50° C. for periods of up to a few months, may be used.

When human serum is used as a source of complement it may itself contain haemolytic antibodies. Anti-A and anti-B are the only antibodies likely to cause trouble, and these can be removed by absorption at 0° C. with an equal volume of packed, washed A_1B cells (cf. Thomsen and Thisted, 1928a).

Samples of human serum used as a source of complement may contribute blood group substances which directly inhibit the haemolytic antibody. For example, if A serum is mixed with group O serum the haemolysis of A cells may be inhibited (Moss, 1910). Similarly, Le^a substance in the serum of Lewis positive subjects (Le(a+b−) or Le(a−b+)) may inhibit anti-Le^a (Cutbush *et al.*, 1956). The use of serum from group O or Le(a−b−) individuals overcomes these difficulties.

Alternatively in detecting haemolysis by Lewis antibodies, the two-stage test may be used and then the Lewis group of the serum used as a source of complement in the second stage is of no importance; see Table XXXIX.

Suitable techniques. One volume of the serum to be tested is mixed with one volume of a 5 per cent suspension of test cells and one volume of fresh serum (as a source of complement) and incubated at 37° C. for 1 hour. The mixture is then shaken and centrifuged, and the supernatant examined for free haemoglobin.

If the titre of the haemolytic antibody is to be determined, serial dilutions of the serum are first made, and then to each tube is added one volume of a 5 per cent suspension of test cells and one volume of fresh normal serum as a source of complement.

It may well be objected that the test in this form is very unsuitable for obtaining quantitative results. The end-point is obviously liable to interference by artefacts; and the amount of complement is not standardized. An alternative method has been recommended by Dr R. E. Rosenfield (personal communication): one volume of a 2 per cent suspension of A or B cells in fresh normal serum is added to a volume of each dilution of the serum being tested. After a period of incubation (1-2 hours at 37° C.) the tubes are spun and complete lysis is recorded for tubes showing no 'button' of cells. This method does at least avoid the possibility of artefacts causing traces of haemolysis in the absence of haemolytic antibody. Nevertheless, it is open to some of the objections mentioned

RCPE0002068_0211

above; thus for a quantitative test the point of 50 per cent haemolysis would doubtless be better. As mentioned earlier, all that is needed in practice is a test to detect those sera which contain exceptionally large amounts of haemolytic antibody, and this can be done with a very simple test.

In order to save carrying out a special test for haemolysins, Gardner and Tovey (1954) used a single test to confirm the presence of anti-A in the serum (as a check on the group) and to reveal the presence of haemolysins. One volume of the serum, taken within the previous 24 hours, was added to one volume of a 5 per cent suspension of cells and left for 1 hour at room temperature (18° C.). The tubes were examined with the naked eye for agglutination, then placed in the incubator at 37° C. for 1 hour and examined for lysis. When the sera of approximately 2000 group O blood donors were examined in this way, 9 per cent contained strong anti-A haemolysins and 1 per cent contained strong anti-B haemolysins.

Use of pig group A (A^P) red cells. Lockyer and Tovey (1960) tested group O sera by mixing one volume of serum with one volume of a 10 per cent suspension of A^P cells in 1.5 per cent saline and incubated the mixtures for 1 hour at 20° C. (not 37° C. because there were then some non-specific reactions). The same sera were tested by the method of Gardner and Tovey (1954) and by the method recommended in M.R.C. Memorandum No. 36, that is, incubation of a 1 in 8 dilution of the serum with a suspension of human group A red cells. Of 2500 sera tested, 450 were positive using Gardner and Tovey's screening test; ninety of these were positive using a 1 in 8 dilution of serum and all these samples haemolysed the A^P cells. Fifteen other samples also haemolysed the A^P cells and were shown to have a haemolysin titre of 1 in 4 to 1 in 6 at 37° C. Thus in all, 105 or 4 per cent of the samples haemolysed A^P cells. In order to detect the few samples of group O serum with haemolytic anti-B, human B red cells (10 per cent suspension) may be added to the A^P cells.

Special methods. Ray and Chaplin (1960) estimated haemoglobin released from lysed red cells, using the very sensitive method of Hanks *et al.* (1960), and they were thus able to use very dilute suspensions of red cells. They found that a particular serum would produce 25 per cent lysis of a 2 per cent cell suspension; but with a 0.02 per cent suspension the serum would produce 25 per cent lysis even when it was diluted to 1 in 128.

It is sometimes convenient to use the release of ^{51}Cr from ^{51}Cr-labelled red cells to detect the presence of a small proportion of group A red cells in a population consisting predominantly of O cells (unpublished observations).

Use of Animal Serum as a Source of Complement

As an alternative to using absorbed group O serum as a source of complement in tests for anti-A and anti-B lysins it is possible to use instead serum from suitable animals. Dennis and Konugres (1959) found that the use of horse serum as a source of complement gave exactly the same results as those obtained when human serum was used as a source of complement. The results of using horse complement in the 'screening' of 25,000 donors were described by Walker and Dennis (1959).

When certain other animal sera are used as a source of complement (for example, rabbit) haemolysis is obtained with practically all human complement-binding blood group antibodies (Mollison and Thomas, 1959) and is obtained with some anti-A sera which fail to lyse when horse or human serum is used as a source of complement (Dennis and Konugres, 1959).

Mollison and Thomas recommended the following technique when using animal serum as a source of complement: one volume of a 20 per cent suspension of red cells was first incubated for 1½ hours with four volumes of inactivated serum containing antibody. The cells were then washed twice with saline and incubated at 37° C. for 2 hours or more with four volumes of fresh animal serum as a source of complement.

The animal serum was absorbed three times, each for 15 minutes, with an equal volume of six times washed human red cells of mixed groups A and O to free it from hetero-agglutinins. During absorption, cells and serum were cooled to 0° C. before being mixed, to avoid haemolysis.

USE OF SERUM CONTAINING IMMUNO-CONGLUTININ (I-K) TO DETECT COMPLEMENT-BINDING BY BLOOD GROUP ANTIBODIES

Preparation of serum. Coombs and Coombs (1953) produced potent I-K sera in rabbits by so-called 'auto-stimulation' (see Chapter VII). Their method was used successfully by Mollison and Polley (1960), from whose paper the following details are taken:

A culture of *Salmonella pullorum* was killed with formalin and the strength adjusted to $60{,}000 \times 10^6$ per ml.; injections of 0.5 ml. were given on days 0, 4 and 7, and test bleeds were taken on the following few days. The best serum was obtained on day 12, that is to say, 5 days after the last injection. The serum was absorbed with human group O and A cells and used at a dilution of 1 in 12. At this dilution the reactions were as good as those of undiluted serum.

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Technique of Tests with Immuno-Conglutinin (I-K) Serum

Red cells are first incubated with serum containing antibody, then incubated with complement and finally tested with diluted I-K serum. One volume of a 20 per cent suspension of red cells is incubated at 37° C. for 1½ hours with four volumes of serum previously inactivated by heating to 56° C. for 30 minutes. The red cells are then washed once in saline (three times if the source of complement is to be animal serum rather than human serum). Four volumes of fresh serum as a source of complement are then added to the cells and incubated with them for 20 minutes. The red cells are then washed once and one volume of cell suspension is mixed with one volume of diluted I-K serum on an opal tile and rocked gently over a lighted surface and after 3 to 5 minutes inspected for agglutination.

Usually, fresh human serum from a group AB donor is used as a source of complement. Stronger reactions are obtained if rabbit serum is used, but false positive reactions are then troublesome; the rabbit serum must be diluted 1 in 2 after being absorbed twice with human red cells, and it may be necessary to dilute the I-K serum further. On the whole it is better to use human serum as a source of complement (Mollison and Polley, 1960).

The serum must either be fresh or stored at −50° C.; serum stored at −20° C. for more than a few days is quite unsuitable as a source of complement in the I-K test (Polley and Mollison, 1961).

Scope of I-K Tests

When human serum is used as a source of complement the test is not as sensitive as the indirect antiglobulin test. The main value of a test with I-K serum is to confirm that complement is bound to the red cells by the particular antibody being tested. Some results of tests with I-K serum are given in Chapter VII.

THE ANTI-HUMAN GLOBULIN TEST (COOMBS TEST)

The principle of this test has been described in Chapter VII. As described there, it is now known that anti-human globulin sera can be used to detect at least three different proteins: 7*S* gamma globulin and 19*S* gamma globulin (antibodies) and a β_1 globulin associated with complement (see p. 240). Antiglobulin sera prepared by injecting whole human serum or separated serum globulin usually react satisfactorily with Rh-sensitized cells indicating that they have an adequate content of anti-7*S* gamma

globulin. They often react much less well with adsorbed complement indicating that they have a less than optimal amount of anti-β_1 globulin and they may fail altogether to react with red cells sensitized with anti-Le^a (without complement) indicating that they have little or no anti-19*S* gamma globulin. It appears that in the routine detection of blood group antibodies there is little need for an anti-19*S* gamma globulin reagent since the only antibodies it commonly detects are those of the Lewis system and these invariably bind complement and so can be detected indirectly by sensitizing red cells with antibody and complement and using an anti-β_1 globulin reagent.

Antiglobulin sera therefore need contain only anti-7*S* gamma globulin and anti-β_1 globulin. Usually the dilution at which the serum reacts best with adsorbed gamma globulin is greater than the dilution at which it reacts best with adsorbed complement. Therefore either the same antiglobulin serum must be used at two different dilutions or two different antiglobulin sera must be used; in practice perhaps the most practical approach is to select one good anti-7*S* gamma globulin serum and one good anti-β_1 and make a mixture containing each antibody at its optimal dilution.

Preparation of Antiglobulin Sera

The antigen. If the object is to obtain a serum with antibodies against many human proteins, including gamma globulin and beta-1 globulin, whole human serum may be injected. To obtain more specific antigens, one of the following methods may be used.

Gamma globulin can be extracted from human serum by one of the various available methods and used as an antigen. A simple method of injecting relatively pure gamma globulin is to take red cells from the animal to be injected, for example a rabbit, incubate them with a sample of human serum, during which time they adsorb human gamma globulin in the form of hetero-agglutinins, and then to inject the red cells back into the same animal (Milgrom *et al.*, 1956; Matuhasi and Usui, 1957).

Complement can be adsorbed on to antigen-antibody precipitates — for example a mixture of potato protein and anti-potato (Ellis and Gell, 1958) or egg albumin and anti-egg albumin (Rosenfield, Haber and Gilbert, 1960). The washed precipitate is mixed with fresh human serum, then washed again and injected into an animal. Another method which apparently succeeds is to inject human group A or B red cells agglutinated by human anti-A or anti-B serum (Matuhasi and Usui, 1957; 1958).

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Use of adjuvants. An alum precipitate may be used, following Proom's (1943) method:

2.5 ml. of the serum is mixed with 5 ml. of distilled water and 9 ml. of 10 per cent potash alum. The pH is adjusted to 6.5 with 5 N NaOH. The precipitate is washed twice with 20 ml. of 1/10,000 merthiolate saline and finally made up to 10 ml. with 1/10,000 merthiolate saline. 10 ml. of the final suspension is equivalent to 2.5 ml. of serum.

Alternatively, Freund's complete adjuvant may be used. One part of antigen solution is well mixed with an equal part of Freund's adjuvant in a mortar. Only small amounts of the mixture (not exceeding 0.2 ml.) should be injected into any one site as otherwise a cold abscess may form.

The dose. Two doses of 0.005 mg. of human gamma globulin are capable of causing the formation of a fairly potent antibody in a rabbit (experiments by Miss M. Cutbush, quoted in Mollison, 1956). Very much smaller doses suffice. Thus, Chaplin and Cassell (1960) produced a good antiglobulin serum in a rabbit by giving four injections, each dose contained approximately 0.005 μg. of protein.

It is customary to give very much larger doses than this, but although there may be some justification for giving a dose of the order of a few milligrammes for the first injection it seems best to avoid large doses thereafter, as this may inhibit antibody production.

The route of injection. Although satisfactory reagents have been produced by the intravenous or intraperitoneal route there is little doubt that more potent reagents can be produced by intramuscular or better still intradermal injection.

The advantage of intraperitoneal injection is that it is extremely simple. By injecting 0.3 ml. intraperitoneally and giving the same dose 1 month later and bleeding the rabbit after a further 10 days, it is often possible to obtain useable reagents.

At the other extreme is the following sophisticated method which can be relied upon to produce potent antisera: Alum-precipitated serum is produced by Proom's method (see above) and is mixed with an equal volume of Freund's adjuvant. 0.05 ml. of the mixture is injected into each of the four footpads of a rabbit. Thirty days later an intrasmucular injection of 5 ml. of alum precipitated serum is given into each thigh. The animals are bled 10-14 days later. This method seems not to stimulate the hetero-agglutinins. (All details from Dr J. F. Mohn, who mentions that the adjuvant he uses is made by adding 20 mg., rather than the usual 5 mg., of dessicated Bacto-*Mycobacterium butyricum* to 10 ml. of incomplete adjuvant.)

Injections into the footpads are painful and the author has found it desirable to give the animal a general anaesthetic. The paws do not seem to become painful subsequently; it is probably very important to keep to the small dose recommended by Dr Mohn.

The animal. In the above description only rabbits have been referred to. They are still preferred by most workers.

Rabbits are best bled by nicking the marginal ear-vein with a sharp scalpel and letting the blood drop into a container. Xylol lightly wiped on the ear helps to distend the veins and pressure on the vein at the base of the ear promotes the flow. Vaseline should be wiped on the vein just before it is nicked to stop the blood from sticking to the fur. Thirty-five to 40 millilitres can be obtained at one time from a large rabbit.

Several other animals have been used successfully for the production of anti-human globulin sera. Goats produce good reagents (Hill and Haberman, 1955; Dunsford and Bowley, 1957), and are kept by many transfusion centres. Horses also produce good reagents — for example, the antiserum produced by the Pasteur Institute — and so do sheep (Stratton, 1956b).

Absorption and Preliminary Testing of Antiglobulin Sera

Removal of Hetero-agglutinins by Absorption

The serum is inactivated by heating to 56° C. for 20 minutes. The serum should then be absorbed with human red cells. It is customary to use a mixture of two or three samples of red cells to include the antigens A, B and O. The red cells must be washed very thoroughly in saline to remove all traces of plasma. For example, if one volume of cells is suspended each time in three or four volumes of saline, about ten washings will be needed. It is convenient to test the washings with salicylsulphonic acid and when this reaction becomes negative to perform one further wash (Pickles, 1949, p. 160). In the author's experience the washings never give a completely negative reaction; nevertheless the test is a useful guide.

Often, a single absorption of antiglobulin serum with an equal volume of washed cells for 1 hour at 4° C. is sufficient to remove all agglutinins active against normal human red cells. This should be checked by testing the undiluted serum against suspensions of washed O, A and B red cells. If any agglutination occurs the serum must be absorbed again.

Dr J. F. Mohn (personal communication) has found that potent hetero-agglutinins are effectively removed by several absorptions with one-tenth

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volume of red cells; thus 1 ml. of serum is absorbed three to five times with 0.1 ml. of packed red cells.

Stratton and Jones (1955) found that absorption with normal red cells reduced the antiglobulin titre against Rh-sensitized cells whereas absorption with trypsin-treated cells did not.

Inactivation of Hetero-agglutinins by Heating

Another method of removing the agglutinins active against human red cells is to heat the antiglobulin serum to 63° C. for 1 to 2 hours, as first described by van Loghem (1950). Alternatively, the serum, diluted 1 in 10 to avoid coagulation, can be heated to 70° C. for 1 hour (Menolasino and Davidsohn, 1954).

Destruction of hetero-agglutinins by heating seems to be entirely satisfactory when the antiglobulin serum is to be used only for detecting incomplete Rh antibodies, but is not satisfactory when the antiglobulin serum is to be used for the detection of complement-binding antibodies. Thus, Hunter and Thomas (1957) tested eight different antiglobulin sera and found that only two out of the eight, after heating, would react as well with complement-coated red cells as before heating. Heating to 63° C. for 30 minutes produced a relatively small drop in the titre of the antiglobulin serum versus complement-coated red cells, and the authors suggested that this should be tried when testing a new antiglobulin serum. They found that this amount of heating did not always completely destroy hetero-agglutinins. They also recommended that the serum should be diluted 1 in 2 before being heated, to minimize the risk of coagulation.

It has since been observed that a satisfactory method of getting rid of hetero-agglutinins is to heat the antiglobulin serum to 63° C. for 30 minutes and then to absorb the sera once only with ten times washed red cells.

Reactions of Antiglobulin Sera with Reticulocytes

Jandl (1960) showed that reticulocytes might be agglutinated by antiglobulin sera and that this was due to a reaction between transferrin attached to the reticulocytes and anti-transferrin in the antiglobulin serum; addition of transferrin to the antiglobulin serum inhibited the reaction.

Nelken (1961) found that five out of six rabbit antiglobulin sera which he tested would give positive reactions with reticulocyte-rich samples of red cells. He found that after absorption of the antiglobulin sera with reticulocyte-rich red cells, the sera no longer gave these positive reactions and that their titre against Rh-sensitized red cells was unchanged.

It seems desirable to test antiglobulin sera with a reticulocyte-rich sample of red cells and if they react, to attempt to absorb out the relevant antibody, as described by Nelken.

Tests with Different Kinds of Sensitized Red Cells

The next step is to test the antiglobulin serum against red cells sensitized with an antibody which does not bind complement, for example, incomplete anti-Rh, and secondly with a complement-binding antibody, for example, normal incomplete cold antibody or anti-Le^a. Rh-positive red cells are weakly sensitized by mixing one volume of a 20 per cent suspension with two volumes of a serum containing a weak Rh antibody and incubating the mixture at 37° C. for 30 minutes. The red cells are now washed three times in normal saline and finally made up as a 20 per cent suspension.

Cells may be sensitized with normal cold incomplete antibody as follows: one volume of packed washed red cells of group O is mixed with twenty volumes of fresh normal serum (preferably from an A_1B or A_1 donor). The mixture is left at 0° C. for 1 to 2 hours. The red cells are then washed in warm saline and are ready for testing. Methods of obtaining optimal binding of complement when red cells are sensitized with anti-Le^a are given in a later section.

The washed, sensitized red cells and washed, unsensitized cells of groups A, B and O are now tested against increasing dilutions of antiglobulin serum. The results of testing two antiglobulin sera in this way are shown in Table XXXVIII. Both antiglobulin sera react satisfactorily with red cells sensitized with anti-Rh, and both show a definite prozone, reacting best at 1 in 16 to 1 in 32. Antiglobulin serum (*a*) reacts well with cells sensitized with normal incomplete cold antibody, but shows no prozone; antiglobulin serum (*b*) reacts with these cells only up to a dilution of 1 in 4. With both sera, cells sensitized with other non-complement-binding antibodies were most strongly agglutinated by diluted antiglobulin sera; cells sensitized with other complement-binding antibodies, such as anti-Le^a, were most strongly agglutinated by undiluted antiglobulin serum. Evidently, the use of serum (*b*) at a dilution of 1 in 32 would be satisfactory for the detection of Rh antibodies, but entirely unsatisfactory for the detection of Lewis antibodies.

With almost all antiglobulin sera the dilution which is optimal for the agglutination of cells sensitized with a non-complement-binding antibody such as anti-Rh is greater than the dilution which is optimal for the agglutination of cells sensitized with a complement-binding antibody. There-

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fore, the antiglobulin serum must either be used at two dilutions or a blend of two different antiglobulin sera must be used; that is, of one serum diluted to the point at which it reacts best with Rh-sensitized cells and of another diluted to the point at which it reacts best with complement-coated cells.

A blend of two antiglobulin sera is in fact probably the most satisfactory reagent for the detection of all kinds of antibodies. If both of the antiglobulin sera are diluted more than 1 in 10 in the final blend, some extra protein should be added; for example when rabbit antiglobulin sera are used, the dilutions may be made in a 1 in 10 dilution of rabbit serum previously absorbed with human red cells or alternatively may be made in 1 per cent bovine albumin.

TABLE XXXVIII

THE RESULTS OF TESTING TWO ANTIGLOBULIN SERA*

	Cells sensitized with	*Dilutions of antiglobulin sera*							
		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Antiglobulin serum (*a*)	Anti-Rh (weak)	+	+	++	++	++	+	+	+
	Normal incomplete cold antibody	+++	+++	++	+	+	(+)	tr	—
	Unsensitized cells	—	—	—	—	—	—	—	—
Antiglobulin serum (*b*)	Anti-Rh (weak)	+	+	+	++	++	++	+	+
	Normal incomplete cold antibody	++	(+)	—	—	—	—	—	—
	Unsensitized cells	—	—	—	—	—	—	—	—

* Both antiglobulin sera are weak. Satisfactory antiglobulin sera react best at one in 200 or one in 500 with red cells weakly sensitized with anti-Rh and at one in 50 or one in 100 with 'complement-coated' cells, for example, cells sensitized with normal incomplete cold antibody.

In this account it has been assumed that an antiglobulin serum which is satisfactory for the detection of incomplete anti-Rh and for the detection of complement adsorbed on to sensitized red cells will be satisfactory for detecting all incomplete blood-group antibodies. That is, it has been assumed that it need contain only anti-7*S* gamma globulin and anti-beta$_1$ (complement) globulin. It is known that red cells sensitized with some 19*S* gamma globulin antibodies, such as anti-Le^a, will react with an antiglobulin serum containing only anti-19*S* but in practice almost all incomplete 19*S* antibodies bind complement and are more readily detected by giving the test cells an opportunity of taking up complement as well as antibody and then testing them with an anti-beta$_1$ globulin serum.

The question remains whether there are qualitative differences between the 7*S* gamma globulins of which various blood-group antibodies are composed. There is evidence that no such differences exist between different examples of incomplete anti-Rh (Cutbush *et al.*, 1955), but nevertheless it may be wise to test antiglobulin sera against a variety of blood-group antibodies before taking them into use. Thus, an antiglobulin serum might well be tested for its ability to react with red cells sensitized with several examples of anti-K and anti-Fy^a in addition to testing it with red cells sensitized with anti-Rh and with normal incomplete cold antibody.

Technique of Antiglobulin (Coombs) Test

In the final stage of the antiglobulin test, well-washed red cells are mixed with antiglobulin serum and observed for agglutination. This final stage can be carried out on flat opal tiles, in test tubes or in capillary tubes. In the author's laboratory the opal tile technique is preferred and will thus be described in more detail than the tube technique.

The best kind of tiles are made of sheets of opal glass divided into a number of squares by thick lines made by sand-blasting; the lines are coloured. It is convenient to have two sizes, one with about twenty squares and one with about sixty. The small tiles are convenient for one or two tests, with controls; the larger ones are valuable for comparing a series of reactions. Suitable tiles may be obtained from Aygee, Ltd, 100 Westminster Bridge Road, London, S.E.1.

The Direct Test

Tests on flat opal tiles. Three or four drops of whole heparinized or oxalated blood are added to 2-3 ml. of saline in a small test tube (e.g. 7.5 cm. × 1 cm.), mixed and centrifuged so as to pack the cells lightly. The supernatant is rejected, fresh saline is added and the sediment resuspended. After three washings a few drops of saline are added to the remaining sediment to make a strong (20 per cent) suspension. Control cells (see below) are treated similarly.

Two drops of this suspension are now placed side by side on an opal tile; one is mixed with a drop of suitably diluted antiglobulin serum (see above) and the other drop is mixed with a drop of saline. The drops are stirred with a plastic rod and spread out to cover a roughly circular area about 2 cm. in diameter. As soon as the drops have been mixed the exact time should be noted.

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Now the opal tile is very slowly and gently rocked over a source of light. If agglutination is observed its degree is noted together with the time that has elapsed; for example, 'very strong agglutination within 30 seconds', or 'weak agglutination at 3 minutes'.

A weak positive control should always be included. Control red cells can be prepared every few days by incubating Rh-positive red cells with a serum containing weak Rh antibody. A few drops of these weakly sensitized red cells are then washed at the same time as the test cells.

If no agglutination of the test cells is observed after 5 minutes, the test should be considered negative, provided that the control red cells are agglutinated within this time. The commonest cause of false negative results is failure to wash the cells adequately, in saline. This is not surprising, since a drop of human serum diluted 1 in 5000 to 1 in 10,000 will inhibit the reaction between a drop of diluted antiglobulin serum and one of weakly sensitized cells. If a relatively large volume of blood is used, it is advisable to wash the cells four times.

Vos and Kirk (1958b) demonstrated the importance of thorough washing of red cells by comparing simple resuspension of centrifuged red cells in saline with vigorous shaking in a machine. In each case the red cells were given three washes with 20 ml. of saline and were finally tested with serial dilutions of antiglobulin serum. With vigorously shaken red cells the titre was higher and there was no prozone; the authors found that prozones were always associated with free anti-Rh in the supernatant and suggested that prozones could be avoided by proper washing of the red cells.

As explained above, the antiglobulin serum must either be used at two dilutions or a special blended antiglobulin serum must be used. In rare cases in which the test is negative but there are grounds for believing that the red cells may be sensitized, the washed cells should be tested against a wide range of dilutions of more than one antiglobulin serum.

Tests in tubes. If the direct antiglobulin test is to be carried out in test tubes, the following technique may be used (Rosenfield, Vogel and Rosenthal, 1951):

Place two drops of a 2-5 per cent suspension of the red cells in a small test tube (8×70 mm.); wash four times with saline. Finally, add to the washed deposit one or two drops of antiglobulin serum, mix and immediately centrifuge lightly (1000 rev./min. for 30-60 seconds). Roll the tube lightly and look for evidence of agglutination; if necessary a hand lens may be used.

This method is very sensitive and for this reason it is particularly important to include the appropriate negative control. For example, an antiglobulin serum may be absorbed to the point where it no longer agglutinates unsensitized cells on a tile but may well agglutinate them after centrifugation.

Tests in capillary tubes. One part of four-times washed red cells is taken up into a capillary tube followed by one part of suitably diluted antiglobulin serum. The tubes are inverted and set up at 45°; the reactions are recorded as negative if no agglutination occurs within 10 minutes (Chown and Lewis, 1957).

Each of the three methods of performing the antiglobulin test has some advantages. The tube method is very sensitive but gives no indication of the speed with which the reaction develops. The capillary method does indicate the speed of agglutination but the method is possibly too sensitive and considerable difficulty may be found in absorbing the antiglobulin serum so as to free it completely from hetero-agglutinins. The special value of tests on tiles is that many tests can be carried out side by side and comparisons are very easily made.

The Indirect Antiglobulin Test

The indirect antiglobulin test is used firstly to detect incomplete antibodies using cells of known group, and secondly to detect red cell antigens, using sera containing incomplete antibodies of known specificity. Cells and serum are left in contact to allow sensitization to occur; the cells are then treated exactly as in the direct test.

For routine use one drop of a 20 per cent suspension of red cells is incubated at 37° C. with four drops of serum.

The period of incubation should normally be about 2 hours; although the great majority of antibodies can be detected after 30 minutes' incubation, this time can be shortened even to 15 minutes in emergency. (A discussion of this point will be found later in this chapter in the section on Compatibility Tests). In detecting traces of antibody Wootton (1951) found that it was an advantage to use a period of incubation as long as 4-6 hours.

Normally, incubation is carried out at 37° C., although with some antibodies, particularly certain examples of anti-P_1 and other cold antibodies, positive results may be obtained only if the cells are sensitized at a lower temperature, such as 25° C.

Suitable negative and positive controls must always be included.

RCPE0002068_0217

Special Techniques for Detecting Complement-binding Antibodies by the Antiglobulin Test

As described in Chapter VII, complement appears to play two different roles in the detection of antibodies by the antiglobulin sensitization test. First it may be easier to detect complement adsorbed on to the sensitized cells than to detect the antibody itself; this is the case, for example, when red cells have adsorbed anti-Le^a and complement. The anti-Le^a, being composed of 19*S* gamma globulin, does not react well with most antiglobulin sera which contain very little anti-19*S* gamma globulin, but the complement is likely to react well since most antiglobulin sera contain a considerable amount of the relevant anti-$beta_1$ globulin.

The second role which complement plays is in promoting the adsorption of antibody or preventing its elution. For example, weak anti-Jk^a antibodies may be poorly adsorbed on to red cells in the absence of complement and may be difficult to detect unless red cells are incubated with antibody and complement simultaneously; even then, the adsorption of the antibody is detected only indirectly, that is, by detecting the binding of complement (Polley and Mollison, 1961).

When absolutely fresh serum is available, one-stage tests, that is incubation of red cells and antibody-containing serum, should give entirely satisfactory results since complement is available for promoting antibody adsorption and for being bound by the sensitized cells.

When only stored serum is available, or if by chance the fresh serum is anti-complementary, the position is more complicated. Provided that the antibody is one which is well adsorbed by red cells in the absence of complement, a two-stage test solves the problem. In the first stage the red cells are incubated with the antibody-containing serum after treating it in some way to prevent the adsorption of any abnormal complement (complementoid) which may be present.

Complement and complementoid may be removed by absorption with kaolin. 1 ml. of serum is added to 0.5 g. of kaolin (e.g. kaolin 'heavy', supplied by British Drug Houses, Ltd) and incubated at 37° C. for 15 minutes. The supernatant serum is then recovered. Some antibodies are absorbed by kaolin treatment and the method therefore cannot be recommended for routine use.

Complement-adsorption may also be prevented either by heat inactivation (heating the sera to 56° C. for 30 minutes) or, better still, by treating the serum with EDTA (4 mg./ml.).

A solution containing 4.45 per cent K_2H_2 EDTA and 0.3 per cent NaOH is prepared. 0.1 ml. amounts of this solution are pipetted into small glass containers

and allowed to dry at 37° C. This provides 4 mg. of EDTA in each container and may be used to treat 1 ml. of serum.

Two-stage test. Four volumes of EDTA-treated serum are mixed with 1 volume of a 20 per cent suspension of red cells and incubated in a water-bath at 37° C. for 1½ hours. The cells are then washed three times and incubated with two volumes of fresh serum for 15 minutes at 37° C. before being finally washed and tested with a suitable antiglobulin serum.

One-stage test. If antibody-binding is poor in the absence of complement, the two-stage test gives poor results. In some instances complement and complementoid can be removed by absorption with kaolin and then

TABLE XXXIX

EFFECT OF LEWIS GROUP OF SERA USED AS A SOURCE OF COMPLEMENT IN 'ONE-STAGE' AND 'TWO-STAGE' ANTIGLOBULIN TESTS WITH CELLS SENSITIZED WITH STORED ANTI-Le^a SERUM (from Polley and Mollison, 1961)

Test	*Fresh serum used as source of complement*	*Dilutions of anti-Le^a (reciprocals)*					
		2	4	8	16	32	64
'One-stage'	Le(a+b−) serum	−	−	−	−	−	−
	Le(a−b+) serum	+	(+)	−	−	−	−
	Le(a−b−) serum	++	++	+	−	−	−
'Two-stage'	Le(a+b−) serum	+++	+++	++	+	(+)	−
	Le(a−b+) serum	+++	+++	++	+	(+)	−
	Le(a−b−) serum	+++	+++	++	+	(+)	−

In 'one-stage' tests Le(a+) cells were incubated with anti-Le^a and fresh serum simultaneously; in 'two-stage' tests Le(a+) cells were incubated first with anti-Le^a and then washed and incubated with fresh serum.

fresh normal serum added as a source of complement; this is sometimes successful in detecting anti-K. Unfortunately kaolin treatment sometimes removes blood group antibodies as well as complement, as in some instances of anti-Jk^a and anti-Fy^a investigated by Polley and Mollison (1961). In such cases the only available method is to add fresh serum directly to the stored serum before incubation with test cells, hoping that sufficient normal complement will be provided to compete successfully with any complementoid present.

Four drops of fresh normal serum are mixed with four drops of antibody-containing serum and added to one drop of a 20 per cent suspension of red cells. The mixture is kept at 37° C. for 1½ hours and the cells are then washed and tested as usual. In one-stage tests with sera containing Lewis antibodies, if fresh serum is

RCPE0002068_0218

used as a source of complement, it must be from a donor of the type Le(a—b—). By contrast, in two-stage tests the Lewis group of the serum used as a source of complement is unimportant (see Table XXXIX).

Prozones in Titrating Antibody-Containing Sera using the Indirect Antiglobulin Test

In rare cases a prozone may be encountered in titrating an antibody-containing serum by the indirect antiglobulin technique. Thus red cells incubated with undiluted antibody-containing serum and then washed may be agglutinated weakly or not at all by antiglobulin serum, whereas cells incubated with diluted antibody-containing serum may be strongly agglutinated (Freisleben and Knudsen, 1957; Giblett *et al.*, 1958).

In the case described by Freisleben and Knudsen, the serum contained anti-M which agglutinated red cells as well as sensitizing them to an antiglobulin serum; the authors suggested that cells exposed to undiluted serum might take up agglutinating antibody predominantly which was eluted on washing. In the case described by Giblett *et al.* (1958), the antibody was anti-s, which agglutinated cells in saline to a titre of 8 but had an indirect antiglobulin titre of 1000 with a definite prozone.

Dr E. R. Giblett (personal communication) suggests that the prozone in this case may have been due to the fact that cells exposed to undiluted serum took up an excess of antibody which was loosely-bound — perhaps due to relatively few antigen sites — and the ready elution of this excess antibody neutralized the antiglobulin serum. This explanation is supported by the finding that when the cells were washed six to eight times before being tested against the antiglobulin serum, the prozone was abolished.

Very occasionally samples of red cells from normal subjects give a positive direct Coombs reaction. If such samples are tested for the presence of some blood group antigen, for example D^u, using incomplete anti-D, it may be falsely concluded that the antigen is present. If, as a routine, the cells are tested directly with an antiglobulin serum, before incubation with the serum containing the blood group antibody, this mistake can be avoided.

Indirect Antiglobulin Test for the Detection of Incomplete Anti-A (or Anti-B)

A large proportion of group O sera can be shown to contain incomplete anti-A (or anti-B). Since such sera also contain anti-A and anti-B agglutinins, it is necessary to neutralize these agglutinins before carrying out an indirect antiglobulin test on the serum. It is necessary to discover, for each

serum, the amount of A (or B) substance which will just prevent agglutination of a saline suspension of cells and then to discover whether these cells, after washing, give a positive antiglobulin test.

One volume of undiluted saliva (from a group A (or B) secretor) will usually completely abolish the power of an equal volume of anti-A serum to agglutinate A_1 cells in saline; one volume of a 1 in 10 dilution of mixed A and B substances as prepared[1] for the neutralization of isoagglutinins or one volume of 'purified A substance',[1] has an equivalent inhibitory effect (personal observations). If a serum has a high titre in saline, considerably greater quantities of A substance are required. If more than two volumes of saliva are added to the serum the saliva should first be rendered isotonic as otherwise it will lyse the red cells. According to Ege (1945) the freezing-point depression of saliva is 0.2° C., so that the most watery saliva may have a tonicity equivalent to about 0.3 per cent NaCl. The addition of one-tenth volume of 7 per cent NaCl to each volume of saliva should ensure an adequate margin of safety.

A simple and convenient method of revealing incomplete anti-A is to add a relatively small volume of immune anti-A serum to whole blood (Crawford and Mollison, 1951). For example, the addition of one volume of immune anti-A to ten volumes of whole blood will not cause agglutination directly but will usually sensitize the red cells to agglutination by an antiglobulin serum.

Antiglobulin Test with Enzyme-treated Cells

Unger (1951) claimed that if Rh-positive cells were treated with trypsin before being exposed to anti-Rh serum, and if the antiglobulin test was subsequently carried out on the cells, very faint traces of antibody could be detected.

The sensitivity of this technique has since been confirmed by others; moreover it is sometimes useful in separating the reactions of two antibodies; thus in a serum containing anti-Fy^b and anti-Jk^b, anti-Fy^b failed to react with papain-treated cells, whereas anti-Jk^b reacted well (Dr R. R. Race, personal communication).

The combination of treatment with ficin and the antiglobulin test has been found valuable in detecting the Le^a antigen in adults of the apparent phenotype Le(a—b+) (Cutbush, Giblett and Mollison, 1956). To obtain reliable results it is, however, essential to include a number of negative controls in antiglobulin tests on enzyme-treated cells. The best 'negative' control is provided by incubating each sample of enzyme-treated cells with a serum known to be free from antibodies. It has been found that

[1] Obtainable from Messrs. Sharp & Dohme.

RCPE0002068_0219

only very 'mild' treatment with ficin is required in this test, for example five volumes of a 20 per cent cell suspension are incubated at 37° C. for 15 minutes with one volume of a 0.5 per cent solution of ficin in Hendry's iso-osmotic buffer, pH 7.4. The amount of enzyme-treatment is critical and it is advisable that the dilution of ficin should be prepared freshly.

Mixed Antiglobulin Reaction

The principle here is to use an antiglobulin serum to link up two types of sensitized cell, each of which has adsorbed incomplete antibody; for example, leucocytes may be incubated with a serum suspected of containing leucocyte antibodies. The cells are then washed and mixed with an antiglobulin serum and with red cells sensitized with incomplete Rh antibody. The mixture is examined under a phase-contrast microscope. When the result is positive, agglutinates consisting of a mixture of white cells and red cells are seen. This test has been used successfully to demonstrate iso-antibodies against leucocytes and platelets by Chalmers *et al.* (1959).

Gamma-Globulin Neutralization Test

As already mentioned, if a sufficient amount of gamma globulin is added to an antiglobulin serum, the serum will no longer agglutinate red cells sensitized with Rh antibody or with some other blood group antibodies. The reactions of a suitably diluted antiglobulin serum with Rh-sensitized red cells are usually completely inhibited by first adding to the antiglobulin serum, gamma globulin in a final concentration of 0.01 per cent.

The gamma-globulin neutralization test may be used to classify cases of acquired haemolytic anaemia into warm and cold types. Thus in the majority of cases of the warm antibody type the reaction of the red cells with antiglobulin serum is readily inhibited by the addition of gamma globulin; in these cases, the cells react best with diluted, rather than undiluted, antiglobulin serum, i.e. a prozone is observed. In the cold antibody type the cells react best with undiluted antiglobulin serum (no prozone); the reaction is not inhibited by the addition of gamma globulin, because the antiglobulin serum is reacting with complement (beta globulin) bound to the patient's red cells, and the patient's serum contains potent cold agglutinins.

Estimation of Gamma Globulin

Wiener, Hyman and Handman (1949) pointed out that the antiglobulin test can be used for the assay of human serum globulin. They made dilutions of normal serum and of the material to be tested (e.g. normal

spinal fluid) and investigated the inhibitory effect of these dilutions on the reaction between antiglobulin serum and Rh-sensitized cells. The method was applied to the investigation of patients with hypogammaglobulinaemia by Wiener (1955) and de Muralt *et al.* (1957). Grubb (1956a) investigated the specificity and sensitivity of a similar method and concluded that amounts as small as 0.1 μg. could be detected.

The following method was recommended in the second edition of this book, and has since been used in testing a large number of patients with hypogammaglobulinaemia (see below).

Eight dilutions, over a range 1 in 3000 to 1 in 10,000, are prepared from normal serum. The patient's serum is diluted over the range 1 in 50 to 1 in 1000. An aliquot of each dilution of serum is now added to an equal volume of diluted antiglobulin serum and then mixed with red cells sensitized with Rh antibody. Suppose that a 1 in 5000 dilution of normal serum and a 1 in 100 dilution of the patient's serum both just inhibit the reaction of the antiglobulin serum. Then, knowing the concentration of gamma globulin in the normal serum to be 1000 mg./100 ml., the concentration in the patient's serum is $\frac{100}{5000} \times 1000$, or 20 mg./100 ml.

This method was used in parallel with two other methods, namely, free electrophoresis and the immuno-chemical method in agar-gel plates described by Gell (1957). It was found that the method of inhibiting an anti-gamma globulin serum was the most sensitive (Kekwick *et al.*, 1961).

Immunological methods and electrophoretic methods of estimating gamma globulin do not measure exactly the same components of serum. Thus, electrophoresis measures the component of serum which migrates most slowly at pH 8.6. The serological method measures any protein which reacts with anti-gamma (7*S*) globulin; it is known from immuno-electrophoresis that such protein extends from the γ_2 region all the way into the α_2 region (for references, see Burtin, 1960). When the gamma globulin is measured by a precipitation method against an anti-gamma (7*S*) globulin serum, the amount found in normal serum is about 40 per cent greater than that estimated by electrophoresis (Slater, 1955). Similarly the amount of gamma globulin in normal serum, as estimated by the method described above (inhibition of antiglobulin serum method), is found to be about 50 per cent higher than the amount estimated by electrophoresis. This discrepancy is found only if gamma globulin is used as a standard. If one wants to obtain results which agree with those of electrophoresis, it is necessary to use normal serum as a standard and to take its gamma globulin concentration to be that determined by electrophoresis (see Kekwick *et al.*, 1961).

Inhibition of the reaction between anti-19*S* gamma globulin and Le^a-sensitized red cells has been used to estimate 19*S* gamma globulin (Polley *et al.*, 1961).

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COMPATIBILITY TESTS

The term 'compatibility test' usually refers to an investigation of the patient's serum for the presence of antibodies which react with the donor's red cells *in vitro*. This is also sometimes called a direct matching test. When no antibodies are found the red cells are said to be compatible and are expected to survive normally, although their survival may be curtailed if they induce the formation of isoantibodies in the recipient following transfusion.

In practice it is taken for granted that when a compatibility test is performed, donors of the same ABO and Rh groups as the recipient are first selected, and these red cells are then tested directly against the recipient's serum.

In interpreting the phrase 'of the same Rh group' all laboratories would agree that Rh-positive (D-positive) blood should not normally be given to Rh-negative recipients, but many would see little objection to giving Rh-negative blood to Rh-positive recipients, were it not for the fact that supplies of Rh-negative blood are usually limited.

Limitation of supply of Rh-negative blood does sometimes make it necessary to consider giving Rh-positive blood to Rh-negative recipients. If the recipient is a male who has not been transfused before, and particularly if the recipient is elderly, the transfusion of Rh-positive blood cannot be considered to be very undesirable. No Rh antibody is likely to be formed for several weeks after the transfusion, so that if further transfusions are needed within a few days of the first transfusion more Rh-positive blood can be given (provided that direct matching is carried out with a sample obtained on the day of transfusion). Although it may sometimes be defensible to give Rh-positive blood to Rh-negative males, it must be an absolute rule that Rh-positive blood is never transfused to an Rh-negative female who is below the age of the menopause.

If Rh-negative blood is transfused to Rh-positive recipients there will be a small risk of provoking the formation of anti-c, although this will, of course, be possible only when the recipient is of the phenotype CC. In such recipients the same risk arises if a transfusion of blood of the phenotype Cc is given, and some transfusion centres consider that the red cells of all female recipients should be tested with anti-c, and that c-negative recipients (i.e. those of the phenotype CC) should be transfused only with CC blood. This is a very desirable practice, because if a woman once forms anti-c the risk that any child born to her subsequently will have haemolytic disease of the newborn is very great, because the gene c is so frequent in the population.

The risk from the antigen E is much less, mainly because the gene *E* is much less frequent than *c*.

The blood group antigen K is taken into account by some transfusion centres in selecting blood for female recipients, but all other antigens cause trouble so much less frequently that they can be ignored in the preliminary selection of donors, and need be taken into account only when the recipient forms the corresponding antibody.

When blood is obtained from a transfusion service which uses elaborate checks to avoid errors in ABO and Rh grouping it is reasonable to assume that the blood is of the ABO and Rh group stated, and it is necessary only to test the ABO and Rh group of the recipient, as a preliminary to 'direct matching'.

Before discussing direct matching tests the question of the minor cross-match will be disposed of.

The 'minor' cross-match. In some transfusion laboratories it is the custom to test the donor's plasma against the recipient's red cells (the 'minor' cross-match) as well as to test the donor's cells against the recipient's serum (the 'major' cross-match).

The 'minor' cross-match serves primarily to detect errors in ABO grouping, but these certainly should not occur if ABO groups are determined by testing both red cells and serum, and the direct matching test ('major' cross-match) acts as a further safeguard.

The 'minor' cross-matching test should in principle also reveal the presence of immune blood group antibodies, such as anti-K, in the donor's plasma, but unless the indirect antiglobulin test is used for the minor cross-match, the presence of such antibodies may be overlooked. Because it is desirable to make direct matching tests as simple as possible, it is much better to 'screen' the plasma of all donors for the presence of blood group antibodies at the Blood Collecting Centre rather than at the actual time of transfusion. It is suggested that the serum of all donors should be tested against red cells (if necessary a pool derived from several donors) which contain the antigens D, c, E and K. Donors whose serum gives a negative indirect antiglobulin test can safely be used without minor cross-matching. Even this is somewhat perfectionist and the simple screening of serum from Rh-negative donors for anti-D, to guard against the possibility that an emergency transfusion of such blood to an Rh-positive recipient might prove harmful, is a reasonably adequate safeguard.

Anti-D is in fact quite commonly encountered in blood donors; for example Dr F. Stratton (personal communication) found anti-D in fifty out of 8807 consecutive Rh-negative blood donors — an incidence of 0.57 per cent.

RCPE0002068_0221

When group O blood is matched for transfusion to a group A patient, the 'minor' cross-match will always be incompatible, and it is far more appropriate to test the donor's plasma for immune anti-A.

There is one remote risk which the 'minor' cross-match would help to guard against. Suppose that a recipient of group AB had developed polyagglutinable cells. This would not be detected in ABO grouping, nor in cross-matching, but transfusion of normal AB blood might be dangerous if it contained potent anti-T' agglutinins. This risk seems too remote to justify the performance of a test which adds considerably to the complexity of cross-matching.

Recommended Procedures

A special form should be used for requesting blood for transfusion; an example is shown as Table XL. An important feature of the form is that it

TABLE XL

REQUEST FOR BLOOD FOR TRANSFUSION*

Patient's Name.....Sex .....Age .....
(Block letters — Surname first)
Ward .....Physician .....No. .....
Diagnosis:

Degree of Urgency:
(*a*) Desperate (blood needed 'at once' — without matching)
(*b*) Urgent (blood needed as soon as matching can be done)
(*c*) Non-urgent
(*d*) Reserve for operation, etc.
Date of operation .....
Number of bottles required.....
What is patient's blood group?
O, A, B, AB, Rh-negative, Rh-positive, Not known
Has patient been previously transfused? Yes/No/Not known
Any untoward reaction to previous transfusion? Yes/No
If patient is a woman, has she ever been pregnant? Yes/No
Any evidence of haemolytic disease of the newborn? Yes/No
Time and Date of Despatch
of this Form from Ward .....
Signed .....
(Legible signature essential)
A sample of blood from the patient (5 ml. clotted) should always accompany this Form; when blood is needed 'at once' (or 'urgently'), send 5 ml. of oxalated blood as well.

* Based on a form used at Hammersmith Hospital.

enables the clinician to indicate how urgently blood is required. Another very important feature is that the laboratory is provided with information about the recipient's previous history. The knowledge that a patient has received a previous transfusion will warn the laboratory that extra care must be taken in direct matching.

Routine Method of Direct Matching

No single test will detect all blood group antibodies. Thus, although the indirect antiglobulin test is an almost infallible method of detecting 'incomplete' antibodies, it will not detect weakly agglutinating antibodies, i.e. those which agglutinate weakly in saline.

The indirect antiglobulin test will not detect weak saline agglutinins, since these are removed from the cells during the washing process. Strong agglutinins are not completely removed, and thus when the cell suspension is finally tested, the cells are seen to be agglutinated in the drop to which saline is added, as well as in the drop mixed with antiglobulin serum.

The agglutination test. Because the antiglobulin test fails to detect weak saline agglutinins, an additional test for such agglutinins must be made. One volume of a 2 per cent suspension of the donor's cells should be added to one volume of the recipient's serum and left at room temperature (18-25° C.) for at least 1½ hours; this period of time is determined by the time taken for the cells to fall to the bottom of the tube.

Although 'room temperature' (18-25° C.) is specified in the preceding paragraph, there is in fact some uncertainty about the best temperature to use for the agglutination test. Most agglutinating antibodies react more strongly at room temperature than at 37° C. and react even more strongly at 4° C. Thus the test is certainly made more sensitive by the use of a relatively low temperature. On the other hand, by using a relatively low temperature (18-20° C.) some weak agglutinins (e.g. anti-P_1) which are of no clinical significance will be detected. At even lower temperatures (4-12° C.) many cold auto-agglutinins will be detected.

There is evidence that antibodies which react *in vitro* only at temperatures below about 30° C. are incapable of causing destruction *in vivo* (Cutbush and Mollison, 1958), and it might therefore seem best to carry out direct matching at about this temperature (30° C.). On the other hand, antibodies which agglutinate weakly at 30-37° C. are much more easily detected at 20-25° C.; moreover there are some antibodies (e.g. some Lewis antibodies) which give a weakly positive indirect antiglobulin test at 37° C. but also agglutinate cells at 20-25° C. The use of an agglutination test at 20-25° C. provides an additional method of detecting such antibodies.

In interpreting the results of agglutination reactions occurring at room temperature it is valuable to know whether the patient's serum agglutinates his own cells under similar conditions; for example, when the patient's serum contains cold auto-agglutinins his cells may be agglutinated at room temperature. When this is observed it is possible to discount a

RCPE0002068_0222

similar degree of agglutination in the tube containing the patient's serum and the prospective donor's cells.

If the patient's serum agglutinates one or more samples of red cells at 20–25° C. and does not agglutinate the patient's own cells, an attempt should be made to identify the antibody responsible and the result of the indirect antiglobulin test should be scrutinized with special care. If the indirect antiglobulin test is negative and if there is difficulty in finding donors whose red cells are not agglutinated at 20–25° C., the agglutination tests are repeated at 30° C. Provided that no agglutination is observed at this temperature, the blood can safely be released for transfusion.

Indirect antiglobulin test. The standard method, described earlier in this Chapter, is used. As always, a weak positive control must be included when using the antiglobulin test; cells mixed with serum containing a weak incomplete antibody should be incubated at the same time as the cells and serum under test. As a negative control the donor's red cells alone should be used.

As a rule, the patient's serum should be incubated with the donor's cells for 2 hours, although in urgent cases this period may be reduced, as described below in the section on 'Urgent Cases'.

Provided that fresh serum from the patient is used, it may be assumed that sufficient complement is present to enable one to detect complement-binding antibodies such as anti-Le^a, anti-Jk^a and some examples of anti-K and anti-Fy^a; but if serum which has been kept at 4° C. for more than 24 hours, or if plasma is used, complement must be supplied. This is best done by a two-stage test (see above) in which the cells are first incubated with the patient's serum or plasma for 1½–2 hours and are then incubated with fresh AB serum (or other compatible serum) for 15 minutes. It is convenient to keep supplies of fresh-frozen AB serum for this purpose; this is best stored at −50° C. or lower, but if −20° C. is the lowest temperature available, samples will keep satisfactorily at this temperature for several weeks.

Urgent Cases

Even when blood is urgently required it is usually possible to wait for 45 minutes; during the waiting period plasma or a plasma substitute such as dextran can be transfused. If it is really necessary, within 45 minutes the patient's ABO and Rh group can be determined and a direct matching test can be done. The risk of error is increased by performing the tests in a hurry and clinicians take the responsibility for this extra risk when they declare that the blood is needed 'urgently'.

The first step is to determine the patient's ABO and Rh groups; rapid methods are described earlier in this chapter. It is convenient to use heparinized blood for these tests, and for this reason clinicians should be asked to supply heparinized blood as well as clotted blood from the patient. Heparinized blood is useful for rapid grouping on slides and is also more convenient than clotted blood for the preparation of cell suspensions; it is also useful as a source of plasma for the direct matching test in cases of urgency, since it may be difficult to obtain enough serum from the clotted sample until clot retraction is well advanced. Nevertheless serum rather than plasma should be used whenever possible.

Blood of the same ABO and Rh groups as the patient is selected. The direct matching tests are then set up exactly as in the routine procedure, but in the agglutination test the mixture of cells and serum is incubated for only 15 minutes before being lightly centrifuged so as just to bring down the cells to the bottom of the tube, and the cells are then spread on a slide and examined microscopically in the usual way. Similarly, in the indirect antiglobulin test mixtures are incubated only for 15 minutes.

French (1958) examined a large number of antibody-containing sera with the object of seeing if the antibody could be detected by the indirect antiglobulin test after only 15 minutes' incubation. The sera examined included thirty containing anti-D, eleven containing anti-K, four containing anti-Le^a, and various others. When necessary, fresh serum was added as a source of complement.

The presence of an antibody was detected by the 'standard' indirect antiglobulin test using undiluted serum and an incubation period of 2 hours. Dilutions of the sera in AB serum were also tested, and the sera were regarded as 'maximally diluted' when they would still just give a positive antiglobulin test after 120 minutes' incubation.

Ninety-seven per cent of the sera tested, when used undiluted, gave a positive indirect antiglobulin test after 15 minutes' incubation. When the sera were 'maximally diluted', 39 per cent gave a positive indirect antiglobulin test after 15 minutes' incubation. It was concluded that 15 minutes' incubation suffices to detect all but the weakest blood group antibodies.

Desperate Cases

These are cases in which the clinician decides that blood must be given at once if the patient's life is to be saved. It must be emphasized that few cases fall into this category, since all but the most severe cases of blood loss can be temporarily treated with infusions of plasma or plasma substitute while compatibility tests are being performed.

If blood must be given without any preliminary tests, group O blood

RCPE0002068_0223

must be used. For women who have not passed the age of child-bearing, Rh-negative blood must be used.

It must be emphasized that even when the degree of urgency is great a blood sample must be taken from the patient when transfusion is begun. This sample is the safest to use for direct matching if the patient requires further blood in the next 48 hours and it may prove invaluable if the patient develops an adverse reaction and investigations are required.

Compatibility Tests when Very Large Amounts of Blood have to be Transfused, as in Cardiac Surgery

When the blood of many donors is to be transfused and particularly when the bloods are to be mixed together in a pump-oxygenator before transfusion, the question arises whether the samples ought to be cross-matched with each other. Presumably the only risk to be guarded against is that the plasma of one donor may contain a potent antibody, for example anti-K, and that this may become mixed in the pump-oxygenator with the red cells of a K+ donor and that the subsequent transfusion of the sensitized red cells may be followed by rapid destruction *in vivo* with the release of some haemoglobin into the circulation (see Chapter XIII). In practice it is very unusual for the plasma of a normal subject to contain antibodies potent enough to bring about significant red cell destruction in this way and in the author's opinion simple screening of the plasma of all donors as suggested earlier in this Chapter, is a sufficient safeguard.

For those who prefer to carry out cross-matching between samples, the experiments of Allen *et al.* (1959) are very instructive. These authors considered the reliability of using pooled red cells and pooled sera in blood grouping tests; for example, one part of D-positive red cells was mixed with one to four parts of D-negative red cells and the resulting pools were tested with anti-D serum. It was found that even with small pools the detection of the D-positive cells was much more difficult, although reliable results were obtained so long as the tests were read microscopically. When sera were pooled there was very little loss of sensitivity provided that an adequate volume of the serum pool was added to the cell suspension. Thus even when six or seven sera were mixed, provided that the amount of each individual serum added to the cells was the same as that which would have been used if each serum had been added separately, there was no loss of sensitivity.

Compatibility Tests in Patients having Repeated Transfusions

In patients who are having blood transfusions every day or two there is a temptation to obtain a large sample of serum and use this for cross-matching for several successive transfusions. One must remember that any one of a series of transfusions may stimulate the formation of an immune blood group antibody and that this antibody may rapidly increase in titre once it has appeared. Because of this it is wise to have some arbitrary limit and to insist on having serum taken within 24 hours of transfusion whenever possible.

A case reported by Salmon (1959) illustrates the point. In a patient known to have anti-c and anti-Jk^a in the serum, four bottles were cross-matched. Three were given and two of these were subsequently shown to be S+. Six days later the fourth bottle which was also S+ was transfused without further cross-matching. This transfusion produced a haemolytic reaction and it was shown that anti-S had developed in the interval since the first transfusion was given.

Compatibility Tests in Newborn Infants

At birth the only iso-antibodies present in the infant's serum are those which have passed through the placenta from the mother's circulation. As a generalization, incomplete antibodies pass readily and agglutinating antibodies fail to pass.

It can be assumed that a sample of red cells compatible with the mother's serum will also be compatible with the serum of her infant. Thus, if the mother is group B and the infant group O, group B red cells will be compatible with the infant's serum, and if transfused at the time of birth will survive normally.

In one such case, reported in previous editions of this book, a group O infant of a group B mother was given an exchange transfusion using B, Rh-negative blood. Forty-three days after transfusion the corrected survival was 55 per cent. No anti-B was found in the infant's serum 20 weeks after birth, but it was present at 33 weeks.

Similar cases have been described by Jervell (1924), Wiener (1943, p. 74) and Pickles (1949).

Although the mother's red cells are always compatible with her infant's serum at the time of birth, they should not be used if the mother is Rh-positive and the infant is Rh-negative.

Although there are no records of an infant having been sensitized to a foreign red cell antigen by a transfusion in the first few weeks of life, it cannot be assumed that infants cannot be sensitized at this age. For example, it has been found that infants aged 6-8 weeks can be successfully immunized with alum-precipitated diphtheria toxoid provided that the level of naturally conferred antitoxin in the baby is low enough at the time of injection (Barr *et al.*, 1950).

RCPE0002068_0224

Haemolytic transfusion reactions are almost unknown in newborn infants because their serum so seldom contains high titre iso-antibodies. For this reason, many experienced transfusionists do not consider it necessary to carry out direct matching tests.

Whilst admitting that intravascular haemolysis is very rare following transfusion to newborn infants, it has been described following the transfusion of adult A_1 blood to an infant with haemolytic disease of the newborn due to anti-A (see Chapter XVII). Moreover increased jaundice may follow the transfusion of Rh-positive blood to an infant with haemolytic disease of the newborn due to anti-Rh. In practice the main risk of omitting cross-matching is that the infant may be suffering from undiagnosed haemolytic disease of the newborn and may rapidly destroy the transfused red cells.

Although, if haemolytic disease of the newborn has been excluded, the risk of omitting cross-matching tests in infants during the first month or two of life is extremely small, the following procedure is recommended as a routine:

Obtain a few drops of blood from the infant by skin-prick and determine its ABO and Rh group. Provided that the infant's red cells are compatible with the mother's serum (e.g. group O, mother group A) the mother's serum may be used for cross-matching. The advantage of using the mother's serum is two-fold. Firstly, it is usually more convenient to obtain serum from the mother than from the infant and secondly, any blood group antibodies which are present in the infant's serum will be present in the mother's serum in at least equal titre. If the infant's red cells are incompatible with the mother's serum, it will be necessary to obtain serum from the infant for cross-matching if blood of the same ABO group as the infant is going to be transfused. Alternatively the mother's serum can still be used if AB substance is first added to it to neutralize anti-A and anti-B.

Cases in which All Donors seem Incompatible

There are three circumstances in which it may be very difficult to find a donor whose red cells do not react with the patient's serum. Firstly, when the patient's serum contains a potent cold auto-agglutinin; in most cases the agglutinin is active only up to a temperature of about 30° C., and thus confusion can be avoided by working strictly at 37° C. Cell suspension and serum should be warmed separately to 37° C. before being mixed, and tests should be read on warm slides. In carrying out the indirect antiglobulin test, cells should be washed in warm saline.

Secondly, in acquired haemolytic anaemia of the warm antibody type the patient's serum may react with all samples of red cells when the indirect antiglobulin test is used. In such cases it is sometimes possible, by testing the serum with a panel of red cells (see below) to recognize some definite specificity and then to select compatible cells (see p. 198), but such cases are exceptional. More often the serum reacts to some extent with all samples of red cells. If transfusion is considered absolutely necessary, for example so that the patient can undergo splenectomy, and time permits, it is desirable to study the survival of a small sample of red cells, using a donor whose cells react less strongly with the patient's serum than those of others. In such cases it is usual to find that following the transfusion most of the red cells are removed from the circulation within a few days, and the beneficial results of the transfusion are therefore very short-lived.

In acquired haemolytic anaemia of the warm antibody type the serum may contain no free auto-antibody. The ideal procedure then is not only to cross-match red cells against the patient's serum to detect incompatibility from any iso-antibodies which may be present, but also to test the red cells against an eluate made from the patient's own red cells in case the auto-antibodies have some definite blood group specificity. Even when free auto-antibody is present in the patient's serum, it may be worth cross-matching against an eluate from the patient's red cells because such an eluate may contain more antibodies than are present in the patient's serum. Thus, Weiner, Whitehead and Walkden (1956) described a patient of the probable genotype *cDE/cde*, suffering from acquired haemolytic anaemia, whose serum contained anti-E; an eluate prepared from the red cells contained in addition anti-c.

Thirdly, when a patient's serum does not agglutinate his own cells but contains an antibody reacting with almost all blood samples it may be very difficult to find a compatible donor except by testing large numbers of random donors. Under these circumstances it is always worth testing the brothers and sisters of the patient, since relations have a greatly increased chance of being suitable donors.

It may be worth remarking that when the patient's serum clumps all samples of red cells in direct matching tests the cause is most often rouleaux formation; methods of dealing with this have already been discussed.

ISOLATION AND IDENTIFICATION OF ANTIBODIES

Isolation of Antibodies

Often a serum contains several iso-antibodies. If the serum is to be used as a specific reagent, depending upon the action of a single antibody, the unwanted antibodies must be removed or neutralized. The isolation of an antibody can be accomplished in three main ways and the processes are

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best illustrated by an example. Suppose a serum contains anti-Rh and anti-A and the object is to prepare an anti-Rh serum, free from anti-A activity. One of the following methods can be used:

Absorption

Principle. The antibody to be removed is adsorbed on to red cells which contain the corresponding antigen but lack antigens against the antibody which is to be left. Thus, in the present example A_1, Rh-negative cells are used. Absorption is carried out at the temperature at which the antibody reacts most strongly. Thus, for absorption of anti-A, 0° C. is used (absorption at 0° C. will remove all anti-A, whereas, if an anti-A serum is absorbed at 37° C. it will still react at lower temperatures). In practice, several absorptions with A cells may be necessary to remove all traces of anti-A.

Technique. In order to avoid haemolysis of A cells by anti-A, either the serum must first be heated to 56° C. for 30 minutes to inactivate complement or the absorption must be carried out strictly at 0° C.; the latter method will be described.

For the absorption of 10 ml. of serum, obtain by venepuncture 50 ml. of fresh A_1, Rh-negative blood and mix immediately with 5-10 ml. of 3 per cent trisodium citrate. Divide the blood amongst four 15 ml. centrifuge tubes, fill up the tubes with 0.85 per cent saline, mix and centrifuge. Remove the supernatant and add saline again. After three washings remove as much saline as possible. Each of the four tubes should contain approximately 5 ml. of packed cells.

The pooled cells and the serum to be absorbed must now be separately chilled by placing the tubes in crushed ice. Then 5 ml. of the serum to be absorbed is added to two of the tubes reserving the other two for a second absorption, if this should be necessary. Mix the serum and cells and leave at 0° C. for half an hour. Then centrifuge the mixture for 5 minutes and take off the supernatant serum.

The serum should now be tested to see whether it still contains anti-A. This is best done by adding one small volume of the serum to an equal volume of a 2 per cent suspension of the original A_1, Rh-negative cells, in a precipitin tube, leaving for 30 minutes and then centrifuging for 1 minute at 1000 r.p.m. The deposit should be examined under the low power of a microscope. If there are no agglutinates the serum is suitable for the Rh testing of cells of all groups. If there are agglutinates, the serum must be submitted to a second absorption.

The process must be repeated until the serum gives no trace of a reaction with A_1 cells. It is desirable to use the least number of absorptions which will suffice, since there is a tendency for the activity of the serum to be slightly reduced by each absorption, due to dilution by saline mixed with the 'packed' cells.

Milgrom and Layrisse (1958) have described the use of red cell stroma for absorbing antibodies and preparing eluates. The stroma was prepared from out of date (21-day-old) stored blood by washing red cells in saline and then lysing them in distilled water, and washing them in distilled water at 0° C. The sediment was then lyophilized and was used either to absorb anti-A and anti-B or to remove hetero-agglutinins from antiglobulin sera or to prepare eluates. For example, 50-200 mg. of powder from Rh-positive red cells were incubated with 10-100 ml. of anti-Rh serum; the powder was washed three times in saline and then the antibody was eluted into about 1 ml. saline by heating for 5-7 minutes at 56° C.

Gold, Lockyer and Tovey (1958) have described the use of formol-treated red cells, particularly for the absorption of anti-A and anti-B agglutinins.

'Coming-back' phenomenon. When absorbed sera are stored there is a tendency for the antibody which has apparently been removed completely, to reappear. This phenomenon is sometimes referred to as 'coming-back'. Race and Sanger (1954, p. 280) refer to this in connection with a serum containing anti-D; after thirteen absorptions the antibody appeared to have been removed but after a few days' storage the serum once more became capable of agglutinating Rh-positive red cells. Boyd *et al.* (1945) encountered the phenomenon in absorbed anti-M and anti-N sera. Crawford and Mollison (1951) also noted that when they absorbed an antiglobulin serum with particular sensitized red cells the antiglobulin serum lost the ability to react with the same cells; however, after storage at −20° C. the absorbed serum regained the ability to react. They supposed that during absorption some globulin became detached from the red cells and formed a complex with antibody and that the dissociation of this complex on storage liberated antibody.

Use of enzyme-treated cells for absorption. Sometimes advantage can be taken of the fact that some antigens are destroyed by enzymes. For example Eaton *et al.* (1956) observed that the antigen Yt^a was destroyed by enzymes; they used enzyme-treated AB Yt(a+) cells to remove anti-A and anti-B, but not anti-Yt^a, from a serum containing all three antibodies.

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Neutralization

Occasionally it is difficult to find appropriate red cells for removing anti-A or anti-B; for example, suppose a serum contains anti-B and anti-c, and one would like to remove anti-B. The red cells needed are those of group B, anti-c negative (e.g. CCDee). If a donor with these groups is not readily available it is possible to use the serum for testing all ABO groups by first 'neutralizing' the anti-B with B substance. As a source of B substance one can use either the saliva of a group B secretor or animal B substance prepared from horse stomach (available commercially from Sharp & Dohme). This is fairly satisfactory if the neutralized serum is to be used for agglutination tests, but if the anti-c is 'incomplete' and the serum is to be used for indirect antiglobulin tests, it may prove difficult to add enough B substance to abolish completely the indirect antiglobulin reaction with group B cells; this will be particularly likely if there is much incomplete anti-B in the original serum.

Elution

Principle. A 'wanted' antibody is removed from a serum by adsorption on to suitable cells, which are then separated from the serum. The cells, still 'holding' the wanted antibody, are washed free from serum and are then made to 'give up' the antibody into saline.

For example, to obtain anti-Rh from a serum containing anti-Rh and anti-A, the method described below could be used. (This example is chosen simply for ease of illustration. In practice, absorption is the method of choice for isolating anti-Rh from anti-A. An example of a circumstance in which elution was the appropriate method of isolating an antibody is given later in this Chapter).

Technique (after Landsteiner and Miller, 1925). Add 5 ml. of serum to 1 ml. of packed, thrice-washed, O Rh-positive cells and leave for 1 hour at 37° C. (In Landsteiner and Miller's original method, 5 ml. of serum was added to five drops of packed cells; room temperature rather than 37° C. was used because the method was described for the elution of anti-A and anti-B.) After incubation, centrifuge the mixture and discard the supernatant. Wash the cells three times in saline at room temperature.

Now add 1 ml. of saline to the packed sensitized cells (the smaller the volume added, the 'stronger' will be the eluate; Landsteiner and Miller used a volume of 0.6 ml.).

Place the mixture in a water-bath at 56° C. for 5 minutes, shaking repeatedly. Have ready: water at 56° C.; place the tube containing the cells in a centrifuge bucket containing this water. Spin rapidly (3000 rev./min. for 2 or 3 minutes) and immediately remove the supernatant saline, which will appear cherry red due to partial haemolysis of the cells during heating. This supernatant fluid should contain anti-Rh but not anti-A.

Since antibodies deteriorate rapidly in a medium of saline, the antibody can instead be eluted into group AB serum instead of saline in the last stage of the process, i.e. immediately before placing the cells in a water-bath at 56° C.

An example of a circumstance in which elution was very helpful will serve to illustrate the value of the method:

> The original Duffy serum contained four antibodies, anti-A, anti-B, anti-Rh and the antibody under investigation, later called anti-Fy^a. In order to remove anti-A, anti-B and anti-Rh, it was necessary to find a blood donor of group AB, Rh-positive who was also 'Duffy negative'. This could not be done directly, for the serum reacted with all Rh-positive bloods.
>
> Accordingly a sample of O, Rh-negative, 'Duffy positive' blood was selected by simply testing the serum against a panel of O, Rh-negative bloods. The sample of O, Rh-negative, Duffy positive blood was now mixed with the original serum and, by the process already described, the Duffy antibody was eluted from the cells and thus isolated.
>
> The eluate was now used for testing AB, Rh-positive bloods, and a Duffy negative sample was selected which could be used for absorption.

Elution should be used only in special circumstances, since it is difficult to avoid considerable loss of potency in the process. Absorption should be the standard method of isolating antibodies.

Other methods of elution. The simple method described above often fails to produce a satisfactory eluate from the red cells of patients with acquired haemolytic anaemia. In such cases methods which involve elution from red cell stroma are more successful as Kidd (1949) first showed.

The following method (Weiner, 1957) for eluting antibodies from stroma has proved very satisfactory.

Wash sensitized red cells and pack tightly. Lyse by freezing and thawing. Add ten volumes of 50 per cent ethyl alcohol (pre-cooled to −6° C.). Mix well, then leave at −20° C. for about 1 hour. Spin at 3000 rev./min. for 5 minutes and remove the supernatant. Break up the sediment well and wash once in an excess of distilled water. Add to the sediment a volume of saline equal to the volume of eluate required. If the cells were strongly sensitized add two to three volumes of saline; if the cells were weakly

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sensitized, add half or quarter volume of saline. Leave at 37° C. for half an hour. Centrifuge. Take off the supernatant which is the eluate.

Jensen (1959) compared the above two methods of elution with each other and with five other methods. He concluded that of the seven the method of Landsteiner and Miller was the quickest (15 minutes) and gave relatively good eluates; Weiner's method took 3–4 hours but gave stronger eluates. None of the other methods tested were as satisfactory.

Elution of Complement-binding Antibodies

Complement-binding antibodies may be difficult to elute if they are adsorbed on to red cells in the presence of complement. It may then be preferable to sensitize red cells with EDTA-treated serum as a preliminary to recovering the antibody by elution. For example, eluates of anti-Le^a made from EDTA-treated serum are much more potent than those made from cells sensitized with untreated, fresh serum (Polley and Mollison, 1961; see also Stratton, 1959).

Preparation of Concentrated Eluates

Culp and Chaplin (1960) prepared concentrated eluates of incomplete anti-D by first sensitizing D+ cells with an equal volume of potent anti-D, then preparing stroma by a modification of Ponder's method (1948), eluting from the stroma at 56° C., dialysing the eluates against distilled water, lyophilizing the eluate, and finally resuspending the residue in a small volume of saline.

A convenient method of concentrating eluates is to dialyse them against 'carbowax' (obtainable from Union Carbide Ltd, London).

Identification of Antibodies

When a sample of red cells is found to be incompatible with a patient's serum during cross-matching, a choice must be made between two courses of action. Either the red cells of other donors may be tested, and samples which fail to react may be regarded as compatible and released for transfusion, or the antibody may first be identified, when blood of theoretically compatible group can be selected for cross-matching. There can be no doubt that the second course is by far the safest and should be followed wherever possible. If the antibody is first identified there will be a double check on each donor. For example, suppose the antibody is found to be anti-c. Then c-negative blood can be obtained either from a transfusion centre or by testing any available units with known anti-c serum. This blood can then be matched directly against the patient's serum. The

identification of antibodies is greatly simplified by knowing the full blood groups of the patient. Thus if the patient's groups are O, CcDEe, MN, S, P_1, Lu(a+), K—, Le(a—b—), Fy(a—) and Jk(a+), the only likely blood group antibodies in the serum are anti-K, anti-Le^a, anti-Le^b and anti-Fy^a.

The patient's serum should be tested against a panel of cell samples whose full blood groups are known. Tests with a few carefully selected blood samples will often enable one to make a good guess at the identity of the antibody. For example, tests with the panel shown in Table XLI will separate the reactions due to the commonest antibodies. Thus, anti-Le^a would react only with sample number 3, anti-K only with sample number 2, and anti-c would react with all samples except number 1.

Tests with a few well-selected blood samples serve to suggest the identity of a blood group antibody but are seldom sufficient for proof. Thus if the antibody appears to be anti-K, because it reacts with the only

TABLE XLI

PHENOTYPES OF SIX BLOOD SAMPLES WHICH WOULD MAKE A USEFUL 'PANEL' FOR THE IDENTIFICATION OF BLOOD GROUP ANTIBODIES

Of these six donors only one (number 6 of the probable Rh genotype *cDE/cDE*) is rare

1.	O	CCDee	MMS	P+	Lu(a+)	K—	Le(a—b+)	Fy(a+b+)	Jk(a+b+
2.	O	CcDee	MNss	P+	Lu(a—)	K+	Le(a—b+)	Fy(a—b+)	Jk(a+b—)
3.	O	ccddee	MMS	P+	Lu(a—)	K—	Le(a+b—)	Fy(a+b+)	Jk(a+b—)
4.	O	ccDEe	NNss	P+	Lu(a—)	K—	Le(a—b+)	Fy(a+b—)	Jk(a—b+)
5.	O	ccddee	MNS	P—	Lu(a—)	K—	Le(a—b+)	Fy(a—b+)	Jk(a—b+)
6.	O	ccDEE	MMss	P+	Lu(a—)	K—	Le(a—b+)	Fy(a+b+)	Jk(a+b+)

K-positive sample in the panel, tests should be made with several known K+ and K— samples (three of one and four of the other) to establish its identity beyond reasonable doubt.

Misleading conclusions are sometimes reached because an antibody reacts only with homozygous samples or only with strongly reacting samples. For example a serum might react only with samples 2 and 3 shown in Table XLI and appear at first sight to be a 'new' antibody; further tests would show that it reacted only with Jk^aJk^a samples and not with Jk^aJk^b or Jk^bJk^b samples.

Inhibition by group specific substances. In identifying antibodies it is sometimes helpful to demonstrate that they are specifically inhibited by a group specific substance. In practice because anti-P_1 is such a common antibody a neutralization test with hydatid cyst substance is often valuable. As Table XLII shows, anti-P_1 serum is inhibited by adding an equal volume of a 1 in 1000 dilution of a preparation of hydatid cyst fluid.

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As mentioned earlier, some anti-P_1 sera agglutinate P_1-negative as well as P_1-positive red cells at temperatures up to 18-20° C. These reactions are not neutralized by hydatid-cyst substance. The best way of identifying anti-P_1 in such sera is to use the highest temperature at which the serum reacts – usually 25-30° C.; the serum then usually agglutinates only P-positive red cells and these reactions are inhibited by hydatid substance.

Hydatid substance does not inhibit the reactions of other common cold agglutinins such as anti-M, anti-Le^a, anti-Le^b and anti-H(O).

There is usually no difficulty in identifying Lewis antibodies but it may sometimes be useful in sera containing other antibodies in addition, to neutralize the Lewis antibodies by adding suitable saliva.

The antibodies anti-H and anti-O are readily identified by their positive reactions with all group O red cells and weak or negative reactions with

TABLE XLII

SPECIFIC INHIBITION OF ANTI-P_1 BY HYDATID CYST FLUID

	Dilutions of hydatid cyst fluid substance (reciprocals)						
	5	50	500	1000	2000	5000	Control
Anti-P_1	–	–	–	–	(+)	++	++
Anti-I	(+)*	++	++	++	++	++	++

* Some anti-I sera are inhibited by a 1 in 5 dilution of hydatid substance; others are not inhibited at all.

A_1 red cells. Addition of 'secretor' saliva to the serum inhibits the reactions of anti-H but not of anti-O (see Table XX). In the author's experience agglutinins capable of producing strong agglutination of group O (but not group A_1) red cells at room temperature are usually not inhibited by the addition of 'secretor' saliva.

Characteristics of Antibodies as an Aid to Identification.

Often the behaviour of an antibody *in vitro* provides valuable clues to its identity. Thus if a serum haemolyses red cells at 37° C. the reaction may be due to anti-Le^a but cannot be due to any antibody of the Rh system. If a serum agglutinates a saline suspension of red cells at room temperature but not at 37° C., the antibody may well be anti-P_1 and is most unlikely to be anti-Rh, anti-Fy^a or anti-K. An antibody which can be detected only by the indirect antiglobulin test is likely to be anti-Rh but may be anti-K, anti-Fy^a or anti-Jk^a. An antibody which reacts with cells, using

the indirect antiglobulin test, but does not agglutinate enzyme-treated cells, may well be anti-Fy^a.

It is sometimes very helpful to add gamma globulin to the antiglobulin serum in order to distinguish antibodies which bind complement from those which do not. Thus red cells sensitized with anti-Jk^a will, because

TABLE XLIII

INDIRECT ANTIGLOBULIN TESTS WITH A SERUM CONTAINING ANTI-c AND ANTI-JK^a

The reactions of the two antibodies can be distinguished thus: (1) those due to anti-Jk^a are given only by fresh and not by heated serum and red cells sensitized with anti-Jk^a, because they have also adsorbed complement, react with antiglobulin serum to which gamma globulin has been added; (2) those due to anti-c are given by heated and by fresh serum and the reactions are abolished by adding gamma globulin to the antiglobulin serum

Test cells	*Patient's serum*	*Reactions with antiglobulin serum*	
		alone	*+gamma globulin*
c+Jk(a+)	Fresh	+	+
	Heated	+	–
c+Jk(a–)	Fresh	+	–
	Heated	+	–
c–Jk(a+)	Fresh	+	+
	Heated	–	–

they have adsorbed complement (beta$_1$ globulin) react with an antiglobulin serum to which gamma globulin has been added. On the other hand red cells sensitized with anti-c, because they have only adsorbed gamma globulin, will not react with an antiglobulin serum to which gamma globulin has been added. Table XLIII shows tests made in a case in which a patient's serum contained these two antibodies.

DETECTION OF WHITE CELL ANTIBODIES

The simplest method is a test for leuco-agglutinins (Dausset *et al.*, 1954). Freshly drawn blood is defibrinated in an Erlenmeyer flask by agitating about 30 ml. with ten glass beads for 10 to 15 minutes; the defibrinated blood is mixed with one-fifth its volume of 3.5 per cent PVP (or Dextran; see Goudsmit and Van Loghem, 1953a). The mixture is then left at 37° C. in an inclined tube for 30 minutes; the pink supernatant is pipetted off and should contain 3000 to 5000 white cells per c.mm. Now 0.1 ml. of the

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patient's serum previously inactivated at 56° C. for 30 minutes is mixed with 0.05 ml. of white cell suspension. Further tests are set up with a 1 in 2 and 1 in 4 dilution of the patient's serum in saline. The mixtures are left for 90 minutes at 37° C.; then 0.1 ml. of 1 per cent acetic acid is added to haemolyse the red cells and a thick drop of the mixture is examined under the low power of a microscope. It is advisable to keep sera sterile since infected serum may give false positive results (Goudsmit and van Loghem, 1953b).

The one serious disadvantage of the test for leuco-agglutinins is that the cells must be fresh. Conversely the one real attraction of the mixed antiglobulin technique described by Chalmers *et al.* (1959) is that it can be used with stored white cells. In this method the white cells are first fixed in 1 per cent v/v formalin in saline at 4° C. for 18 hours and are then suspended in one volume of glycerol mixed with four volumes of buffered saline containing one in 200 normal rabbit serum. The white cells are then frozen and stored at −79° C. When needed, they are thawed and incubated with the serum suspected of containing white cell antibodies. The white cells are then washed and mixed with antiglobulin serum and with washed Rh-sensitized red cells – the 'detector' cells. Mixed agglutination of white cells and red cells, identified under phase-contrast, indicates that the white cells have taken up an antibody from the patient's serum.

According to van Loghem, van der Hart *et al.* (1958) incomplete white-cell antibodies can also be detected by the antiglobulin consumption test, which they performed as follows:

> 0.1 ml. of a three-times washed sediment of white cells (from dextran-sedimented blood) is incubated for 1 hour at 37° C. with 0.15 volumes of the serum under investigation, which has previously been heated to 56° C. for 30 minutes. The white cells are then washed twelve times in large volumes of saline. The washed, packed white cells are now incubated with an equal volume of antiglobulin serum. The titre of the antiglobulin serum, before and after absorption, is determined by testing with Rh-sensitized red cells. Using this technique, incomplete white cell antibodies were found three times more frequently than leucocyte agglutinins.

CHAPTER THIRTEEN

INCOMPATIBILITY: OR SHORTENING OF THE LIFE-SPAN OF RED CELLS BY ISO-ANTIBODIES

Transfused red cells are regarded as incompatible if their survival in the recipient's circulation is curtailed by antibodies. Several kinds of incompatibility have to be considered; in the most straightforward cases, the red cells undergo immediate destruction because they encounter incompatible antibodies; in other cases the red cells may be compatible at the time of transfusion and at first survive normally but may then provoke the formation of iso-antibodies which bring about their destruction.

In addition to those cases in which the antibody responsible for the rapid destruction can be readily detected and identified, there are cases in which the red cells may be removed from the circulation at an abnormal rate although no antibody can be detected. As described below, there are strong grounds for believing that this phenomenon has an immunological basis.

Transfused plasma may be regarded as incompatible if, by virtue of its content of iso-antibodies, it is capable of destroying the recipient's own red cells.

METHODS OF STUDYING THE EFFECTS OF INCOMPATIBILITY ON RED CELL SURVIVAL

A. *Observations after Transfusions of Incompatible Red Cells*

Knowledge of the effects of transfusing relatively large volumes of incompatible blood to human subjects is derived from accidents, and is necessarily incomplete. Thus information about the exact amount of blood transfused, the characteristics of the antibody concerned, and of the rate of elimination of the transfused red cells is often lacking because the value of measuring these variables is not realized until it is known that a mistake has been made and it is then usually too late to collect all the information. The practice of collecting a sample of serum from the patient immediately before every transfusion and of keeping this for at least 1 week provides one means of always having part of the information required.

Exact measurement of the rate of elimination of red cells after an incompatible transfusion is only occasionally possible. For example, if

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Rh-positive red cells have been inadvertently transfused to a subject whose serum contains Rh antibodies and if by chance the donor is type N and the recipient type M (or MN), quantatitive estimates of the survival of the Rh+ cells can be made using the technique of differential agglutination with an anti-M serum. If donor and recipient belong to the same MN type, it will still be possible to make qualitative estimates of the survival of Rh-positive red cells either by tests with an anti-Rh serum or by the direct antiglobulin test since any surviving Rh+ cells will be 'coated'.

Although knowledge gained from incompatible transfusions provides an incomplete picture of the exact quantitative aspects of the subject, it provides an ample account of the clinical effects of transfusing incompatible blood and these effects are considered at the end of the chapter.

Biological tests. This term is associated particularly with the test introduced by Oehlecker (1940) which consisted of observing the reaction of a conscious patient to the relatively rapid injection of up to 50 ml. of blood. The test is considered further in the following chapter, as is the test introduced by Wiener *et al.* (1942) which was also designed to detect gross signs of red cell destruction, rather than to measure precisely the rate of elimination of injected red cells.

The most satisfactory way of assessing the survival of injected red cells is to make direct measurements. Until convenient methods of labelling with radioactive isotopes were introduced, the only method available was that of differential agglutination. As discussed in Chapter V, relatively large volumes of red cells (at least 50 ml. of blood) must be injected before quantitative estimates can be obtained and the injection of such quantities is likely to provoke unpleasant and even dangerous reactions. Therefore, the introduction of a method which permits the satisfactory labelling of amounts of less than 1 ml. was a great advance, and its application during the last few years has considerably increased our knowledge of the effect of blood group antibodies *in vivo*.

B. *Observations after Transfusions of Plasma containing Incompatible Antibodies*

In the past a great many transfusions of group O blood have been given to recipients of other groups and many haemolytic reactions attributable to potent anti-A or anti-B in the donor's plasma have been described. This information has been supplemented by several studies in which group O plasma has been deliberately transfused to volunteers of other groups; see pp. 486-488.

C. *Deliberate Injections of very small Volumes of Incompatible Red Cells*

The injection of 1.0 ml. or less of incompatible red cells does not as a rule cause any symptoms whatever. The only exception encountered by the present author was a case in which the recipient had a very potent haemolytic antibody; further details are given on p. 497.

Apart from its virtual freedom from causing reactions, the injection of a very small volume of incompatible red cells has theoretical advantages in demonstrating the effects of different antibodies. For example, there can be no question of altering the titre of antibody in the circulation by injecting so small an amount nor can there be any question of overloading the reticulo-endothelial system. When only 1 ml. of incompatible red cells is injected the effect of the following variables can be assessed:

(*a*) The 'grade' of antigen in the red cells (e.g. A_1 or A_2).
(*b*) The characteristics of the antibody (e.g. agglutinating or haemolysing).
(*c*) The potency of the antibody.

In theory, there are other variables to take into account, such as the amount of complement in the serum, although this is unlikely to be of any importance in practice.

The most satisfactory label is ^{51}Cr. A dose of 50 μc. is ample and this will be equivalent to 0.5-5 μg. Cr, depending on the specific activity of the material. For following the survival of red cells over a few hours it is probably entirely satisfactory to use doses up to 20 μg. Cr per ml. of red cells; taking this latter figure and assuming a specific activity of only 10 μc. per μg. Cr, the volume of red cells to be labelled would be $\frac{50}{10} \div 20 =$ 0.25 ml. With higher specific activity ^{51}Cr this volume could be considerably reduced.

Except that the volume of red cells is kept to less than 1 ml., the technique of labelling, washing and injecting the cells is exactly the same as that already described in Chapters II and V. Because destruction of the injected red cells may begin within 2 or 3 minutes of injection, that is to say, within the mixing time, the patient's red cell volume must be estimated if quantitative estimates of survival are to be obtained. This is most conveniently done by labelling a sample (2-3 ml.) of the subject's own red cells with ^{32}P and then injecting them as a mixed suspension with the ^{51}Cr-labelled cells.

To define the pattern of elimination of the injected cells, several blood samples must be taken during the hour following injection. For most purposes, it is sufficient to take three blood samples; suggested times are

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6 minutes, 12 minutes and 25 minutes after the injection. All three samples can be used for ^{32}P counts to estimate red cell volume and they will also, from the ^{51}Cr counts, give a good idea of the pattern of any red cell destruction which occurs. When the patient's red cell volume is known, ^{32}P can be used as a second label for incompatible cells but is suitable only for short periods of observation (1 hour); corrections to apply for ^{32}P loss are given in Chapter II.

(Results obtained with the method will be found below, chiefly on pp. 454-466.)

D. *Injections of Agglutinated Red Cells*

A few experiments of this kind have been reported and are described below in a separate section (see p. 488). Their main value has been to illustrate the reversibility of agglutination and thus to explain the relative harmlessness of agglutinating antibodies in transfused plasma.

E. *Injections of Red Cells Sensitized with Incomplete Antibodies*

The experimental injection of 'sensitized' red cells, that is, of red cells which have adsorbed antibody but have not formed agglutinates, provides a means of studying the relationship between the degree of sensitization and the rate of red cell destruction and also of studying the difference between the effect of complement-binding and non-complement-binding incompatible antibodies. The results are considered below in the section on the effects of the potency and other characteristics of antibodies (pp. 466-473).

F. *Experiments in Animals*

Experiments in animals have contributed much to what is known about the untoward effects of transfusing large amounts of incompatible blood, and are referred to below in considering the relationship between incompatible transfusions and the production of anuria.

The detailed studies of cross-transfusion in dogs, by Dr L. E. Young and his colleagues in Rochester, New York, have also helped in the understanding of many aspects of incompatible transfusion – for example, by demonstrating the absorption of all circulating antibodies by a large transfusion of incompatible red cells or the destruction of the recipient's red cells by a transfusion of incompatible plasma. These results are referred to in the appropriate sections below.

It must also be remembered that the first transfusions given to human subjects were of blood from animal donors (e.g. Denis, 1667) and that the demonstration by Blundell in 1824 that transfusion of the blood of one species to a recipient of another was always unsuccessful was a very important step.

Ponfick (1875) provided extensive confirmation of Blundell's findings; he showed that when donor and recipient belonged to the same species, as much as 60 ml./kg. of blood could safely be transfused but that when they belonged to different species the transfusion of as little as 4 ml./kg. might be fatal. He also found that animals transfused with the blood of another species developed haemoglobinuria, not haematuria as had previously been supposed. He further showed that if blood was haemolysed *in vitro* by freezing and thawing and then injected into an animal of the same species, haemoglobinuria could also be produced. Moreover, the amount of lysed blood which would produce haemoglobinuria (about 1.2 ml./kg. of the recipient's body weight) was almost exactly the same as the amount of intact blood of a foreign species which would produce haemoglobinuria.

Ponfick's observations showed clearly enough that transfused red cells of a foreign species were lysed *in vivo*. At about the same time, Landois (1875) showed that if the red cells of one animal were mixed with the serum of another animal *in vitro* and incubated at 37° C. they might be lysed within 2 minutes.

TYPES OF RED CELL DESTRUCTION OBSERVED

Predominantly Intravascular Destruction

If a very small volume of group A red cells is injected into a subject whose serum contains anti-A with haemolytic activity *in vitro*, 40-90 per cent of the red cells are promptly haemolysed within the blood stream. If the red cells have been labelled with ^{51}Cr almost all the ^{51}Cr will be found in the plasma, since ^{51}Cr is bound to haemoglobin and dissociates from it relatively slowly. Thus for about 1 hour after injection the amount of ^{51}Cr in the plasma indicates the degree of haemoglobinaemia (Jandl *et al.*, 1957; Mollison, 1959d). Thereafter the level of ^{51}Cr declines more slowly than the level of Hb (Jandl *et al.*, 1957).

Provided that red cells and plasma are counted separately, the occurrence of intravascular lysis will be easily recognized but if whole blood samples are counted on the assumption that the radioactivity is all contained within red cells the wrong interpretation may easily be reached.

Fig. 59 shows some estimates (top three curves) of the amount of ^{51}Cr found in the plasma after injecting ^{51}Cr-labelled red cells of incompatible ABO group.

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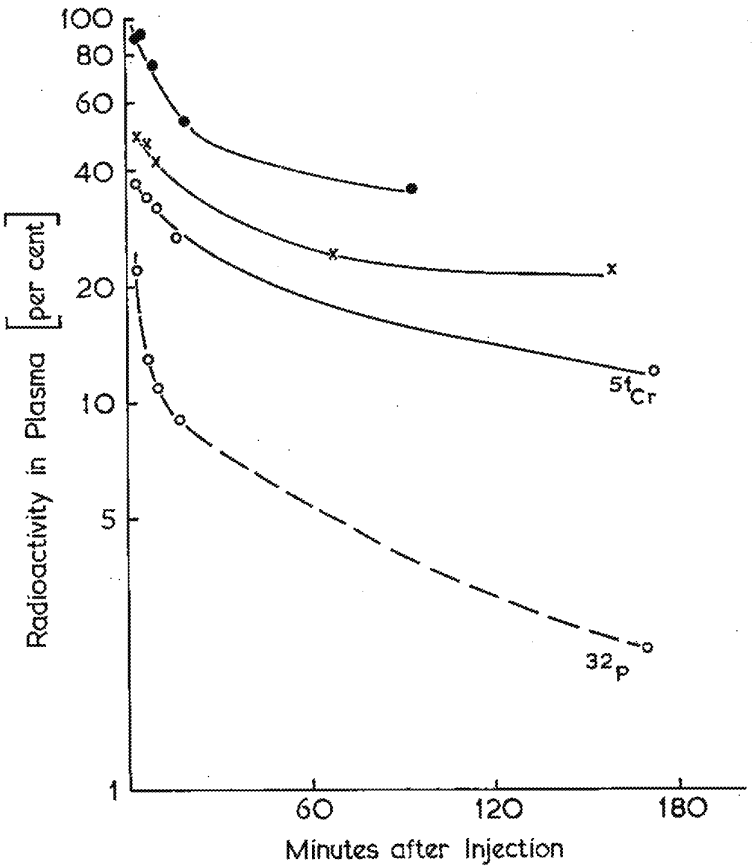

FIGURE 59. Radioactivity in the plasma, as a percentage of the amount injected as labelled red cells, following the injection of ABO-incompatible cells.

●—● ^{51}Cr-labelled group B red cells injected into a group A recipient whose serum had an anti-B haemolysin.

×—× ^{51}Cr-labelled A_1 red cells injected into a group O recipient whose serum contained anti-A agglutinin (titre 32) but no readily demonstrable haemolysin.

○—○ ^{51}Cr-labelled A_2 red cells injected into the same group O recipient as above, at a time when the agglutinin titre against A_2 cells was 64.

○---○ ^{32}P-labelled A_2 red cells injected into the same recipient and at the same time as the ^{51}Cr-labelled A_2 red cells.

(From Cutbush and Mollison, 1958.)

Three minutes after injection the amount of ^{51}Cr in the plasma varied from 40 to 90 per cent of the amount that would have been present if all the injected ^{51}Cr had immediately been liberated from the red cells into the plasma. At this time the amount of ^{51}Cr in the red cells was only 1-3 per cent of the amount injected. If whole blood samples had been counted instead of counting plasma and red cells separately, and the assumption had been made that all ^{51}Cr present was contained in surviving red cells, it would have been concluded that between 40 and 90 per cent of the injected red cells were still present in the circulation 3 minutes after injection, and that thereafter they were cleared from the circulation relatively slowly.

Under these circumstances, for the first 60 minutes or so after injection the amount of ^{51}Cr in the plasma gives a good estimate of the amount of haemoglobin which is present in the plasma because the ^{51}Cr is initially bound to the haemoglobin and dissociates it from it relatively slowly (Jandl *et al.*, 1957; Mollison, 1959d).

Fig. 59 also shows the results of using ^{32}P as a red cell label when there is intravascular lysis. The ^{32}P liberated from the lysed red cells is removed from the circulation far more rapidly because, unlike the ^{51}Cr, it is not bound to protein.

Extravascular Destruction

Most blood group antibodies bring about the removal of intact red cells from the blood stream rather than causing intravascular lysis. In man the chief sites of destruction are the liver and spleen; by using the technique of surface counting the relative amounts removed in these two organs can be ascertained, although the method gives only semi-quantitative results (see Jandl *et al.*, 1957; Mollison and Hughes Jones, 1958).

When destruction occurs predominantly in any one organ, the rate of removal of red cells from the circulation is limited by the rate of blood flow through that organ. For example, if the organ is the liver, the clearance of half the red cells from the circulation will require not less than about 2 minutes, and if it is the spleen, not less than 15-25 minutes. Further information is contained in the section below on the effect of the quality of the antibody on red cell destruction.

When incompatible red cells are injected, it is not uncommon to find that only some of the cells are removed rapidly, the rest surviving for very long periods and even surviving normally; Fig. 60 shows examples in which the antibodies concerned were anti-A_1, anti-P_1 and anti-Lu^b. This phenomenon is discussed in a later section.

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FACTORS AFFECTING THE DESTRUCTION OF INCOMPATIBLE RED CELLS

Four main factors affect the rate of destruction of transfused red cells by iso-antibodies:

(*a*) the quantity of red cells transfused;
(*b*) the 'grade' of the antigen;
(*c*) the amount (titre) of antibody in the circulation;
(*d*) the characteristics of the antibody.

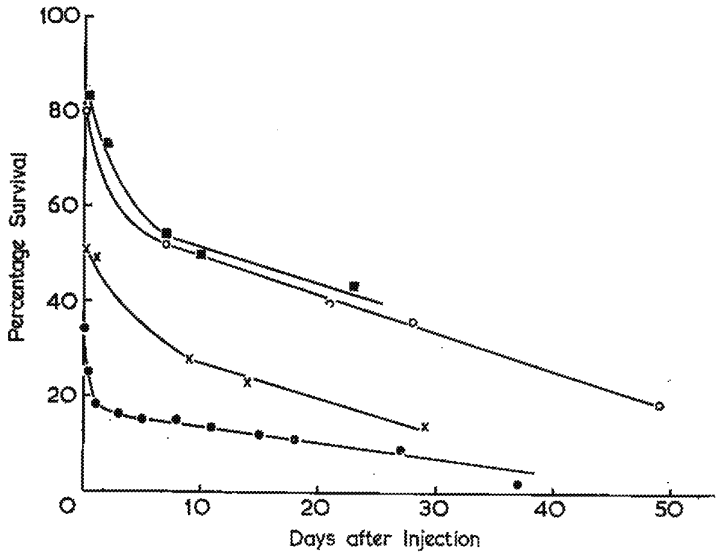

FIGURE 60. Prolonged survival of a proportion of incompatible red cells.
■—■ A_1 red cells in an A_3B recipient.
○—○ Lu^aLu^b red cells } in an Lu^aLu^a recipient.
●—● Lu^bLu^b red cells }
×—× P+ red cells in a P-recipient.
In all three cases the relevant antibody in the recipient's serum was only very weakly active at 37° C. *in vitro* (from Cutbush and Mollison, 1958).

The Quantity of Red Cells Transfused

Absorption of Antibody

If a sufficient volume of red cells is transfused, the antibody in the recipient's serum may for the time being be completely absorbed. When this happens red cell destruction is virtually arrested until there has been

time for new antibody to be formed. Agglutinating antibodies are more easily absorbed than incomplete antibodies. Table XLIV shows the results of some absorption experiments *in vitro*. When sera containing agglutinating antibodies of moderate potency (examples of anti-A and anti-B), were absorbed with one-tenth of their volume of cells, there was

TABLE XLIV

ABSORPTION EXPERIMENTS *in vitro*

Examples 1 to 3: anti-A and anti-B tested for their ability to agglutinate red cells suspended in saline. Examples 4 to 6: anti-Rh tested by the indirect antiglobulin test.

Example no.	*Dilution of serum (reciprocal)*						*Score*
	1	4	16	64	256	1024	
1. Anti-A (unabsorbed)	+ + + +	+ + +	+ + +	+ +	+	—	34
Absorbed with 0.1 vol. cells	+ + + +	+ + +	+	—	—	—	21
Absorbed with 0.2 vol. cells	+ + +	+ + +	(+)	—	—	—	17
Absorbed with 0.5 vol. cells	+ + +	+	—	—	—	—	11
2. Anti-B (unabsorbed)	+ + + +	+ + +	+ + +	+ +	(+)	—	32
Absorbed with 0.1 vol. cells	+ + +	(+)	—	—	—	—	9
Absorbed with 0.2 vol. cells	+ + +	(+)	—	—	—	—	9
Absorbed with 0.5 vol. cells	+ +	—	—	—	—	—	5
3. Anti-A (unabsorbed)	+ + + +	+ + +	+ +	+	—	—	26
Absorbed with 0.1 vol. cells	+ +	—	—	—	—	—	5
Absorbed with 0.2 vol. cells	+	—	—	—	—	—	3
Absorbed with 0.5 vol. cells	(+)	—	—	—	—	—	1
4. Anti-Rh (unabsorbed)	+ + +	+ + +	+ +	+	+	—	27
Absorbed with 0.1 vol. cells	+ + +	+ + +	+ +	+	+	—	27
Absorbed with 0.2 vol. cells	+ + +	+ + +	+ +	+	(+)	—	25
Absorbed with 0.5 vol. cells	+ + +	+ +	+	+	—	—	19
5. Anti-Rh (unabsorbed)	+ +	+	+	—	—	—	11
Absorbed with 0.1 vol. cells	+	+	(+)	—	—	—	7
Absorbed with 0.2 vol. cells	+	(+)	—	—	—	—	4
Absorbed with 0.5 vol. cells	(+)	—	—	—	—	—	1
6. Anti-Rh (unabsorbed)	+	(+)	—	—	—	—	4
Absorbed with 0.1 vol. cells	(+)	—	—	—	—	—	1
Absorbed with 0.2 vol. cells	—	—	—	—	—	—	0
Absorbed with 0.5 vol. cells	—	—	—	—	—	—	0

a considerable reduction in titre. On the other hand, when serum containing an incomplete antibody of comparable potency (the first example of anti-Rh in Table XLIV) was absorbed with one-tenth of its volume of cells, there was no observable fall in titre. Absorptions of the same sera with half of their volume of packed cells produced a distinctly greater

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effect on the agglutinating anti-A and anti-B than on the incomplete anti-Rh.

Table XLIV also shows that when the antibody was relatively weak

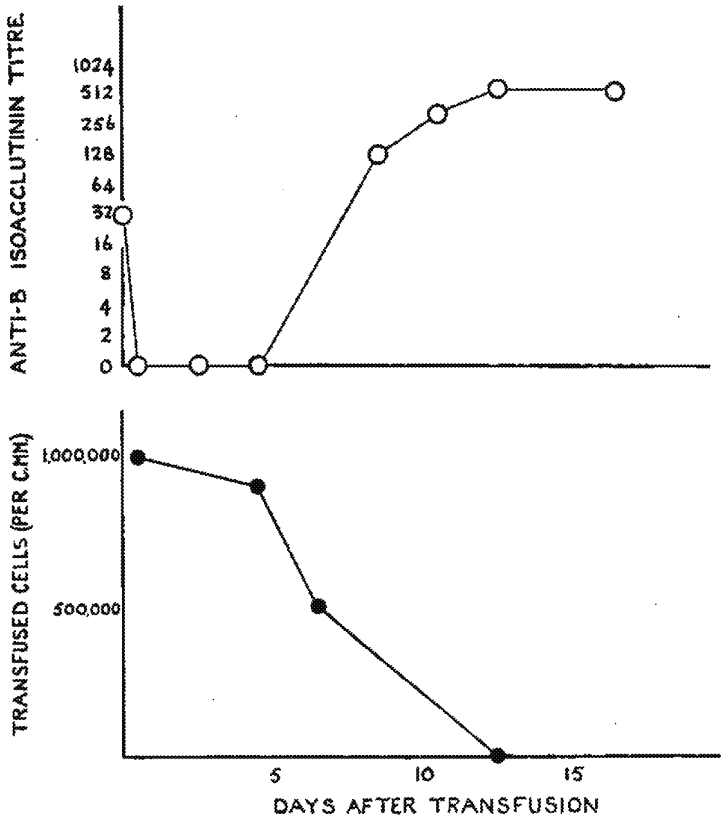

FIGURE 61. Transfusion of 1000 ml. of citrated group B blood to a group A patient. For 4 days after transfusion the B cells survived well and anti-B could not be detected in the patient's serum. The anti-B then reappeared and rapidly increased in titre; between 6 and 8 days after transfusion the B cells were rapidly eliminated and the patient became slightly jaundiced. (This diagram was made by Mr R. E. Davis working in Professor J. V. Dacie's laboratory. I am indebted to them for permission to publish their findings.)

(examples 5 and 6) it was sometimes completely absorbed even by one-fifth of its volume of red cells.

Examples of absorption of antibody *in vivo* are shown in Figs. 61 and 62. Thus, Fig. 61 shows an example of a case in which the transfusion of

1000 ml. of group B blood to a group A patient, with an anti-B iso-agglutinin titre of 32, led to the complete absorption of anti-B for a period of about 5 days after the transfusion.

Fig. 62 shows the complete absorption of a weak incomplete Rh antibody by a transfusion of only 500 ml. of Rh-positive blood. Three days after transfusion some of the transfused Rh-positive red cells were still present; thus when a sample of red cells was taken from the recipient and

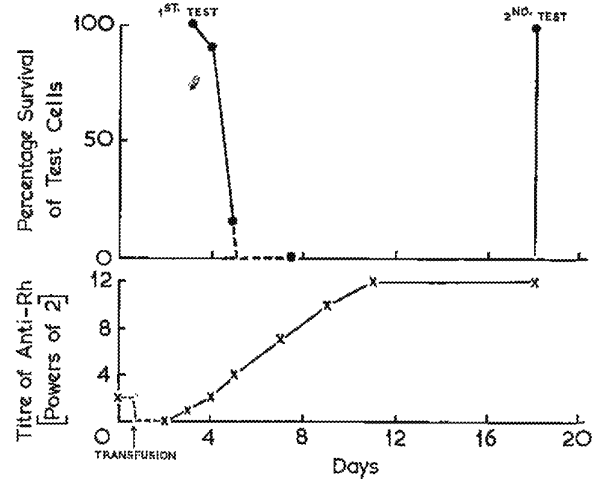

FIGURE 62. Changes in the titre of anti-Rh and in the survival of labelled Rh-positive red cells during the 18 days following a transfusion of Rh-positive blood. The pre-transfusion sample had a titre of only 2 and for 2 days following the transfusion anti-Rh could not be demonstrated in the recipient's serum. Three days after transfusion, when the anti-Rh titre was 1, a small volume of ^{51}Cr-labelled red cells was injected. Only 10 per cent of the labelled cells were removed from the circulation during the first 24 hours after injection but during the 24 hours after that almost all of them were destroyed. As the figure shows, at this time the titre of anti-Rh was beginning to rise rapidly. Eighteen days after transfusion, when the anti-Rh titre had reached 2000, a second injection of ^{51}Cr-labelled red cells was given and these red cells were eliminated rapidly from the circulation (half-time approximately 30 minutes) (from Mollison and Cutbush, 1955).

tested with an antiglobulin serum, a proportion of them were agglutinated. At this time Rh antibody had reappeared in the circulation (titre 1). A test injection of ^{51}Cr-labelled Rh-positive cells was given and there was no measurable destruction in the following 2½ hours. Next day 90 per cent of the injected cells was still present but on the following day, that is the fifth day after the original transfusion, the number had fallen to 14 per cent and 2 days after this, there were no surviving Rh-positive cells. This

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rapid decrease in the number of Rh-positive cells surviving corresponded with the rapid increase in the amount of Rh antibody in the patient's circulation; 15 days after the first test a second injection of 1 ml. of ^{51}Cr-labelled Rh-positive red cells was given; this time the titre of Rh antibody had risen to 2000. The cells were eliminated from the circulation exponentially with a half-time of about 30 minutes.

When a very large transfusion of incompatible red cells is given the antibody may all be absorbed and not re-appear in the circulation for many days after transfusion.

Mrs C. developed ante-partum haemorrhage towards the end of her sixth pregnancy. She had been transfused once before, 5 years previously, and had developed jaundice on the following day; the cause of this reaction had not been determined.

Red cells from two bottles of blood appeared to be compatible by a test in albumin and were transfused. The patient developed a rigor. The post-transfusion sample gave a positive direct antiglobulin test and further tests revealed the presence of an antibody in the patient's serum detectable only by the indirect antiglobulin test; this was identified later as anti-Fy^a. The patient had meanwhile had a very severe haemorrhage and had been diagnosed as having the syndrome of afibrinogenaemia. It became necessary to transfuse large volumes of blood and since only two out of fourteen bottles available were compatible by the indirect antiglobulin test, it was decided to give some that were incompatible. This decision seems in retrospect to have been right. After one or two incompatible bottles of blood have been given the amount of antibody remaining in the patient's serum is likely to be too small to cause rapid red cell destruction. Even if no incompatible blood had been given already, the giving of some could have been justified on the grounds that the patient was in immediate danger of dying of haemorrhage and any blood was almost certainly better than no blood. In the end fourteen bottles of incompatible blood were given. Serum taken after the transfusion of the first two bottles was examined later and found to have no free anti-Fy^a. Antibody appeared again 5 to 7 days after transfusion; during the following few days the patient's haemoglobin concentration fell and the serum bilirubin concentration reached a peak on the tenth day. Fy(a+) cells which had been present in the circulation in large numbers up to about the seventh day after transfusion, as shown by a positive direct antiglobulin test, rapidly disappeared between about the seventh and ninth days.

When the serum contains a potent incomplete antibody, the transfusion of even four bottles of blood (about 2000 ml. of citrated blood or 1600 ml. of whole blood) may fail to remove all antibody from the circulation. The following case is an example:

Mrs L. had a difficult labour, followed by a severe post-partum haemorrhage. She had been typed during pregnancy as Rh-positive and this was considered to justify the transfusion of four bottles of Rh-positive blood without matching, in view of the patient's serious condition. The patient did not respond well to transfusion, her systolic pressure remaining at approximately 70 mm. Hg. for many hours. A few hours after delivery the infant was noticed to be jaundiced and investigations were requested. A sample of venous blood was taken from the mother 12 hours after transfusion and the findings were as follows: the sample was agglutinated both by anti-Rh and anti-A sera; the appearance with an anti-Rh serum was that of small clumps of cells in a suspension of unagglutinated cells. Similarly the direct antiglobulin test showed strong sensitization of a proportion of the cells. Differential agglutination with an anti-A serum showed that there were 1,200,000 group O cells per c.mm. out of a total red cell count of 3,700,000 per c.mm. The patient's serum contained Rh antibody. It was now plain that the patient was group A, Rh-negative. The number of O, Rh-positive cells present was only about two-thirds of the number expected if all had survived normally. At 48 hours the number of surviving red cells had fallen to 480,000 per c.mm. These estimates are plotted in Fig. 63 (Case A).

In experiments in dogs, Christian *et al.* (1951) showed that the rapid destruction of red cells occurred only so long as both iso-antibody and complement were available in the recipient's plasma. It seems rather unlikely that exhaustion of complement plays any role in human haemolytic transfusion reactions. Thus if fresh human serum is absorbed with an equal volume of red cells sensitized with a complement-binding antibody it is found that about five successive absorptions are required to remove all complement activity from the fresh serum (unpublished observations). Thus complement would seem unlikely to be a limiting factor except possibly in a patient whose serum complement level was already low before transfusion.

The Saturation of the Reticulo-endothelial System

Miescher (1956a) showed that when non-viable red cells were injected into guinea-pigs the half-time of clearance from the blood stream was inversely proportional to the number of cells injected.

Halpern *et al.* (1957) showed that this was also true for the clearance in rabbits of nucleated (pigeon) red blood cells and was true for particles varying in size from 25 to 250 Å; and that when repeated doses were given the clearance diminished progressively. Similarly Francis *et al.* (1957) showed that the rate of removal of foreign protein (horse serum) injected into immunized rabbits was faster with smaller doses than with large.

In man it has also been shown that the rate of clearance of 'non-viable' stored red cells varies inversely with the dose; thus when the dose of cells

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was approximately 0.25 ml./kg. half of the cells were removed in 30 minutes, but when the dose was approximately 1.5 ml./kg., the clearance of half the cells took more than 3 hours (Noyes *et al.*, 1960).

An example of the relatively slow clearance of a large volume of

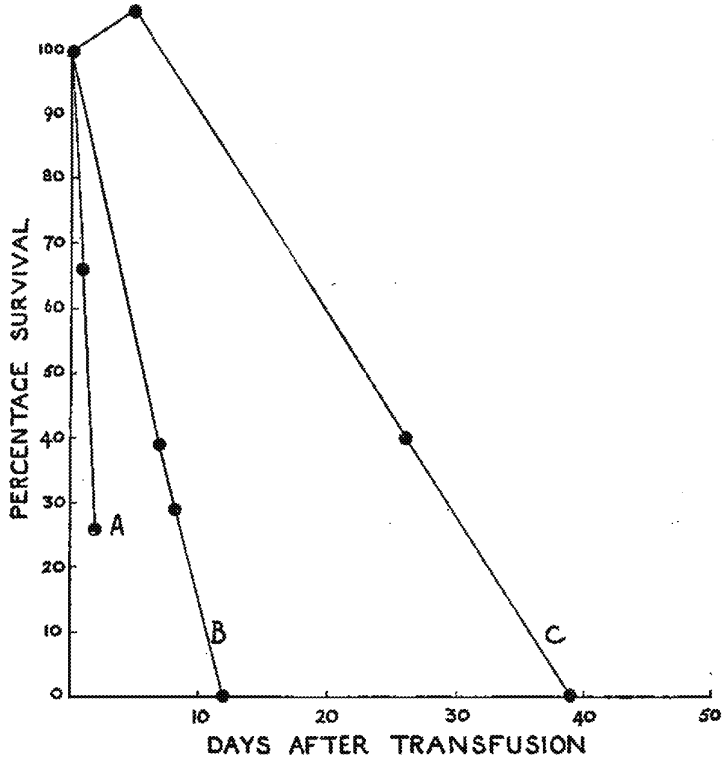

FIGURE 63. Survival of Rh-positive cells in three different Rh-negative patients.
A. Potent anti-Rh present at the time of transfusion
B. Very weak anti-Rh present at the time of transfusion
C. No anti-Rh present at time of transfusion but developing about one month after transfusion.

incompatible red cells despite the presence of a powerful antibody has been mentioned above; see case A in Fig. 63. This patient received a transfusion of four bottles of O Rh-positive blood; despite this, her serum still contained 'free' antibody capable of sensitizing cells strongly to anti-globulin serum. By the end of 48 hours after transfusion, the number of

surviving Rh-positive cells had fallen to 480,000 c.mm., so that probably almost three-quarters of the transfused red cells had been eliminated. This represents the destruction of approximately 550 ml. of red cells in 48 hours or 11 ml. of red cells per hour or very approximately 0.2 ml. of red cells per hour per kg. of bodyweight.

In the case described by Mohn *et al.* (1961) in which a relatively large volume of very potent Rh antibody was injected into an Rh-positive volunteer, the average rate of red cell destruction may be calculated to have been approximately 0.15 ml./kg./hour, although the maximum was probably about 0.25 ml./kg./hour (420 ml. of red cells destroyed in 24 hours in a man who may have weighed 70 kg.).

It may be concluded that the maximum capacity of the R.E. system for red cells in a normal adult is of the order of 300-400 ml. of red cells per day.

To speak about the maximum capacity of the reticulo-endothelial system is to over-simplify the truth. If a relatively small volume of 'non-viable' red cells (less than 0.3 ml./kg.) is injected into an animal, all the red cells will be removed within a few minutes. If a much larger dose (approximately 2 ml./kg.) is given, a proportion (very approximately half) disappear within minutes and the remainder are cleared more slowly. On this basis the maximum capacity of the R.E. system could be defined in two ways: firstly the volume of cells which it can remove immediately; secondly the volume which it can remove per hour after the initial loading dose. Even this would be far from being a complete account because, for example, the volume of non-viable red cells removed 'immediately' is greater with large doses than with small ones. Experiments with stored red cells show clearly that the capacity of the R.E. system to remove 'non-viable' red cells is limited but they also show that the limits are determined by a considerable number of variables (Hughes Jones and Mollison, 1961).

Circumstances in which a small Dose of Incompatible Red Cells is Cleared no more Rapidly than a large Dose

When the amount of red cells injected is too small to cause any important change in the titre of antibody in the recipient's circulation and when the rate of clearance is too slow to tax the capacity of the R.E. system, a large dose of incompatible red cells may be cleared as fast as a small dose. Fig. 64 shows the survival of c-positive red cells on two occasions in a patient whose serum contained incomplete anti-c. On the first occasion approximately 200 ml. of red cells were transfused. The titre of anti-c fell only slightly (from 8 to 4); half the red cells were removed in about 11 days. Five weeks after this transfusion, when the titre of anti-c was scarcely higher (8 to 16), 1 ml. of red cells was injected and the cells were

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removed from the circulation only slightly more rapidly; thus 50 per cent were removed in about 7 days. Evidently the rate of removal of the larger dose was not limited by the capacity of the R.E. system but was limited simply by the low titre of the antibody concerned. The rate of removal of the 1 ml. dose was presumably governed by the same factor.

In a patient whose serum contained anti-P_1, two doses of P-positive red cells from the same donor were given, the first of 1 ml. and, 30 minutes later, the

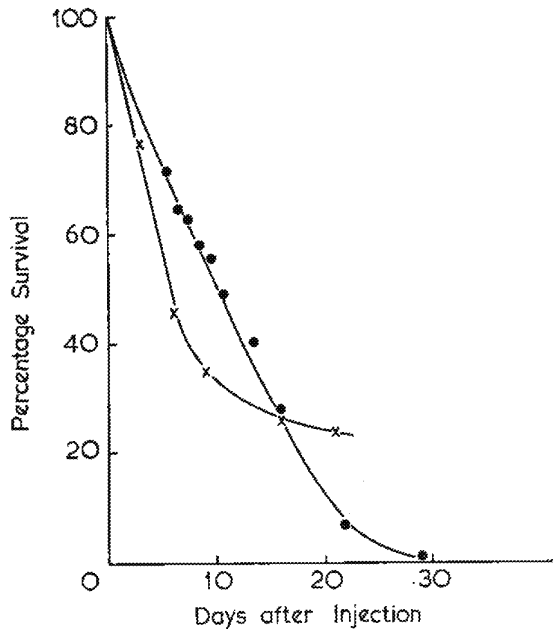

FIGURE 64. Survival of c+ red cells injected on two occasions in a patient whose serum contained anti-c. The first injection (●—●) was given a few hours after the patient had been inadvertently transfused with 500 ml. of c+ citrated blood (equivalent to 200 ml. red cells). The second injection (×—×), of 1 ml., was given 36 days after the first. For further details, see text.

second of 22 ml. In each case approximately 20 per cent of the cells were removed with a half-time of 2–4 minutes. This was not surprising since even in the case of the larger dose the absolute volume of red cells removed rapidly was only about 4 ml., that is less than 0.1 ml./kg. and, as mentioned above, it is evident that this is an amount which the R.E. system can deal with immediately.

An important conclusion about results obtained with very small doses of incompatible red cells is that the proportion of red cells destroyed is the

same as when a large volume of blood is transfused. The only difference is that the *rate* of removal of the large volume may be reduced either because the titre of antibody in the recipient's circulation is temporarily reduced or because the capacity of the R.E. system is temporarily overloaded.

Effect of Antigen Content of Red Cells

Sub-groups of A. There are a number of clinical observations which suggest that A_1 red cells are more rapidly destroyed than A_2 red cells when injected into recipients of group O. For example, Wiener, Samwick *et al.* (1953b) injected, over a period of 1 hour, 30–40 ml. of A_1 or A_2 blood, diluted in saline, to group O recipients. In all of five subjects injected with A_1 blood, haemoglobinaemia (80–100 mg./100 ml.) was observed and three of the five recipients developed reactions. In five subjects injected with A_2 blood no haemoglobinaemia was observed and no reactions developed.

In a case studied by the author, when 0.5 ml. of A_1 red cells was injected a sample taken 3 minutes after injection contained only 0.5 per cent of the injected cells. Fifty-four per cent of the injected radioactivity was free in the plasma. When 0.5 ml. of A_2 red cells was injected a sample taken 3 minutes after injection contained 2.5 per cent of the injected red cells and only 38 per cent of the injected radioactivity was free in the plasma (Mollison, 1959d). The rather small difference between the amount of intravascular destruction in the two cases was thought to be due to the fact that the A_2 donor was a relatively 'high grade A_2', i.e. a relatively strong reactor.

In another case, a patient of group O was seen after she had been inadvertently transfused with 400 ml. of blood of sub-group A_2. A few hours after the transfusion had been given, 1 ml. of ^{51}Cr-labelled red cells of sub-group A_1 was injected. The A_1 red cells were removed within a matter of minutes but the A_2 cells were slowly removed over a period of 3 days.

Further evidence of the relative insusceptibility of A_2 cells is provided by cases in which a recipient of sub-group A_2 is transfused with A_1 blood and then with group O blood and is found to destroy the A_1 red cells preferentially. Thus in a case reported by Ervin and Young (1950), the recipient, subgroup A_2 received a transfusion first of A_1 blood, then of group O blood containing immune A antibodies. The transfused A_1 cells (from five bottles of blood) were eliminated within approximately 2 days of transfusion and an anti-A_1 agglutinin could be demonstrated in the patient's plasma.

In a case reported by Hartmann (1957), a patient of subgroup A_2B was

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transfused with three bottles of A_1B blood during an operation followed by one bottle of group O blood; 'haematuria' was observed. During the next 24 hours two more bottles of group O blood were transfused and after each bottle 'haematuria' was noted. The anti-A titres of the bottles were relatively low (64-128). Two months later the patient's circulation contained group O cells and none of A_1B. Anti-A_1 was present but had disappeared after a further month.

Although red cells with a weak A antigen are relatively insusceptible to damage by anti-A, a haemolytic reaction following the transfusion of A_x blood has been reported in a patient whose serum contained potent anti-A; the pre-transfusion titre was 1000 vs. A_1 cells and 8 vs. A_x cells (Schmidt, Nancarrow *et al.*, 1959).

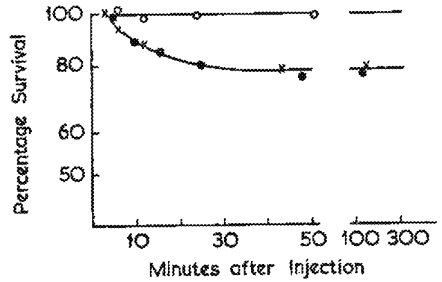

FIGURE 65. Survival of red cells from a group O donor, M. C., of the apparent phenotype Le(a—b+), labelled with ^{32}P on one occasion (●) and with ^{51}Cr on another (×), in a patient, W. B., whose serum contained potent anti-Le^a. The survival of red cells from a donor, A. R. T., of group A_1, Le(a—b+), (○), labelled with ^{51}Cr and injected on a different occasion is also shown (from Cutbush *et al.*, 1956).

Differences in the P and Lewis antigens. It is well known that there are considerable differences between the 'strength' of P antigen in different P-positive blood samples; these differences observed in tests *in vitro* are correlated with the amount of red cell destruction *in vivo*. For example, in one patient whose serum contained a strong anti-P_1, the red cells of one P-positive donor were injected and the proportion destroyed rapidly was 49 and 51 per cent on two different occasions. In between these two tests the red cells of another P-positive donor, whose red cells reacted a little more strongly *in vitro*, were injected and 60 per cent were destroyed rapidly *in vivo*.

Another example of the relationship between the strength of reactivity *in vitro* and the proportion of red cells destroyed rapidly *in vivo* is provided by tests made in a patient whose serum contained anti-Le^a. In this patient

the red cells of adult donors of the phenotype Le(a+b—) were removed with a half-time of approximately 2 minutes whereas those of infant donors of the phenotype Le(a+b+) (later to become Le(a—b+)) were removed with a half-time of 3-5 minutes. When red cells of adults of the phenotype O Le(a—b+) were injected only a proportion were removed rapidly (see Fig. 65); these red cells, after enzyme-treatment, could be shown to react weakly *in vitro* with anti-Le^a (Cutbush *et al.*, 1956).

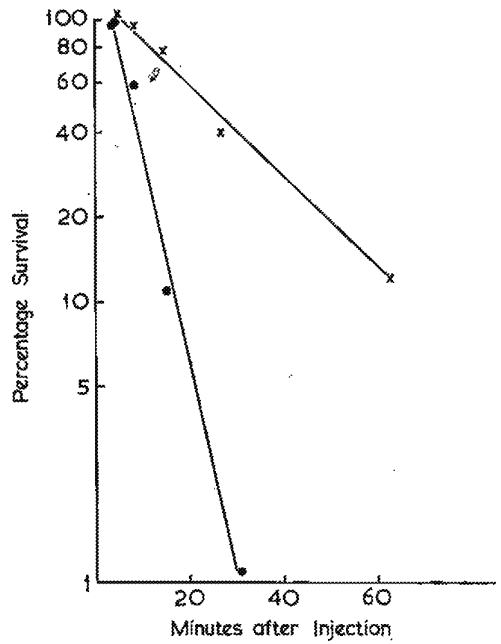

FIGURE 66. Destruction of Jk^aJk^a red cells (●—●) and of Jk^aJk^b red cells (×—×) in a patient whose serum contained anti-Jk^a (from Cutbush and Mollison, 1958).

Differences between homozygous and heterozygous cells. In some blood group systems red cells from donors known to be homozygous for a particular blood group gene react *in vitro* distinctly more strongly than red cells from heterozygotes; one well-recognized example is the difference between the reactions of Jk^aJk^a and Jk^aJk^b cells. As Fig. 66 shows, in a subject whose serum contained anti-Jk^a, homozygous red cells were destroyed more rapidly.

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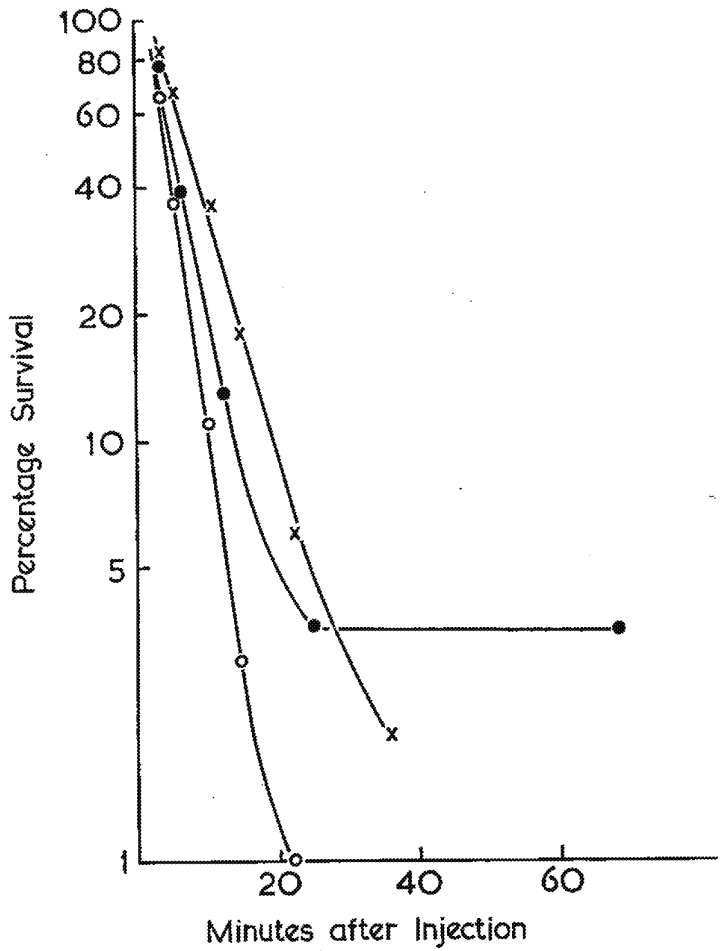

FIGURE 67. Destruction of incompatible red cells by anti-c behaving as a saline agglutinin (titre 128). ●—● *cde/cde* red cells; ○—○ *cde/cde* red cells injected on a second occasion two years later; ×—× *CDe/cde* red cells (from Cutbush and Mollison, 1958).

The difference between MM and MN red cells may be more striking. In a case described by Cutbush and Mollison (1958), when red cells of type *MM* and *MN* were injected into a recipient with anti-M, there was an astonishing difference between the survival of the two types of cells, *MM* cells being eliminated with a half-time of a few minutes and *MN* cells with a half-time measured in days. The case is described in more detail in a later section because of the help it gives in understanding the clinical significance of the thermal range of antibodies.

An example of the slightly greater rate of elimination of *cc* cells compared with *Cc* cells in a patient whose serum contained anti-c is shown in Fig. 67; *in vitro*, the reactions of the homozygous cells were distinctly stronger than those of the heterozygous cells.

Evidence for Heterogeneity of Antigen Distribution within a Single Red Cell Population

As mentioned earlier in this chapter, when a sample of incompatible red cells from a single donor is transfused, some of the red cells may be destroyed within a few minutes or hours whereas others may survive for many weeks (see Fig. 60). This phenomenon was attributed by Mollison and Cutbush (1955) to heterogeneity of the red cell population. It was pointed out that the insusceptibility of some red cells to destruction was only relative and that when the same red cells were exposed to a sufficiently potent antibody all were rapidly eliminated (see Fig. 73). The phenomenon seems to be associated particularly with naturally-occurring antibodies for the examples given by Mollison and Cutbush were due to anti-P_1 and anti-Le^a and the further examples given by Cutbush and Mollison (1958) were of anti-A_1, anti-Le^b, anti-M and anti-Lu^b.

Akeroyd and O'Brien (1952) reported that following the transfusion of 11 pints of group AB blood to a group A recipient, there were signs of red cell destruction including jaundice, but that although the patient's serum contained anti-B with a haemolysin titre of 4 and an agglutinin titre of 128, some B cells persisted and could be demonstrated for 6 weeks after transfusion.

The most reasonable hypothesis seems to be that a certain minimum amount of antibody must be taken up by the red cells to determine their accelerated removal from the circulation and that this in turn demands a certain minimum number of antigen sites.

Similar results have been observed in dogs, particularly when the transfused red cells contain a 'weak' antigen; see Swisher and Young (1954).

Characteristics of the antibody (including potency)

Antibodies which are readily Haemolytic in vitro

The commonest antibodies of this kind are some examples of anti-A and anti-B and these bring about the intravascular haemolysis of a large

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proportion of the injected cells. Within a few minutes the plasma may contain haemoglobin representing from 40 to 90 per cent of the amount injected as intact red cells (Jandl *et al.*, 1957; Cutbush and Mollison, 1958); an example has been given above (Fig. 59).

Jandl *et al.* (1957) found that some examples of anti-A and anti-B would bring about complete lysis *in vitro* only when the ratio of serum to cells exceeded 500 to 1 (v/v). Sera which even in this excess would not cause any lysis of red cells produced predominantly extravascular destruction, with only 10 per cent of the injected radioactivity being found in the plasma. A further description of the effect of non-haemolytic anti-A and anti-B is given in the following section.

There can be little doubt that other antibodies which are readily haemolytic *in vitro*, for example most examples of anti-P+–P_1 (anti-Tj^a) could cause predominantly intravascular lysis of small samples of red cells but no quantitative evidence on the subject has been presented.

Muir and McNee (1912) called attention to discrepancies between the effects produced by lytic antibodies *in vitro* and *in vivo*. They prepared an immune anti-rabbit red cell serum and found that whereas 1.5 ml. would haemolyse red cells from 7.5 ml. of blood *in vitro*, a similar amount of serum injected intravenously destroyed the red cells from 60 ml. of blood; moreover the destruction *in vivo* was spread out over 1–2 days.

Wasastjerna (1948) confirmed that an immune haemolytic serum produced much more red cell destruction *in vivo* than *in vitro*. He found that the osmotic fragility of red cells was not increased by incubation with the haemolytic serum *in vitro* although *in vivo* fragility increased in proportion to the severity of the red cell destruction. He found that the amount of red cell destruction was smaller in splenectomized animals, a finding which by itself emphasized the difference between events *in vivo* and *in vitro*.

Antibodies which Agglutinate Red Cells in vitro *but do not Bind Complement*
Agglutinins active in vitro *at* 37° C. As mentioned above, according to Jandl *et al.* (1957) non-lytic examples of anti-A and anti-B bring about predominantly extravascular destruction. In one case in which the serum had an agglutinin titre of 64 but was incapable of lysing red cells *in vitro* they found that 90–95 per cent of injected incompatible cells disappeared with a half-time of 4 to 5 minutes. Most of the radioactivity was present in the liver.

Occasionally Rh antibodies provide good examples of antibodies which agglutinate strongly *in vitro* and certainly do not bind complement. Fig. 67 shows the results of some tests in a subject whose serum contained anti-c with an agglutinin titre against cells suspended in saline of 128. Homozygous cells (*cc*) were removed with a half-time of 2 to 3 minutes and surface counting showed that about 70 per cent were removed by the liver.

When the agglutinin titre is very low, the cells may be removed predominantly by the spleen rather than the liver; Cutbush and Mollison (1958) described two examples, one in which the antibody was anti-Mi^a, present as an agglutinin with a titre of only 2 at 37° C., and one in which it was anti-Lu^b, also only weakly active at 37° C.

The findings of Chaplin (1959) also suggest that in cases of hypogammaglobulinaemia with very low iso-agglutinin titres, destruction of incompatible cells occurs to a large extent in the spleen.

Jandl and Kaplan (1960) showed very elegantly that the effects produced by an antibody depended not only on its characteristics but on the dose in relation to the number of red cells involved. In a series of experiments carried out in a patient of group A with hypogammaglobulinaemia whose serum contained no detectable anti-B, ^{51}Cr-labelled B cells were injected and this was followed, after a few days, by the injection of varying amounts of anti-B. When the ratio of serum to B cells was 0.01 the red cells were removed predominantly by the spleen. When the ratio reached 0.59 a considerable proportion of the cells was removed by the liver; at a ratio of 10 to 1 destruction was predominantly intravascular (*in vitro* the serum was weakly haemolytic).

In cases of hypogammaglobulinaemia in which no iso-agglutinins can be detected in the serum, red cells which would normally be incompatible survive for long periods. For example in a patient of group B described by Wiener and Gordon (1957) A_2 cells survived for more than 60 days. In a case described by Chaplin (1959) 'incompatible' cells had a $T\frac{1}{2}$Cr of 9 days and in the group A patient of Jandl and Kaplan (1960) referred to above the $T\frac{1}{2}$Cr of group B red cells was 5 days (see Jandl *et al.*, 1957).

Injections of agglutinated red cells. When agglutinated red cells are injected, a proportion may be removed by the liver but most of the cells survive normally (Jandl *et al.*, 1957; see also Loutit and Mollison (1946)). Such experiments demonstrate the reversibility of agglutination and help to explain why the transfusion of plasma containing incompatible agglutinins is relatively harmless. These experiments are referred to again later in this chapter.

Agglutinins active in vitro *only at temperatures of* 34° C. *or less.* It is common to find agglutinins with the specificity anti-P_1, or anti-A_1, which

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in vitro agglutinate red cells only up to a temperature of about 30° C. and fail to bring about agglutination if the temperature is kept strictly at 37° C.

Such agglutinins are often reported to be active at 37° C. when in fact they are not. If red cells are incubated at 37° C. with a serum containing cold agglutinins active at 30° but not at 37° C. and the mixture is spread on a microscope slide and examined, agglutinates rapidly form because the temperature has fallen to 30° or thereabouts. If instead the mixture is spread on a warmed slide and immediately placed on a chamber warmed strictly to 37° C. no agglutination is observed.

When a serum is capable of agglutinating red cells at 30° C. but not at 37° C., it is of course difficult to define precisely the thermal range of the

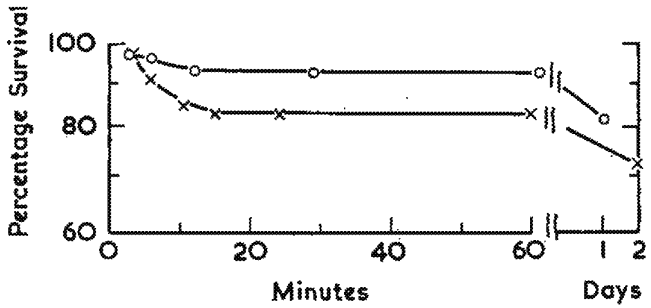

FIGURE 68. Survival of A_1 red cells in two different subjects of group A_2B whose serum contained anti-A_1, definitely active *in vitro* at 30° C. but dubiously active at 37° C.

antibody. Fig. 68 shows the survival of 1 ml. of sub-group A_1 red cells injected into two subjects whose serum contained anti-A_1 definitely active *in vitro* at 31° C. but giving only very dubious agglutination at 37° C. As the figure shows, 10–20 per cent of the red cells were rapidly destroyed but the remainder survived normally.

Very occasionally agglutinins are found which react definitely at 34° C. but not at 37° C. In Fig. 69 some results observed in a case of this kind are illustrated. The antibody was anti-M and *in vitro* agglutinated *MM* cells only at a temperature of 34° C. When *MM* cells were injected more than 50 per cent were removed with a half-time of a few minutes.

The serum of the same patient agglutinated *MN* cells only at a temperature of 31° C. or less. As Fig. 70 shows, when *MN* cells were injected there was no measurable destruction in the first few hours after injection;

50 per cent of cells were destroyed with a half-time of 4 to 5 days and the rest were destroyed still more slowly.

It was shown that the temperature of the suspension of red cells injected did not influence the amount of destruction *in vivo*. Thus two suspensions of red cells from an *MM* donor were prepared and one, labelled with ^{51}Cr, was warmed to approxi-

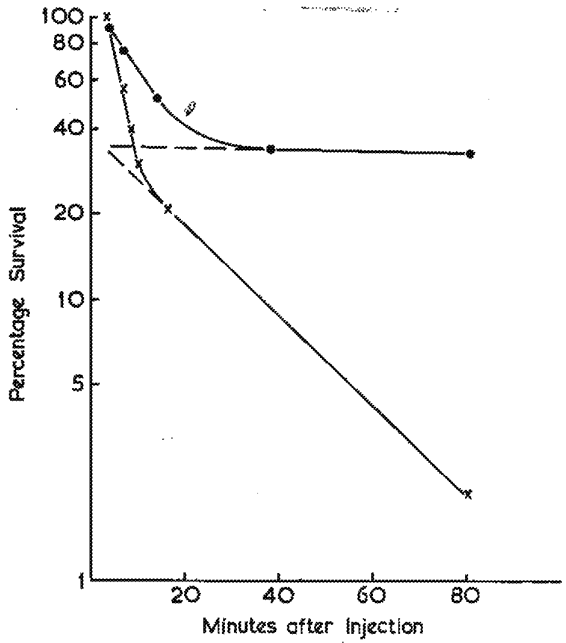

FIGURE 69. Survival of *MM* red cells injected on two different occasions in a subject whose serum contained potent agglutinating anti-M.
●—● First injection
×—× Second injection 12 days after the first. The antibody on this second occasion was slightly more potent than the first but it was still not active *in vitro* above a temperature of 34° C.
(From Cutbush and Mollison, 1958.)

mately 40° C. immediately before injection and the other, labelled with ^{32}P, was cooled to 0° C. immediately before injection. The curve of disappearance of the two samples was identical (see Fig. 71), about 25 per cent of the cells being destroyed in the first 30 minutes. At the time of the test the antibody was considerably less potent than at the time when the tests illustrated in Figs. 69 and 70 were made (Mollison, 1959b).

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One important conclusion suggested by this case is that in direct matching tests it is unsafe to rely on absence of agglutination at 37° C. It seems safer to perform the test at 30° C. or even 25° C.

Antibodies behaving in vitro *as Agglutinins and also Binding Complement*

Antibodies of the P and Lewis systems characteristically agglutinate red cells better at low temperatures (such as 12° C.) than at 37° C. Indeed

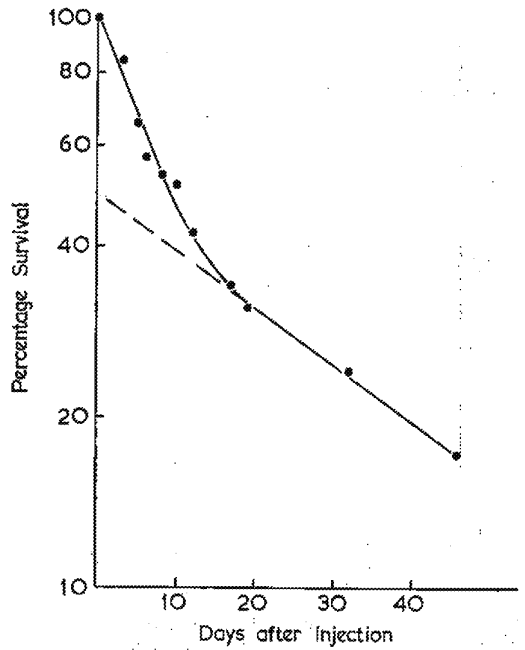

FIGURE 70. Survival of *MN* red cells in the same patient as in Fig. 69. Note that whereas in Fig. 69 the time scale is in minutes, here it is in days. The curve of disappearance of the red cells appears to have two components.

most examples of anti-P_1 are inactive *in vitro* above a temperature of about 25° C. and only a few examples of anti-Le^a produce good agglutination at 37° C.

The most potent examples of anti-P_1 bind complement and almost all examples of anti-Le^a, and many examples of anti-Le^b do so; the author has encountered an example of anti-O(H) which behaved in the same way. Complement-binding can be demonstrated by incubating red cells with

fresh serum at 37° C. and showing that the red cells are then sensitized to an anti-β_1 globulin serum and that this sensitization does not occur if complement-binding is prevented, e.g. by adding EDTA to the antibody-containing serum.

It seems that all naturally occurring antibodies which are potent enough to cause red cell destruction, tend to destroy only a proportion of injected incompatible red cells, whether or not they bind complement. Examples will be found in various figures in this chapter. Thus anti-A_1, Figs. 60 and 68; anti-O(H), Fig. 72; anti-P_1, Figs. 60 and 74; anti-Lu^b, Fig.

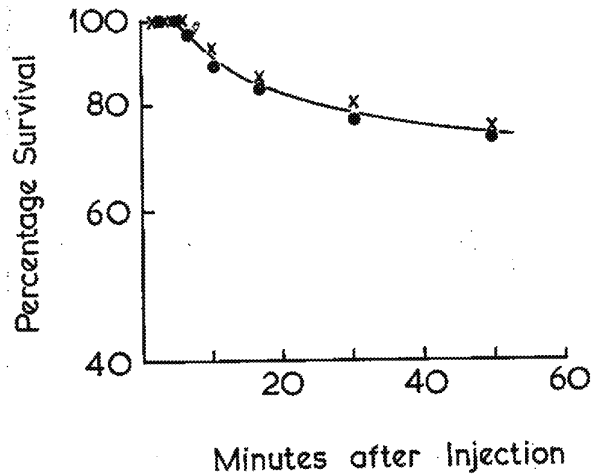

FIGURE 71. Survival of *MM* red cells injected as a warm suspension (38° C.) and as a cold suspension (0° C.) in a patient whose serum contained anti-M active *in vitro* at 34° C. but not at 37° C.
× = warm suspension
● = cold suspension
(From Mollison, 1959b.)

60; and anti-Le^a, Figs. 65 and 73. A case in which anti-Le^b brought about the removal of 56 per cent of injected red cells with a half-time of 2 minutes has been described elsewhere (Mollison, 1959c).

Fig. 73 shows the effect of variations in the potency of antibody.

Red cells from the same group O Le(a+b−) donor were injected into three different subjects whose serum contained anti-Le^a. In the first subject, whose serum had a potent antibody with an indirect antiglobulin titre of 32, virtually 100 per cent of the red cells were removed from the circulation with a half-time of just under 2 minutes. This subject had developed haemoglobinuria after a previous

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transfusion of Le(a+) blood. In the second subject, whose serum contained anti-Le^a with an indirect antiglobulin titre of 8 approximately 80 per cent of the injected red cells were removed with a half-time of 3 minutes, the remaining red cells being removed much more slowly. This patient had developed jaundice after a transfusion of Le(a+) blood. In the third subject, whose serum contained a very weakly reacting antibody (indirect antiglobulin titre of 1 at 37° C.), 65 per cent of

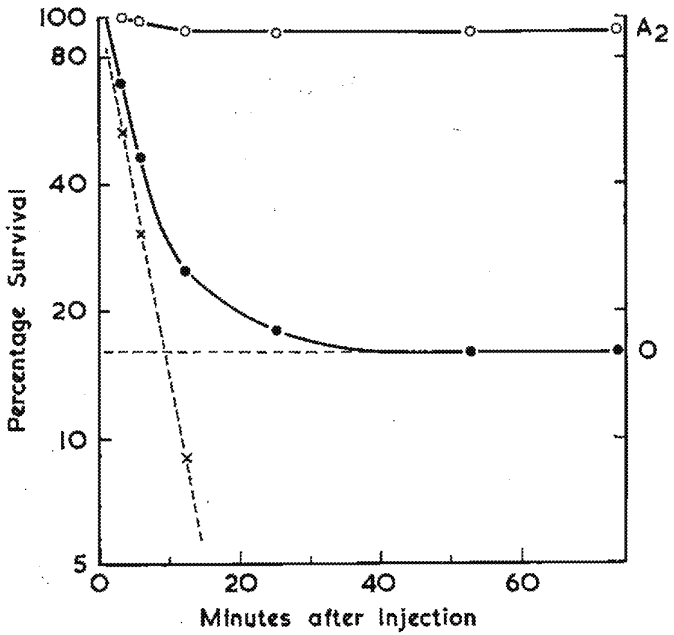

FIGURE 72. Survival of group O and group A_2 red cells in a patient whose serum contained anti-O, acting *in vitro* as a saline agglutinin up to a temperature of approximately 30° C. and behaving at 37° C. as an incomplete complement-binding antibody detectable by the indirect antiglobulin test. The A_2 red cells underwent no significant destruction, but over 80 per cent of the O red cells were rapidly removed from the circulation. The 'rapid component' is plotted separately and has a half-time of removal of approximately 3.5 minutes (from Mollison 1959d).

the cells were removed with a half-time of 6 minutes. The 'tail' of red cells was removed very slowly so that even 3 weeks after injection 23 per cent of the original number injected were still present.

Fig. 74 shows another example of the injection of a warm suspension and a cold suspension of red cells to a patient whose serum contained a 'cold' antibody, that is one reacting more strongly at 0° C. than at 37° C.

As in the case associated with anti-M, reported above, red cells injected as a warm suspension suffer the same degree of destruction as red cells injected as an ice-cold suspension. The explanation of this must surely be that within about 10 seconds of injection into an arm vein the cells have reached the right side of the heart and have been mixed with a relatively large volume of the patient's own blood; thus they are almost immediately warmed to body temperature. Accordingly, the effect which the antibody

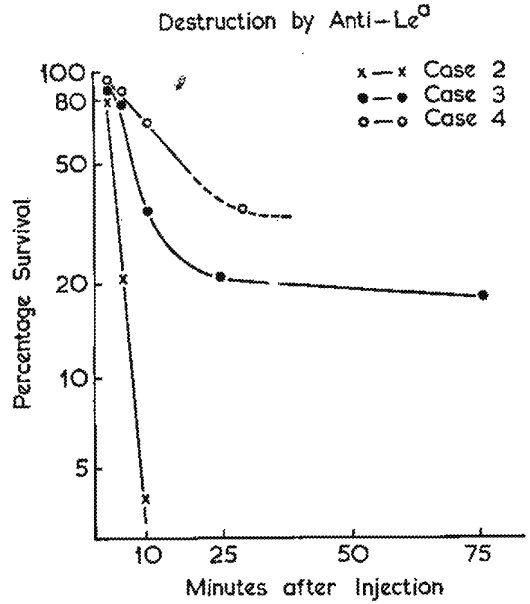

FIGURE 73. Survival of Le(a+b—) red cells from a single donor in three different recipients. Anti-Le^a was present in all three cases but the indirect antiglobulin titre varied greatly amongst the three. Thus, ×—× titre 32; ●—● titre 8; ○—○ titre 1 (from Mollison and Cutbush, 1955).

produces at body temperature decides the fate of the injected cells, whether they are at 0° or 37° C. at the moment of injection.

Incomplete Antibodies which Bind Complement

Some examples of anti-K and of anti-Fy^a and perhaps all examples of anti-Jk^a bind complement and these antibodies do not as a rule agglutinate red cells suspended in saline. Incomplete, complement-binding antibodies usually bring about removal of red cells in the liver although very weak

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antibodies of this type can be associated with predominantly splenic destruction (see destruction of Jk^aJk^b cells in Fig. 66).

The effect of incomplete complement-binding antibodies can also be studied by sensitizing red cells *in vitro* either with the antibody alone or with the antibody and complement and then re-injecting the cells into the circulation. In experiments with a complement-binding anti-Fy^a, it was found that when one volume (approximately 1 ml.) of Fy^a plus red cells

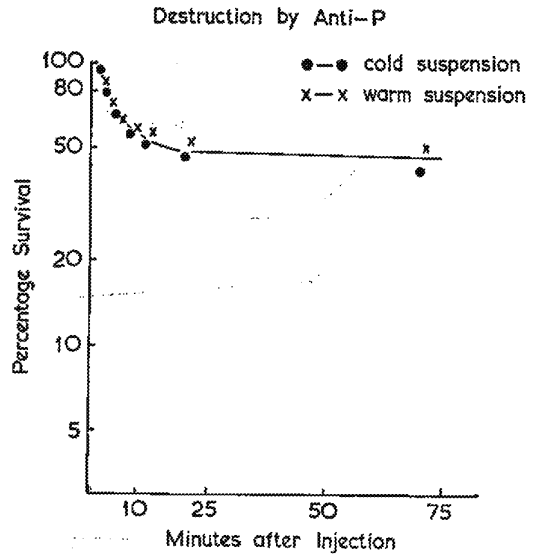

FIGURE 74. Survival of P+ red cells in a patient whose serum contained anti-P_1. The serum strongly agglutinated P+ red cells at room temperature but produced only very weak agglutination at 37° C. However, at 37° C. it was capable of sensitizing red cells to an antiglobulin serum. P+ cells were labelled with ^{32}P and injected as an ice-cold suspension; red cells from the same donor were labelled with ^{51}Cr and injected as a warm suspension (39° C.) immediately afterwards. The temperature of the suspension at the time of injection did not appear to influence the survival (from Mollison and Cutbush, 1955).

was incubated with 2.5 volumes of anti-Fy^a and the cells were then re-injected into the circulation, 90 per cent of them were cleared with a half-time of about 2 minutes; when the cells were incubated with only an equal volume of anti-Fy^a, only about 30 per cent were rapidly destroyed (see Figs. 75 and 76).

In these experiments, the source of anti-Fy^a was gamma globulin prepared from an anti-Fy^a serum. The preparation had an indirect anti-

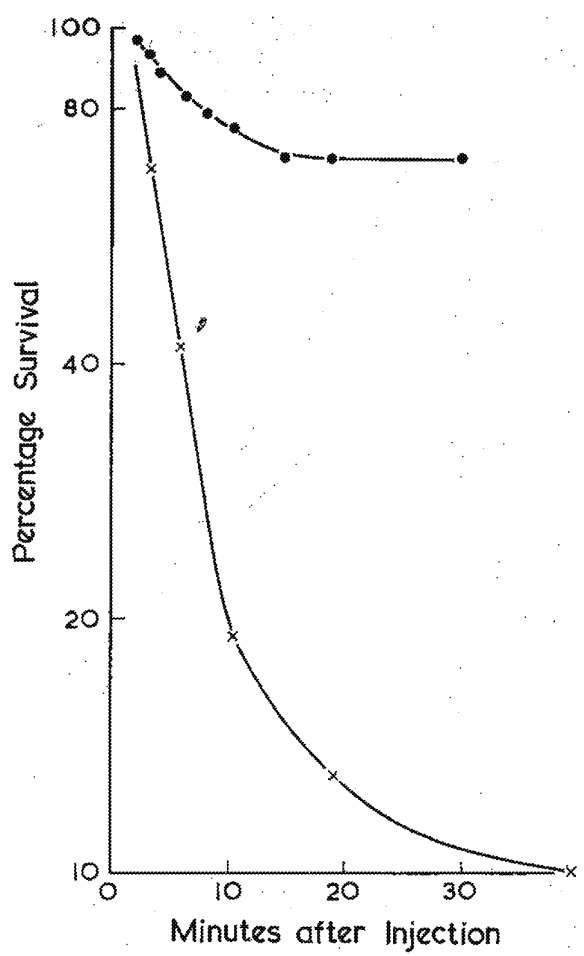

FIGURE 75. Survival of Fy(a+) red cells sensitized *in vitro* with anti-Fy^a and re-injected into the donor.
●—● 1 volume of red cells sensitized with 1 volume of anti-Fy^a
×—× 1 volume of Fy(a+) red cells sensitized with 2.5 volumes of anti-Fy^a
In each case the anti-Fy^a used was a solution of gamma globulin prepared from a serum containing a potent complement-binding anti-Fy^a (from Mollison, 1959d).

globulin titre of 64. It was found that when the cells were treated with antibody but without complement, the destruction of the injected red cells was just as rapid and just as complete as when they were treated with

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antibody and complement before re-injection and this was attributed to the almost immediate binding of complement which would occur when sensitized red cells entered the recipient's blood stream.

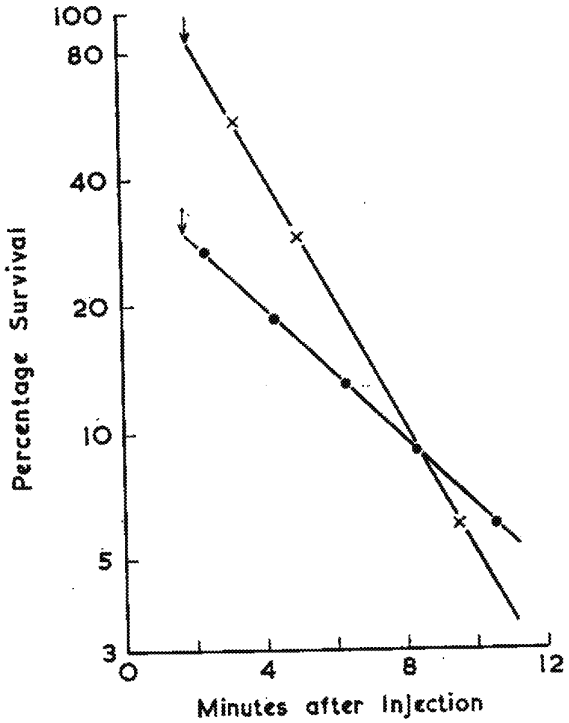

FIGURE 76. Rate of removal of Fy^a sensitized red cells. The figure shows the rapid components of the curves shown in Fig. 75. Red cells sensitized with 2.5 volumes of the preparation of anti-Fy^a (×—×) underwent more extensive destruction (approximately 90 per cent of the cells being removed rapidly), than red cells sensitized with an equal volume of the anti-Fy^a preparation, (●—●) of which only 30 per cent were rapidly destroyed. Moreover, the rate of removal brought about by the larger amount of anti-Fy^a was greater than that brought about by the smaller volume (from Mollison, 1959d).

Incomplete Antibodies which do not Bind Complement

These antibodies, even when they are potent, bring about destruction of red cells predominantly in the spleen. This is a very interesting phenomenon because the blood flow through the spleen is probably only about one-tenth of that through the liver and it follows that red cells coated with

this kind of antibody must continually pass through the liver unscathed although they may be removed on the first occasion on which they attempt to pass through the spleen.

Some more potent examples of this type of antibody, e.g. antibodies with a titre of 64 or more, bring about removal of red cells with a half-time of approximately 20 minutes and this probably represents virtually complete clearance of the blood at each passage through the spleen. Less potent examples, e.g. titre 8, bring about removal of cells with a half-time of 60 to 100 minutes. Still less potent antibodies — titre 2 or 4 — bring about a still slower removal, with a half-time of the order of a few days. (Mollison and Cutbush, 1955; Cutbush and Mollison, 1958.)

When the ratio of Rh antibody to Rh-positive red cells is sufficiently high, some of the cells may be removed by the liver (Jandl *et al.*, 1957; Mollison and Hughes Jones, 1958). This point was investigated further by Jandl and Kaplan (1960). ^{51}Cr-labelled Rh (D)-positive red cells were first injected into the circulation of an Rh-negative subject. After 4 to 6 days various amounts of serum containing incomplete anti-Rh were injected. In two cases in which the ratio of the volumes of anti-Rh to cells was less than 3 to 1 destruction was predominantly splenic but in one case in which the ratio of serum to cells was over 13 to 1 there was appreciable removal in the liver; thus surface counting showed that the liver counts reached half the level of counts over the spleen.

Injections of 'sensitized red cells'. Further information about the destruction brought about by incomplete non-complement-binding antibodies has been obtained by sensitizing red cells *in vitro* and then re-injecting them. Thus Jandl *et al.* (1957) sensitized Rh+ red cells with anti-Rh *in vitro* and injected them into seven subjects. The red cells were cleared from the circulation with a half-time of 18 to 40 minutes. When the ratio of antibody to cells was high (e.g. 2 ml. of serum with an indirect antiglobulin titre of 1000 to 1 ml. of red cells) 95 per cent of the cells were removed rapidly and when the ratio was lower (e.g. 0.5 ml. of serum with a titre of 128 to 1 ml. of cells) only 60 per cent of the cells were destroyed in the 8 hours following the injection.

Observations by the present author are shown in Fig. 77. Red cells were incubated with varying amounts of gamma globulin prepared from a serum containing potent incomplete anti-Rh, the indirect antiglobulin titre of the preparation being approximately 1 in 1000. When the ratio of gamma globulin to cells was at least 1.3 to 1 the cells were removed with a half-time of 15-20 minutes and the rate of clearance was not increased by raising the proportion of gamma-globulin to cells to 3.8 to 1. As the ratio of gamma globulin to cells fell, the rate of destruction diminished

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and at a ratio of about 0.1 of gamma globulin to 1 of cells, there was scarcely any destruction at all even though the red cells gave a moderately strong direct antiglobulin test before re-injection.

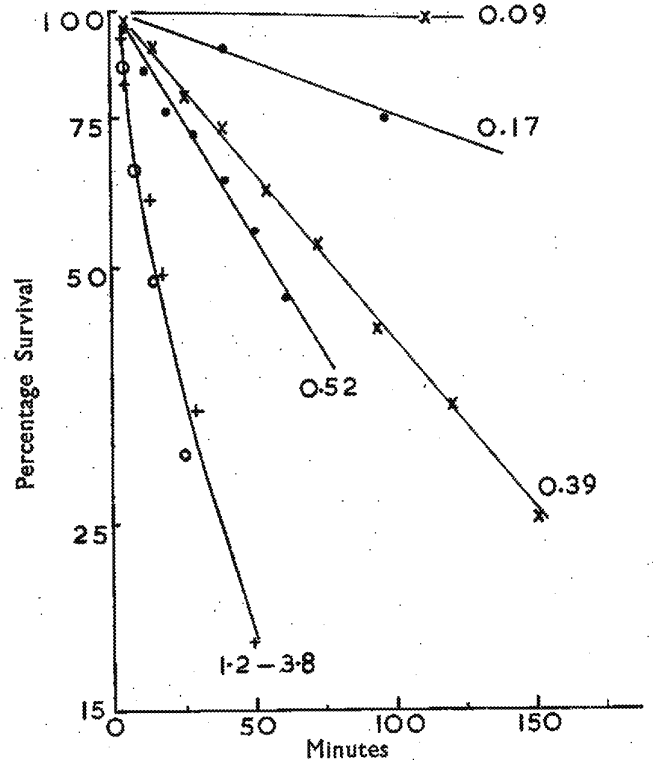

FIGURE 77. Rate of removal from the circulation of Rh-positive red cells sensitized *in vitro* with anti-Rh. Rh-positive red cells were incubated with varying volumes of a preparation of gamma globulin made from a serum containing potent incomplete anti-D. Figures at the end of each curve indicate the relative volume of gamma globulin preparation to red cells, e.g. 0.09 means that 0.09 volumes of gamma globulin were incubated with one volume (approx. 1 ml.) of Rh-positive red cells. This amount of anti-Rh, although it produced a moderately strongly positive antiglobulin test, did not bring about the subsequent rapid destruction of the red cells. With increasing volumes of anti-Rh the rate of destruction increased until at 1.2 volumes (to one volume of red cells) it reached a maximum, with a half-time of approximately 15 minutes (from Mollison, 1959d).

As Fig. 77 shows, in the cases in which considerable destruction occurred but was not complete within 150 minutes, red cell destruction continued

steadily throughout this period. Fig. 78 shows that in this case there was no definite slowing in the rate of destruction until after about 5 hours. In further experiments carried out *in vitro* it was shown that antibody is transferred relatively slowly from sensitized to unsensitized cells and that

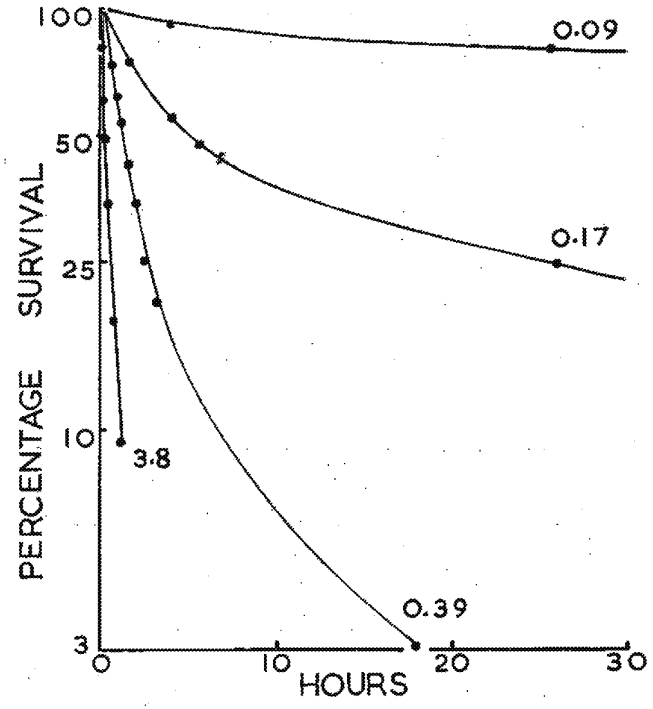

FIGURE 78. In this figure some of the examples shown in Fig. 77 have been followed over a longer period. About 5 hours after the injection of the sensitized red cells the rate of destruction shows definite slowing (from Mollison, 1959d).

this accounts for the rather constant rate of destruction of the sensitized cells for several hours after re-injection.

The transfer of antibody from one population of red cells to another can be studied *in vitro* with the help of differential agglutination. For example, group A Rh-positive red cells can be sensitized with incomplete Rh antibody, then washed and mixed with unsensitized, group O Rh-positive cells. After incubating the mixture for a given period the O Rh-positive cells can be recovered and freed from the A cells by treatment of the mixture with anti-A. The O cells can then be tested

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with an antiglobulin serum to discover whether they have taken up antibody. Such experiments show that when A Rh-positive red cells are initially treated with relatively large amounts of anti-Rh so that they are strongly sensitized, then on incubation with O Rh-positive cells an appreciable amount of Rh antibody will be transferred in 1 hour. On the other hand if the A cells are only moderately strongly sensitized, no detectable amount of antibody is transferred to the O cells (Mollison, 1959d; Evans *et al.*, 1960). The transfer of anti-Rh from one population of red cells to another was also studied by Masouredis (1960a) using ^{131}I-labelled antibody. He found that the maximum transfer occurred at 37-45° C. and that no transfer occurred at 4° C. or at 56° C. In one experiment at 37° C. 9 per cent of the 'donor' cell antibody was transferred within 30 minutes and a maximum of 15 per cent between 120 and 180 minutes.

When red cells sensitized *in vitro* with incomplete Rh antibody have been cleared from the circulation, it can be confirmed by surface counting that they have been destroyed predominantly in the spleen (Jandl *et al.*, 1957; Hughes Jones, Mollison and Veall, 1957; Mollison and Hughes Jones, 1958). If such cells are injected into a splenectomized subject, they are removed from the circulation with a half-time of about 4½ hours and are taken up predominantly by the liver (see Jandl *et al.*, 1957; see also Fig. 79).

Red cells sensitized *in vitro* with Rh antibody may be removed from the circulation at an accelerated rate if the serum contains some abnormal protein which causes the cells to agglutinate. Thus Jandl *et al.* (1957) found that in one patient with multiple myeloma and a serum globulin of 12 g./100 ml., Rh-sensitized cells were removed with a half-time of 5 minutes ('Control' 30 minutes). In one case reported by the author in which Rh-sensitized cells were injected into a patient with rheumatoid arthritis whose serum agglutinated Rh-sensitized red cells *in vitro*, 50 per cent of the cells were removed in 7 minutes (Mollison, 1959c).

When small amounts (1 ml.) of ^{51}Cr-labelled incompatible red cells are removed by the spleen with a half-time of about 20 minutes, from 2 to 5 per cent of the injected radioactivity appears in the plasma (see Fig. 80). It has been shown that during the first hour or so after injection the amount of radioactivity in the plasma is a good indication of the amount of haemoglobin liberated into the plasma, although as time goes on the haemoglobin is cleared from the plasma more rapidly than the ^{51}Cr, which tends to exchange with other proteins in the plasma (Jandl *et al.*, 1957).

In assessing the amount of ^{51}Cr (or Hb) present in the plasma following the extravascular destruction of a small volume of incompatible red cells,

it must be remembered that haemoglobin is being cleared rapidly, particularly at these very low levels of plasma haemoglobin (less than 1 mg. per 100 ml.). The amount actually liberated may be assessed by adding graphically the cumulative amounts which are calculated to leave the circulation in each period of time. Thus, in the case shown in Fig. 80, in which the maximum plasma level of ^{51}Cr (Hb) reached the equivalent of 5 per cent of the injected radioactivity, the amount of haemoglobin

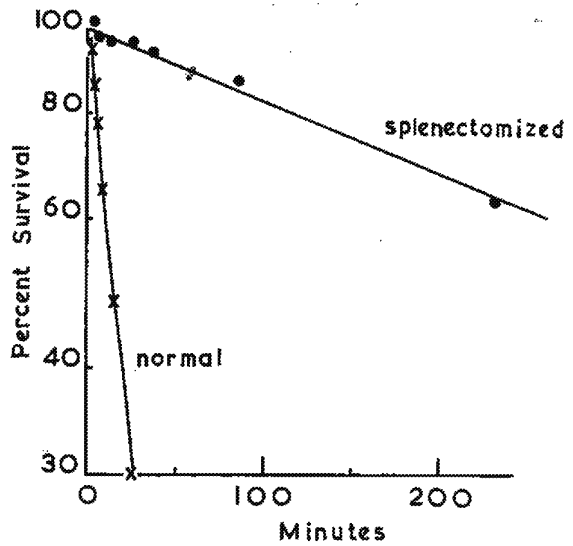

FIGURE 79. The rate of removal of Rh-sensitized cells from the circulation in a splenectomized subject compared with the rate observed in a normal subject. The red cells were maximally sensitized but in the splenectomized subject the half-time of removal was of the order of 5 hours compared with 15-20 minutes observed in normal subjects.

liberated into the plasma may be calculated to have been about 10 per cent of the amount injected as red cells.

When larger amounts of Rh-positive red cells are given to subjects whose serum contains incomplete anti-D, the proportion of haemoglobin liberated into the plasma appears to be greater. For example, Jandl *et al.* (1957) found that in a subject injected with about 20 ml. of red cells, the amount of haemoglobin in the plasma reached the equivalent of 12 per cent of the amount of haemoglobin injected as intact red cells. It seems likely that the higher proportion of the injected 'dose' appearing in the

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plasma after large 'doses' is due at least partly to the limited capacity of the R.E. system to clear the complex of haemoglobin and haptoglobin from the circulation (see Chapter XIV).

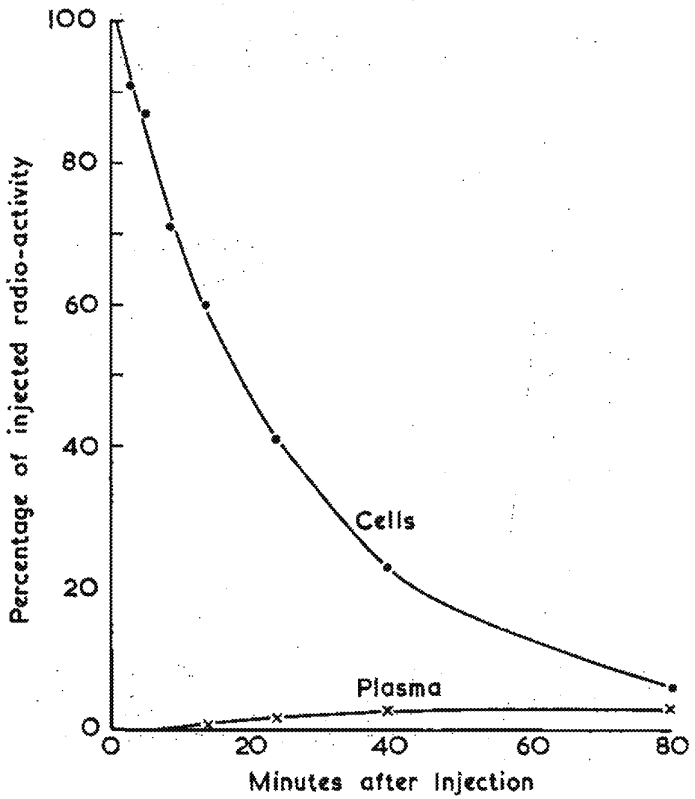

FIGURE 80. Radioactivity found in red cells and plasma following the injection of ^{51}Cr-labelled Rh-sensitized red cells. A small amount of radioactivity appears in the plasma about 10 minutes after the injection and reaches a maximum after about 1 hour. This radioactivity in the plasma represents haemoglobin liberated into the circulation.

The maximum amount of the haemoglobin/haptoglobin complex which can be cleared appears to be of the order of 13 mg./100 ml. per hour (see Chapter XIV). Twenty ml. of red cells contain approximately 7 g. of haemoglobin and if 10 per cent of this were liberated into a plasma volume of 3000 ml. and if the factor of clearance is ignored, the maximum level of plasma haemoglobin would be

approximately 20 mg./100 ml. At this level the rate of clearance of haemoglobin is much slower than at lower levels (see Chapter XIV).

Failure of Steroids to Prevent the Destruction of Red Cells by Incompatible Antibodies

The effect of steroids in slowing red cell destruction in auto-immune haemolytic anaemia is not well understood. It may be due partly to diminished formation of auto-antibodies (Fudenberg *et al.*, 1958) and partly to suppression of the phagocytic activity of the reticulo-endothelial system (Bilbey and Nicol, 1958). In any case, when incompatible antibodies or red cells are injected into the circulation, red cell destruction is scarcely affected by the previous administration of steroids. For example, Dr J. R. Andersen (personal communication) prepared an anti-rabbit red cell serum by injecting rabbit red cells into dogs and then gave regular intramuscular injections of serum (at approximately 48-hour intervals) to the rabbits. This produced a severe chronic haemolytic anaemia which was shown to be unaffected by the administration of cortisone.

Swisher *et al.* (1960) gave incompatible transfusions to dogs and found that the rate of destruction of the incompatible red cells was not influenced in any way by the previous administration of large doses of steroids; and Dr I. W. Brown (personal communication) has observed a severe haemolytic transfusion reaction due to anti-Jk^a in a patient with rheumatoid arthritis on full doses of steroids.

On the other hand the author has given injections of Rh-sensitized red cells to patients with rheumatoid arthritis before and after 5 days' treatment with Cortisone and has found a slight but definite slowing of the rate of destruction after Cortisone (unpublished observations).

Rapid Anamnestic Responses

Several cases have been described in which the patient's serum at the time of transfusion contained either no detectable incompatible antibody or only a very weak one, so that the transfusion was not accompanied by signs of red cell destruction. Five days or more later, signs of red cell destruction developed and the patient's serum now contained readily detectable antibody.

Kraytman *et al.* (1960) described a case in which an elderly lady developed jaundice and anuria 6 days after the transfusion of a bottle of E-positive blood. A simple cross-matching test on a slide had failed to reveal any incompatibility before transfusion. The jaundice developing 6 days later seems very likely to have been due to the fact that the red cells

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were now being rapidly destroyed by anti-E, which was now demonstrable in the patient's serum. It seems uncertain whether the anuria was associated with the red cell destruction or rather was a sequel to the large operation which the patient had undergone at about the same time as the transfusion.

Tenczar *et al.* (1960) described a patient who received a transfusion of eleven bottles of blood during an operation for arthrodesis of the hip. On the seventh day after operation the patient vomited and her urine was found to contain large numbers of red cells. In the next 48 hours the PCV fell from 31 to 17 per cent and the patient became jaundiced. Her serum was shown to contain anti-c and anti-Jk^a. She was transfused again and although her urine continued to contain red cells for a few more days there was no anuria and she recovered.

It is difficult to evaluate the haematuria in this case. This sign is not usually encountered as a sequel of incompatible transfusion although it does occur as a manifestation of the defibrination syndrome associated with severe intravascular destruction. In the present case the authors make no mention of haemoglobinaemia and in the absence of this it must be uncertain whether or not the haematuria was related to the incompatibility.

INCOMPATIBILITY WITHOUT DEMONSTRABLE ANTIBODIES

One may distinguish between two types of incompatibility without demonstrable antibodies: one in which there is rapid red cell destruction beginning at the time of transfusion, so that if a sufficient amount of blood is transfused there will be a haemolytic reaction; and one in which red cell destruction is slow, and never obvious clinically. Although it is likely that there are intermediate cases, it is convenient for the purposes of description to give accounts of these two main types separately.

Rapid Destruction of Transfused Red Cells without Demonstrable Antibody

No Blood Group Antibody Demonstrable at any time

Very occasionally, despite apparent compatibility *in vitro*, transfused red cells are destroyed sufficiently rapidly to produce a haemolytic transfusion reaction. In a case described by Stewart and Mollison (1959) a patient repeatedly developed haemoglobinuria following transfusion, although no blood group antibody could be demonstrated; as Fig. 81 shows, the transfused red cells were eliminated within 24 hours.

The patient was a woman aged 46 suffering from reticulo-sarcoma who had been treated with irradiation; during the year in which she had been ill she had

received several transfusions and these had all been followed by signs of one kind or another. The second transfusion had been followed by jaundice and the fourth and later transfusions by haemoglobinuria. Fig. 81 shows the survival of red cells which were compatible *in vitro* judged by all tests available including the indirect antiglobulin test against enzyme-treated red cells. The figure also shows that the patient's own red cells were removed from the circulation far more slowly, their average life-span being about 11 days.

Very similar cases were reported by Heistö *et al.* (1960) and by Kissmeyer-Nielsen *et al.* (1961) and the author knows of other as yet

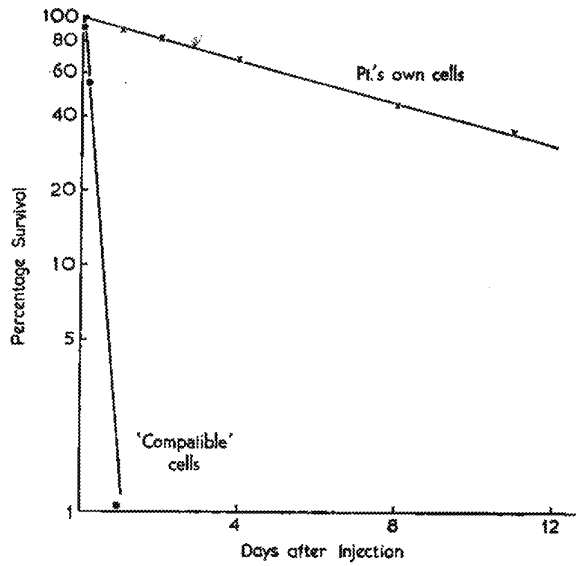

FIGURE 81. Very rapid removal from the circulation of transfused cells, compatible by all the usual serological tests. For comparison, the survival of the patient's own red cells is shown (from Stewart and Mollison, 1959).

unpublished cases (personal communications from Dr H. Chaplin, Jr., and Professor J. J. van Loghem, Jr.).

Blood Group Antibody only Temporarily Absent

Fudenberg and Allen (1957) described two cases in which severe reactions occurred following the transfusion of apparently compatible blood; in both cases a blood group antibody was demonstrated in the patient's serum after transfusion although it could not be demonstrated in the pre-transfusion serum.

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In the first of these two cases a severe haemolytic reaction with haemoglobinuria followed the transfusion of E+ blood. No anti-E could be demonstrated in the pre-transfusion sample although it was present (titre 4) in a sample taken 4 days later. In a second case a patient developed jaundice and oliguria after the last of a series of transfusions and on the day following, anti-K was demonstrated in her serum for the first time. Nevertheless the patient had undergone caesarian section for placenta praevia 48 hours before the last transfusion and the signs may possibly

TABLE XLV

SOME DATA FROM THE PAPER BY FUDENBERG AND ALLEN (1957)

Case no.	*Previous history*	*Blood group antibodies* *Specificity*	*Titre* *At time of injection*	*Max. titre later*	*Half-time of removal of incompatible red cells*
3	Three recent blood transfusions	Anti-s	0	1	55 min.†
4	Thirteen injections of Jk(a+) blood in previous 6 years	Anti-Jk*	1	32*	75 min.†
5	Eight previous blood transfusions but ? any Kp(a+)	Anti-Kp*	2	4*	8 hr.†
6	Twenty-four injections of S+ blood in 3 years	Anti-S	0	8	9 hr.
7	Very many previous injections of Jk(a+) blood	Anti-Jk*	0	0	12 days

* Antibody subsequently disappeared completely.
† Surface-counting showed that destruction occurred predominantly in the spleen.

have been due to her original condition rather than to transfusion (see following chapter).

Fudenberg and Allen (1957) investigated five further cases in which recipients who had previously had demonstrable antibodies in their serum now appeared to have little or no circulating antibody. Table XLV summarizes their findings. The author's guess is that these very weak antibodies were complement-binding because in his experience very weak incomplete non-complement-binding antibodies such as anti-c bring about only very slow destruction *in vivo* (half-time of a few days) when the indirect antiglobulin titre is of the order of 4 or less, whereas an antibody like anti-Jk^a which binds complement may remove red cells from

the circulation with a half-time of 20 minutes even when it is only just demonstrable *in vitro* (see for example the survival of Jk^aJk^b cells in Case No. 17 described by Cutbush and Mollison, 1958). As discussed in Chapter VII the detection of very weak complement-binding antibodies may be interfered with by anticomplementary factors.

Late Development of Antibody

Very occasionally whilst studying the survival of transfused red cells, one may observe the development of an antibody which brings the survival of the red cells to a premature close. The following case was observed in 1942 just after the Rh blood group system had been discovered and before the serious consequences of giving Rh-positive blood to Rh-negative women were appreciated.

Mrs N., an Rh-negative woman, was found to be anaemic during her first pregnancy. Her group was O, Rh-negative and her serum contained no Rh antibody. She was transfused with 500 ml. of Rh-positive blood. Twenty days later she was delivered of normal Rh-positive twins. Three days after delivery a further transfusion of 500 ml. of group O, Rh-positive blood was given. Immediately after the second transfusion, the total count of Rh-positive cells was 720,000 per c.mm., and 5 days later the count was 760,000 per c.mm. This is satisfactory evidence that up to this time Rh-positive cells were surviving normally. However, 21 days later (26 days after the last transfusion), the count of surviving cells was only 300,000 per c.mm. and it was apparent that the cells were now being eliminated at an increased rate. Thirty-nine days after the second transfusion all the donor cells had been eliminated (see Case C in Fig. 63) and Rh antibody could now be demonstrated in the patient's serum.

Moderately Reduced Survival Time of Transfused Red Cells, without demonstrable Antibodies

The existence of this type of incompatibility in man was suspected by Loutit, Mollison and Young (1943) who found that in four recipients out of thirty-five, transfused red cells failed to survive for the expected length of time. Thus, by the end of 60 days after transfusion, two of these recipients had no surviving donor red cells left in their circulation, one had 5 per cent of the original total, and one 19 per cent. By contrast, in eighteen other patients receiving blood stored for the same length of time in the same solution, the average survival at 60 days was 45 per cent.

An attempt was made to investigate this phenomenon further and one recipient was invited to co-operate in carrying out further experiments. This recipient had previously received 500 ml. of a concentrated suspension of red cells prepared from

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the blood of two donors. One of these two donors was now recalled; the blood was freshly drawn into a simple solution of trisodium citrate and transfused to the patient within a few hours. The survival of the transfused cells was very similar to that of the mixed population from two donors which was transfused on the previous occasion (see Fig. 82). It seemed unlikely that the poor survival was related to any peculiarity of the donor, since the blood of both donors had survived

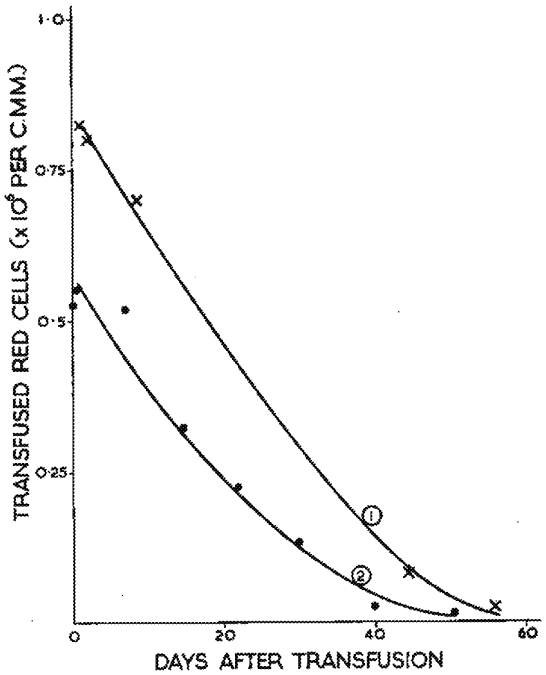

FIGURE 82. Shortened survival *in vivo* without demonstrable antibodies *in vitro*. Red cells from two donors were used for the first transfusion (1); one of these two donors was used for the second transfusion (2), given to the same recipient about 3 months later. For further details, see text.

badly on the first occasion. Moreover, the donor who had been used on two occasions for the recipient gave blood a third time and this was transfused to a different recipient. The survival was strictly normal. Finally, blood was taken from the patient herself and transfused to a patient with anaemia following parturition; the cells survived normally, some 20 per cent being present 84 days after transfusion. (Observations made jointly with Dr J. F. Loutit and recorded by Mollison, 1951, p. 107.)

It has since become clear that this phenomenon is a type of incompatibility and that, as a rule, no antibody can be detected in the recipient's serum by any existing tests. When the survival of relatively large amounts of red cells (from at least 400 ml. of blood) is estimated in recipients in whom survival would be expected to be normal, curtailment of survival is found in about one in twenty cases (Mollison, 1954a).

In some cases it can be shown that the red cells of one donor survive poorly and those of another donor survive normally or at least much

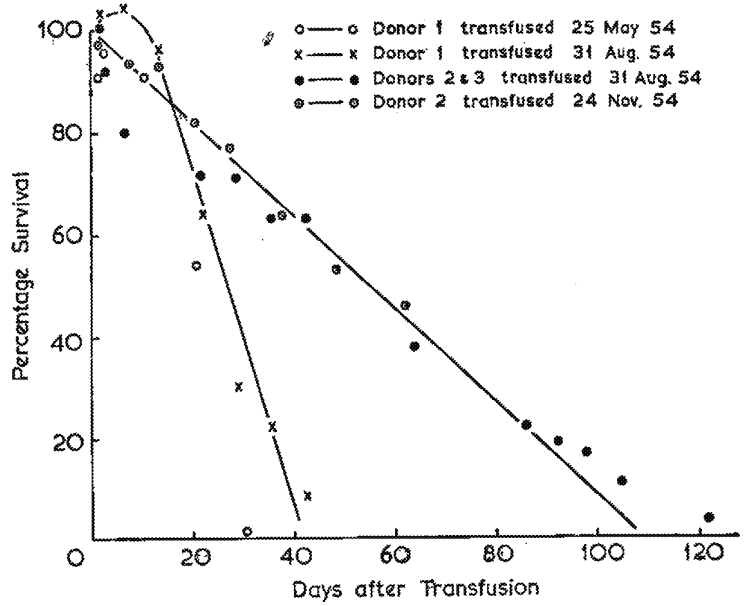

FIGURE 83. Survival of red cells from different donors in a patient with Thalassaemia-haemoglobin C disease. Red cells from 'Donor 2' survived normally on two occasions but red cells from 'Donor 1', which were also apparently compatible, judged by tests *in vitro*, were eliminated in about 1 month.

better; examples are given in Figs. 83 and 85. In other cases it can be shown that the red cells of a donor survive well on one occasion but badly on a subsequent occasion; see, for example, Fig. 23 in Mollison, 1959d; or the survival of the red cells of a given donor may be moderately curtailed on one occasion and drastically curtailed on a subsequent occasion (Jandl and Greenberg, 1957). The converse phenomenon, poor survival on the first occasion with normal survival on a subsequent occasion has been reported

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by Sjölin (1959). When survival is shortened in this way, it is common to find that survival is initially normal but that after about 15 days accelerated destruction begins and all the cells are eliminated by the end of 20–30 days after injection (see Fig. 83).

When the survival of relatively small amounts of red cells, labelled with ^{51}Cr, is estimated, curtailment of survival is found very much more often

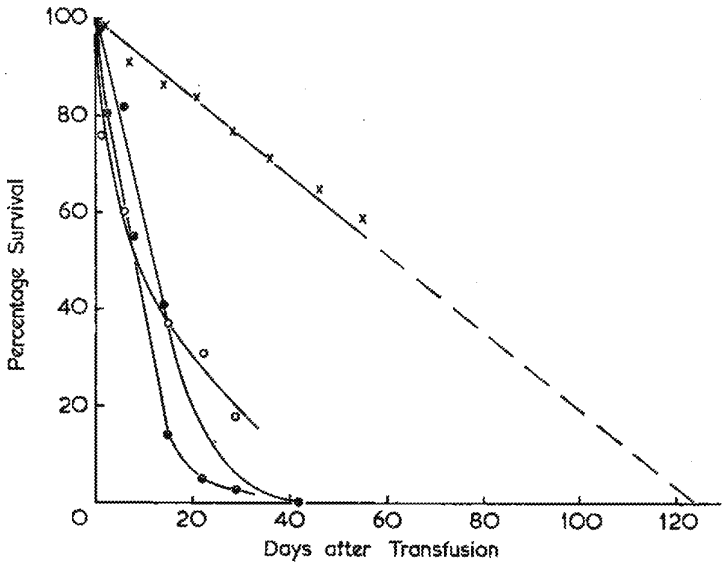

FIGURE 84. Shortened survival of transfused red cells without evidence of incompatibility *in vitro*.
●—● Survival of red cells from a particular donor estimated by the method of differential agglutination.
○—○ Survival of red cells from the same donor on a subsequent occasion estimated by labelling with ^{51}Cr.
○—○ Further estimate using ^{51}Cr as a label.
×—× Survival of donor's own red cells in her own circulation, estimated with ^{51}Cr.
All results corrected for Cr elution.
(From Mollison, 1959d.)

than when the survival of large volumes of transfused red cells is followed using the method of differential agglutination. Adner and Sjölin (1957) found abnormal survival curves in eight out of thirty adult recipients injected with 4–10 ml. of ^{51}Cr-labelled red cells either from cord blood or from adult donors. In all the eight cases survival was at first normal but after 5–18 days there was a sudden increase in the rate of disappearance of

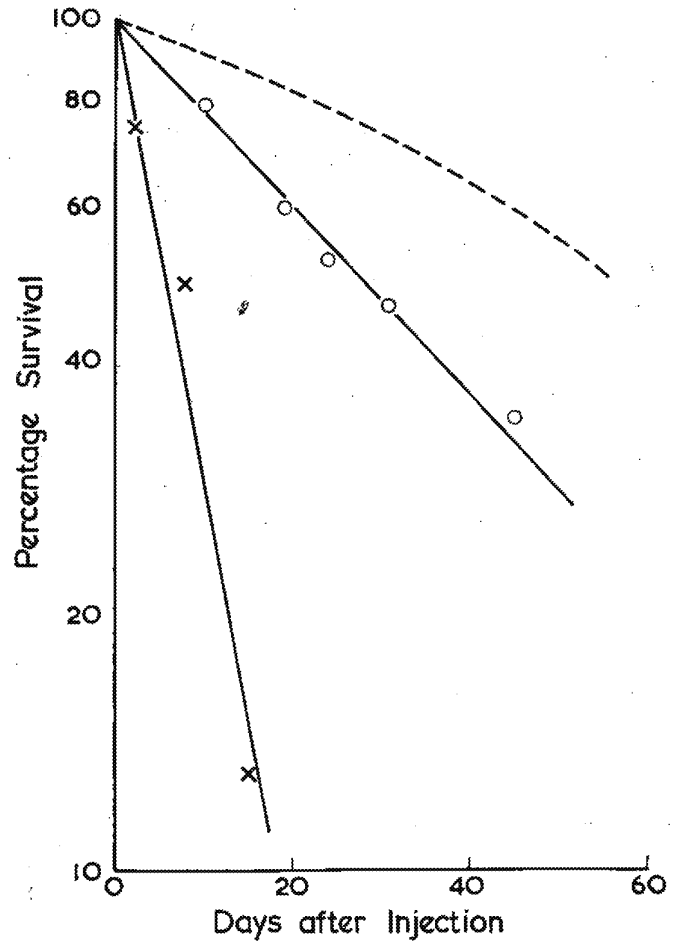

FIGURE 85. Further example of diminished survival of transfused red cells without demonstrable incompatibility *in vitro*. The recipient was the same as in Fig. 84. The red cells of one donor (○—○) survived distinctly better than those of another (×—×); the patient's serum contained no demonstrable blood group antibodies active against the red cells of either donor. The dotted line is the survival curve of normal transfused red cells (from Mollison, 1959d).

the label so that after 20–36 days more than 90 per cent had disappeared. In two of the eight cases an antibody was found (anti-E and anti-Fy^a) but in the remaining six cases no antibody could be demonstrated.

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Sjölin (1959) reported an extension of this work. Among forty-four cases in which 3-9 ml. of red cells from healthy subjects were injected into others, 'collapse' of the injected cells occurred after 6-33 days (average 18 days). Specific antibodies were found in four cases (anti-E in two cases, anti-K and anti-Fy^a in the other two). In one of these cases the antibody (anti-E) was detected 7 weeks after the first injection but in the other three the antibody was detected only after a second injection. In the case in which anti-Fy^a was produced there was 'collapse' at about 18 days following the first injection; after a second injection there was only 50 per cent survival at the end of 24 hours. In the remaining ten cases no antibody could be detected and in one of these 'collapse' occurred 8 days after injection on three successive occasions.

Sjölin had many inexplicable results. Thus in one case in which three successive injections from the same donor were given, on the first occasion there was 'collapse' after 20 days, on the second there was almost normal survival, and on the third complete 'collapse' within a few days of injection. In another case there was 'collapse' at 8 days the first time, almost normal survival the second time and completely normal survival the third time.

The experience of Drs I. W. Brown and G. Eadie (personal communication) has been similar in many respects and they estimate that when small amounts of ^{51}Cr-labelled red cells are transfused from one person to another 'collapse' of the transfused population occurs after about 20 days in 18 per cent of all cases. Among twenty-seven cases in which collapse occurred, they found antibodies in only three (one anti-C, one anti-K and one unidentified).

Kaplan and Hsu (1961) observed that 'collapse' occurred in ten out of forty-one cross-transfusions of small volumes of ^{51}Cr-labelled red cells, '... usually [at] about the 14th day after transfusion and in the next week the remainder of the transfused cells are virtually all eliminated'.

Similar cases were reported by Mollison (1956, pp. 353-5), by Jandl and Greenberg (1957) and by Goudsmit and Krijnen (1957). See also pp. 167-168.

Destruction of Donor's Red Cells by Passively Acquired Antibodies

Young (1945) has described the acquisition of the agglutinin anti-A_1 (alpha$_1$) by transfusion, under the following circumstances:

A patient of subgroup A_2 received 4500 ml. of group O blood and 2000 ml. of pooled plasma, following traumatic amputation of both his thighs. Eight days later, when a further transfusion was being considered, the patient's serum was found to agglutinate A_1 cells, due to the presence of anti-A_1 agglutinins active at 37° C. Anti-A_1 was not present in the pre-transfusion sample of the patient's serum and had evidently come from the group O plasma with which the patient had been transfused. It will be recalled that group O (and group B) plasma contains anti-A_1 as well as anti-A agglutinins. *In vitro* anti-A, but not anti-A_1, is absorbed by A_2 cells. Evidently the transfusion had been followed by absorption *in vivo* of anti-A, but not anti-A_1, by A_2 cells and tissues.

A similar case was reported by Ervin and Young (1950). The recipient, subgroup A_2, received a transfusion first of A_1 blood, then of group O blood containing immune A antibodies. The transfused A_1 cells (from five bottles of blood) were eliminated within approximately 2 days of transfusion and an anti-A_1 agglutinin could be demonstrated in the patient's plasma.

In the case reported by Grove-Rasmussen *et al.* (1951) a patient of subgroup A_2 was transfused with three bottles of group A_1 blood and one of group O. The patient became jaundiced, developed anuria and died. Forty-eight hours after transfusion all the A_1 cells had been destroyed and free immune anti-A was detectable in the patient's serum to a titre of 8 (indirect Coombs test). The donor's serum contained immune antibody; it was of special interest that 10 ml. of group specific substance had been added to the O blood before transfusion but had evidently not rendered it safe.

DESTRUCTION OF RECIPIENT'S RED CELLS FOLLOWING THE TRANSFUSION OF PLASMA CONTAINING POTENT ANTI-A OR ANTI-B

It was Ottenberg (1911) who first suggested that persons of group O could be used as 'universal donors'. He argued that the iso-agglutinins in the donor's plasma, though theoretically capable of damaging the patient's red cells if the patient belonged to groups AB, A or B, would in fact be so diluted in the recipient's plasma as to be harmless. The use of group O blood for transfusion to patients all of groups spread rapidly. At the outset of the Second World War it was accepted that, in practice, the danger of damage to the red cells of recipients of other groups was negligible. Throughout the war, enormous numbers of transfusions of group O blood were given to patients of all groups, without any preliminary matching tests. In the vast majority of cases there was no evidence that undesirable effects were produced. It was well argued that any risks that the procedure carried were small compared with those that would be involved in

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attempting to group recipients and cross-match blood under bad conditions.

Despite this experience it is now known that the transfusion of group O plasma to group A recipients sometimes causes severe red cell destruction; the evidence must now be considered.

Transfusions to Human Volunteers

The first systematic attempt to assess the effect of transfusions of incompatible plasma was made by Aubert, Boorman, Dodd and Loutit (1942). They transfused plasma containing potent anti-A iso-agglutinins to volunteers of group A. By eliminating the complicating factor of the donor's red cells they could be certain that any haemolysis they observed was due to destruction of the patient's own cells. They observed varying degrees of haemoglobinaemia, 'intravascular agglutination' (see below), and hyperbilirubinaemia followed by a progressive reduction in red cell count. The lowest titre of anti-A agglutinin associated with signs of blood destruction was 512. In their hands such anti-A titres were found in the plasma of 40 per cent of group O donors and they therefore concluded that only about 60 per cent of group O individuals could be considered really suitable as universal donors.

Similar findings were reported by Tisdall, Garland, Szanto *et al.* (1946). In their hands, about 23 per cent of group O persons had anti-A (or anti-B) titres of 640 or higher. The transfusion of 250 ml. of plasma with an iso-agglutinin titre of 600 to 4000 frequently caused haemoglobinaemia. In one case a volunteer who received plasma with a titre of only 600 developed a sufficient degree of haemoglobinaemia to produce haemoglobinuria. Many patients had rises in serum bilirubin, though none became jaundiced. Tisdall, Garland, Szanto *et al.* also noted the phenomenon described by Aubert *et al.* as 'intravascular agglutination'; that is to say, saline suspensions of blood taken from patients immediately after the transfusion of incompatible plasma, examined microscopically, exhibited agglutination. In view of the possibility that this reaction occurred only outside the body, they preferred not to describe it as 'intravascular agglutination'.

In a further series of experiments Tisdall, Garland and Wiener (1946) took blood from a group B volunteer who had been immunized by the injection of group A specific substance, so that his anti-A titre was very high (2500). On one occasion, the injection of as little as 25 ml. of this plasma into a group A volunteer produced haemoglobinuria. However, after the addition of group specific substances to the plasma *in vitro*, as much as 250 ml. could be injected without producing signs or symptoms. In a series of cases, 10 ml. of the group specific substances (prepared according to Witebsky's method) were added to 250 ml. amounts of plasma containing potent agglutinins and transfused to volunteers. No signs of blood destruction were observed in any of the recipients. From these experiments it was concluded that only 50 per cent of group O donors could be regarded as safe, but that the blood of all group O donors could be made safe by the addition of a suitable amount of group specific substance.

These conclusions are now known to be unrealistic. Firstly the saline-agglutinin titre of plasma is not a reliable guide to its power of causing red cell destruction *in vivo*; secondly the addition of group specific substance to group O plasma does not necessarily make it incapable of causing red cell destruction.

There is now a great deal of evidence that red cell destruction caused by the transfusion of group O plasma to a group A subject depends mainly and perhaps wholly on the amount of immune anti-A in the donor's plasma.

It is true that potent immune anti-A is found most commonly in plasma which contains potent saline-agglutinins. Thus in most cases plasma with a low saline-agglutinin titre is 'safe'. Nevertheless it is possible to find samples which have a relatively low saline-agglutinin titre and a high titre of immune antibody (Crawford *et al.*, 1952). Cases have been described in which the transfusion of such plasma has caused a serious haemolytic reaction (see below).

Witebsky (1948) first demonstrated the importance of the immune type of anti-A in causing haemolytic disease of the newborn. Ervin, Christian and Young (1950) demonstrated the importance of this type of antibody in transfusion. They reported the results of giving two transfusions of group O plasma to a group A volunteer. The first transfusion was of plasma with a moderately high saline-agglutinin titre (640) but with no immune characteristics; no signs of red cell destruction developed. By contrast when the same volunteer received plasma which had a scarcely higher saline-agglutinin titre (1280) but had, in addition, immune characteristics, there was severe intravascular haemolysis and the haematocrit fell from 42 to 24 per cent in 5 days. Observations by the same authors on patients are described later in this chapter.

The reversibility of agglutination *in vivo* was discussed briefly earlier in this chapter. Loutit and Mollison (1946) referred to experiments in which approximately 200 ml. of red cells from a donor were mixed *in vitro*

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with an equal volume of potent anti-A, left for 1 hour at room temperature and then re-injected into the donor. The survival of the red cells was approximately normal, although immediately before re-injection, the cells were heavily agglutinated.

Jandl *et al.* (1957) performed two experiments with B cells agglutinated *in vitro* with anti-B. In one experiment 26 per cent of the cells disappeared immediately from the circulation following re-injection but almost all of them slowly returned to the circulation and thereafter survived normally. In the other case survival was strictly normal.

Although red cell agglutination appears to be largely reversible *in vivo* it is perhaps unwise to regard it as a totally harmless phenomenon. Pearce (1904) prepared anti-dog red cell agglutinating serum in rabbits and injected this into dogs. Four minutes after injection, heart blood contained large clumps of red cells; at autopsy almost the only lesions were in the liver, where a necrosis was found round the portal vessels within 24 hours of the injection.

Observations on Patients

When the transfusion of group O blood to a group A (or B) patient is followed by a haemolytic reaction it is necessary to obtain evidence that the donor's red cells have not been destroyed before blaming the donor's plasma for the reaction. Wiener and Moloney (1943) investigated a haemolytic reaction in a group A patient transfused with group O blood; using the method of differential agglutination they showed that the transfused red cells had survived and thus obtained proof that the signs of haemolysis were due to destruction of the patient's own red cells.

Before this, several very suggestive cases had been described, particularly by Muller and Balgairies (1936) and DeGowin (1937); it is of some interest that in each of these two cases the donor's serum contained a potent anti-A haemolysin, acting at a dilution of 1 in 10.

Gasser (1945) described haemolytic reactions following the transfusion of plasma. In three group A (or AB) infants, weighing 3.5 to 4.5 kg., who received 50–90 ml. of group O plasma, jaundice developed within a few hours. Examination of films of peripheral blood showed striking microspherocytosis; in one case an increase in osmotic fragility was demonstrated. An interesting point in two cases was that plasma from the same donor had been given on the previous day without producing reactions. This suggests that the capacity of the body to neutralize A iso-antibodies had been saturated by the first plasma transfusion so that a second transfusion within a short time had a more damaging effect.

Ebert and Emerson (1946) also found that the transfusion of group O blood to group A patients frequently produced an increase in the osmotic fragility of the patient's red cells. In each of seven group A patients who received blood with an anti-A titre of 100 or more, there was a definite increase in osmotic fragility after transfusion. In many of the cases, after transfusion, haemolysis of the cells began in 0.65 per cent saline compared with less than 0.50 per cent saline in normal subjects.

Ebert and Emerson also observed a rise in osmotic fragility in patients receiving large volumes of pooled plasma containing only low titre agglutinins, although almost all of the patients had received some group O blood as well. Signs of red cell destruction, including haemoglobinuria, have also been observed in group A patients receiving large amounts of 'small-pool' plasma, that is, plasma prepared from the blood of ten donors of different ABO groups (namely four group O, four group A and two group B or AB). The subjects were patients with burns who had been transfused with an amount of small-pool plasma equivalent to four times their plasma volume (personal communication from Drs J. P. Bull, W. d'A. Maycock, Dorothy Parkin and Elizabeth M. Topley).

Ebert and Emerson noted only about 1 per cent of frank haemolytic reactions in patients of group AB, A or B receiving routine transfusions of group O blood; but they considered that some asymptomatic destruction of recipient's cells was almost invariable after multiple transfusions of group O blood or pooled plasma. They were often able to demonstrate progressive destruction of the patient's red cells for periods up to 1 week after transfusion.

As already mentioned, it was observations made by Ervin *et al.* (1950) that first called attention to the importance of immune characteristics in causing red cell destruction *in vivo*. They investigated four severe haemolytic reactions in group A patients following the transfusion of group O blood and showed that in all four cases the donor's plasma displayed immune characteristics. Signs of red cell damage persisted for relatively long periods after transfusion. Thus osmotic fragility was increased for 8–11 days and microspherocytes were seen in films of peripheral blood for approximately 2 weeks after transfusion.

The following case illustrates many of the features of a severe haemolytic transfusion reaction in a group A patient after the transfusion of blood from a 'dangerous' group O donor:

The patient was a woman aged 23. Following an otherwise normal delivery the placenta was retained and the patient lost some 800 ml. of blood. She was known to be Rh-positive but her ABO group had not been determined. A transfusion of

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group O, Rh-positive blood was begun. Then, under pentothal anaesthesia, the placenta was removed manually. After the first bottle of blood had been given, the transfusion of a second bottle was started.

Some 2½ hours after the beginning of the transfusion, the patient was starting to

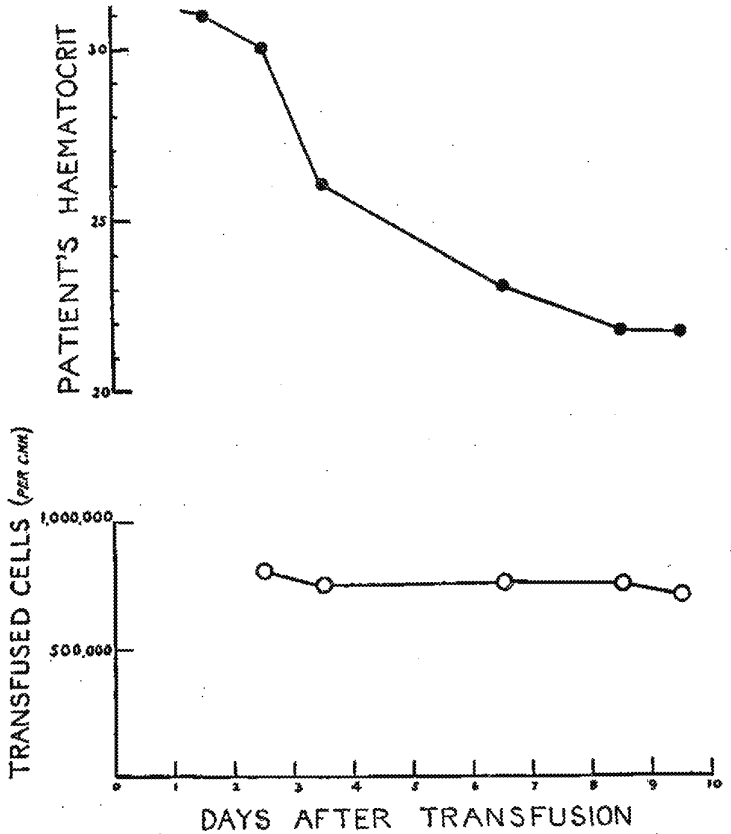

FIGURE 86. Destruction of recipient's own (group A) red cells following the transfusion of group O blood, the plasma of which contained immune anti-A. The transfused cells survived normally and the progressive fall in the recipient's haematocrit was therefore due entirely to the destruction of the patient's own cells.

come round from the anaesthetic. She was heard to be making a grunting noise and was found with her eyes turned up. Her limbs were in spasm and frothy sputum was issuing from her mouth. Her pulse was variable in volume, rate 80 per minute; blood pressure, 80/40; respirations deep and rapid; no cyanosis. The transfusion was stopped, a total of one and three-quarter bottles (approximately 800 ml.)

having been given. The patient was given oxygen to breathe and an injection of morphia (30 mg.). Her condition improved rapidly and soon her blood pressure had risen to 110/70. Twelve hours later some dark brown urine was obtained by catheter and found to contain oxyhaemoglobin. Fourteen hours after the transfusion the patient was noted to be slightly jaundiced. She was treated by a modification of the Borst regime and recovered rapidly (for further details, see Case 1 of Bull *et al.*, 1957).

Investigation of a blood sample taken some 12 hours after the transfusion showed that the patient's serum agglutinated B cells but not A cells, and that her red cells were agglutinated by anti-A and anti-Rh; the direct Coombs test was positive. This raised the possibility that the patient might be Rh-negative with anti-Rh in her serum and that surviving Rh-positive cells were responsible for the positive direct Coombs test. However her infant, though Rh-positive, had a negative direct Coombs reaction. Moreover, a quantitative test on the mother's blood showed that all the cells in her circulation were Rh-positive. On the other hand, a quantitative test with an anti-A serum demonstrated that about 900,000 group O cells per c.mm. were present. It was now clear that all the donor cells had survived and that the haemolytic reaction could have been due only to the destruction of the patient's own red cells.

Suspicion naturally fell on the anti-A content of plasma of the two bottles of blood that had been used, and it was soon found that the first bottle had a quite exceptionally high titre of anti-A. Moreover the plasma had immune characteristics; that is to say, it was strongly haemolytic and, after partial neutralization with A substance, it gave a positive indirect Coombs test with A_1 cells. The patient's own cells continued to give a clear-cut positive direct Coombs test for 48 hours after transfusion. Subsequently, the reaction became progressively weaker; it was faintly positive at 9 days and negative 10 days after transfusion.

There was a progressive destruction of the patient's cells for about a week after transfusion, demonstrated by a progressive fall in haematocrit; this destruction did not affect the transfused group O cells (see Fig. 86). It can be calculated that during the week after transfusion, red cells equivalent to some 800 ml. of whole blood were destroyed. This ignores the red cells destroyed during the 36 hours immediately following the transfusion.

In this case, the 'dangerous' group O donor was an unmarried girl aged 20 who had never had any transfusions or injections of blood. However 38 days before giving blood she had had an injection of anti-tetanus serum, after falling down and cutting her leg. As explained in Chapter VII, the injection of anti-tetanus (horse) serum frequently stimulates the formation of potent anti-A immune bodies. The immune character of the anti-A in this donor was demonstrated not only by showing that it possessed various chraacteristic properties, such as the ability to sensitize A_1 cells after partial neutralization of the serum with A substance, but also by observing that the anti-A titre fell to a comparatively low level 4 to 5 months after the injection of the horse serum.

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Haemolytic Reactions after Transfusions of Plasma Containing only Low Titre Agglutinins but Potent Immune Anti-A

Although it is true that most sera which contain potent iso-agglutinins also contain iso-haemolysins, several instances are now known of the production of a serious degree of red cell destruction by serum having only an average content of saline-agglutinin but a raised content of immune antibody. For example, Grove-Rasmussen *et al.* (1953) described a patient of group A who developed haemoglobinuria following the transfusion of group O blood the plasma of which had an anti-A saline agglutinin with a titre of only 32-64 but contained potent immune anti-A.

Stevens and Finch (1954) described a severe haemolytic reaction in a group A subject transfused in 55 minutes with 350 ml. of citrated group O blood. After 200 ml. had been transfused in 30 minutes the patient complained of tingling in his hands and developed flushing of the face; after 55 minutes he started to shiver; the plasma haemoglobin concentration was 280 mg./100 ml. *In vitro*, the donor's plasma completely haemolysed the recipient's cells in 4 minutes; however, the saline-agglutinin titre was only 16. The donor had received anti-tetanus serum 7 months previously.

Incompatibility in a Case of Parabiosis

Lauer (1941) reported a remarkable case in which the circulations of two sisters, of different blood groups, became joined for a few days.

In a railway accident a girl sustained severe damage to her foot, involving considerable loss of tissue from the sole. With the object of obtaining tissue to repair this injury, the injured foot was temporarily grafted to the thigh of the patient's sister. Although the donor of the tissue belonged to group O and the recipient to group A, the graft was initially successful. During the second half of the week following the grafting, both sisters became ill, and it was clear that a connection had been established between their respective circulations. From the point of view of transfusion terminology, each may be regarded as donor and recipient with respect to the other; for the present purpose the terms are here used with respect to giving and receiving of the graft.

The donor (group O), although subjectively well, became jaundiced and febrile and was found to have haemoglobinuria; her red cell count was found to be 3.6 million per c.mm. At the end of the week the recipient (group A) began to vomit and was found to have become very anaemic (red cell count 1.8 million per c.mm.); a blood smear showed many normoblasts.

On the eighth day venous samples were taken for examination and the blood of both patients was found to contain only group O red cells, with anti-A and anti-B in the serum. The sisters were promptly separated and both recovered uneventfully.

Further examination of the recipient's blood (originally group A) revealed that no agglutination occurred with anti-A sera, even when abnormally potent sera were used. Four days after the separation, group A cells reappeared in the recipient's circulation. After the fourteenth day anti-A agglutinins were no longer demonstrable in the circulation.

Titration of the recipient's serum on the day of separation showed an anti-A titre of 32 and an anti-B titre of 16. The donor's serum was also titrated on the day of separation. Whereas the anti-B titre was found to be 16, the anti-A titre was 2048. The latter remained at this high level for 3 days and then fell gradually. At the end of 6 months it was found to be only 2.

Animal Experiments

Phenomena very similar to those observed in human beings can be produced in dogs by the transfusion of plasma incompatible with the recipient's red cells (Young, Ervin and Yuile, 1949). Haemoglobinaemia may not be maximal until 5 hours after transfusion; some degree of haemoglobinaemia may persist for as long as 72 hours. Red cell destruction continues for far longer; for instance in one case the haemoglobin concentration continued to fall for 9 days, whilst spherocytosis and an increase in osmotic fragility persisted for 20 days. As in the human subject, the maximum change in fragility was not seen until 24 hours after transfusion.

The above experiments were all performed with plasma capable, *in vitro*, of haemolysing the red cells of the recipient dog. Young *et al.* (1950) have performed further transfusion experiments with plasma which, *in vitro*, was not capable of haemolysing red cells and appeared to contain iso-agglutinins only. Transfusion of such plasma produced no haemoglobinaemia, bilirubinaemia or increase in osmotic fragility and no increase in bile pigment excretion.

Summary of Effects of Incompatible Plasma (anti-A and anti-B)

The following summary of the main features observed in group A patients transfused with plasma containing immune anti-A is based partly on observations reported above and partly on other material not yet referred to (unpublished observations made in collaboration with Dr H. Chaplin, Miss H. Crawford and Miss M. Cutbush).

Clinical Signs of Red Cell Destruction

Haemoglobinuria occurs only in the most severe cases; that is, when the haemoglobin-binding capacity of serum haptoglobin is exceeded. In other

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cases even when definite evidence of red cell destruction is obtained, there is only mild transient haemoglobinaemia.

Thus, in a group A subject (M. D.) who was transfused in 75 minutes with 275 ml. of group O blood, the plasma of which had a haemolysin titre of 24 (agglutinin titre 128) the plasma haemoglobin concentration 5 minutes after the end of the transfusion was 47 mg./100 ml.; 3 hours later it was only 8 mg./100 ml. From measurements of red cell volume it was estimated that 200 ml. of red cells were destroyed in the following 5 days. Osmotic fragility increased from 0.44 per cent NaCl (median corpuscular fragility) before transfusion to 0.51 per cent NaCl on the following day.

In a case observed by Colonel J. H. Akeroyd (personal communication), a group A_1 volunteer was transfused with plasma containing potent immune anti-A. Although there was negligible haemoglobinaemia (10 mg./100 ml.) immediately after the infusion, during the following 3 days the packed cell volume fell from 50 to 37 per cent.

Jaundice occurring within a few hours of transfusion was observed in infants by Gasser (1945) and has occasionally been noted, rather later, in adults. Thus in Colonel Akeroyd's volunteer referred to in the previous paragraph the serum bilirubin concentration rose slowly after transfusion to reach a maximum of 8 mg./100 ml. at 60 hours; the subject had by then become jaundiced and remained so for 4 days.

Progressive anaemia is the most commonly observed sign of red cell destruction; the packed cell volume may continue to fall for at least 1 week after transfusion; see Fig. 57 for an example.

Effects on Red Cells Demonstrable by Laboratory Tests

Spontaneous agglutination of whole blood samples withdrawn from the recipient is an invariable feature. It occurs even when plasma containing relatively weak immune anti-A is transfused:

Thus in one patient transfused in 20 minutes with 340 ml. of blood from a donor whose serum had a saline agglutinin titre of 64 and a haemolysin titre of 4, a sample of blood taken immediately after transfusion showed strong spontaneous agglutination; this was best demonstrated by spreading a drop of blood on an opal tile. Three hours later the sign was still present, but was weaker; the next day it was not present. In patients transfused with potent anti-A spontaneous agglutination of blood samples *in vitro* may persist for more than 24 hours.

The direct antiglobulin test becomes positive in group A subjects transfused with plasma containing even weak immune anti-A. Thus in the subject referred to in the previous paragraph, the direct antiglobulin test

was definitely positive immediately after transfusion, was weakly positive the next day and was negative thereafter. In patients transfused with potent immune anti-A the direct antiglobulin test may remain positive for as long as one week.

The osmotic fragility of the patient's red cells increases when incompatible plasma is transfused. As reported by Ervin *et al.* (1950) the changes are greater 24 hours after transfusion than immediately after transfusion. Personal observations have shown that the changes are almost maximal 3 hours after transfusion. As reported by Ervin *et al.*, osmotic fragility may remain elevated for at least 11 days and during this period films of peripheral blood show microspherocytosis.

Destruction of Recipient's Red Cells by Antibodies of Blood Group Systems other than ABO

Haemolytic reactions caused by the transfusion of plasma containing a blood group antibody outside the ABO system are almost unknown. The explanation lies partly in the fact that antibodies other than anti-A and anti-B are relatively rare in donors and partly in the fact that when these antibodies do occur they are seldom sufficiently potent to cause appreciable red cell destruction.

The commonest potent immune antibody in human serum, apart from anti-A and anti-B, is anti-Rh. The destructive power of this antibody can be assessed in two ways: first by experiments in which the antibody is used to treat small samples of red cells *in vitro* before re-injecting them into the circulation and then following their survival, and secondly by transfusing plasma containing the antibody to a suitable subject. Experiments of the first kind have been described above and it was pointed out that if red cells are treated with an equal volume of a preparation with an anti-Rh titre of 1000, all the cells will be rapidly destroyed, whereas if they are treated with one-tenth of their volume of the same preparation, destruction will be negligible. If these findings are translated into terms of transfusion, it can be calculated that in a recipient having a total circulating red cell volume of a 1000 ml. (as in anaemia) the transfusion of more than 100 ml. of plasma containing an anti-Rh titre of 1000 might cause some red cell destruction. In practice such a titre is rare and titres even as high as 256 are not encountered very often. Even if the titre were 256 it appears that about 400 ml. of plasma would be required to produce appreciable destruction. These conclusions agree reasonably well with experiments carried out by Jennings and Hindmarsh (1958). Thus the transfusion of

RCPE0002068_0259

200 ml. of plasma containing incomplete anti-D with an indirect antiglobulin titre of 256 produced a direct antiglobulin reaction at 24 hours with spherocytosis and the recipient's haemoglobin concentration fell by 2.5 g./100 ml. during the following week. In the second case in which 250 ml. of plasma with a titre of 128 was transfused the recipient's bilirubin concentration rose to 2.5 mg. at the end of 5 hours and the recipient's haemoglobin concentration fell by 2.6 g./100 ml. in 1 week.

In several recipients transfused with plasma containing antibodies with titres between 8 and 16 there was no definite evidence of red cell destruction.

In a series of cases published by Mohn *et al.* (1961) in which normal volunteers received 250 ml. of plasma containing moderately potent anti-D (or anti-c or anti-K or anti-M), there were no definite signs of red cell destruction (due partly perhaps to the fact that the red cell volume of the recipients was considerably greater than in the subjects studied by Jennings and Hindmarsh (1958)). In one subject transfused with a serum containing an exceptionally potent anti-Rh (indirect antiglobulin titre of 1000; serum albumin titre of 1 in 400,000) the packed cell volume fell from 48 to 22 in 12 days and spherocytosis and mild haemoglobinaemia persisted for 2 weeks (Bowman *et al.*, 1961).

SYMPTOMS AND SIGNS PRODUCED BY INCOMPATIBLE TRANSFUSIONS

It is convenient to consider together the signs of increased blood destruction, from whatever cause, and this will be done in the following chapter. This section will be concerned solely with those symptoms and signs which are peculiar to the transfusion of incompatible blood.

Symptoms and Signs during the Transfusion of Incompatible Blood

The commonest of these are as follows: feeling of heat along the vein into which the blood is being transfused and flushing of the face; pain in the lumbar region and a constricting pain in the chest. The following three descriptions emphasize these points:

The first is from a report by Denis (1667-78) of the transfusion of calf blood to a human subject; after 6-7 oz. (about 200 ml.) of blood had been transfused, the patient 'found himself very hot along his arm and under the armpits'. After a second larger transfusion on the following day (of about 1 lb. or approximately 450 ml. of blood) 'he felt the like heat along his arm and under his armpits which he had felt before ... and he complained of great pains in his kidneys and that he was not well in his stomach and that he was ready to choke unless they gave him his liberty'.

The second case was that of a young woman of group O to whom 45 ml. of group A blood had been inadvertently transfused on the previous day:

Almost immediately after the beginning of the transfusion the patient felt her face 'flaming'; then she had a very severe throbbing pain in her lumbar region and felt faint. The pain in the back was intense. The transfusion was then stopped. Afterwards she shivered for about an hour and then became very hot and complained of headache. Her further progress was uneventful.

The third case was that of an elderly woman of group O who had inadvertently been transfused with group A blood 3 weeks previously. She agreed to receive test injections of very small amounts of A blood for research purposes. In view of the fact that her serum contained a potent anti-A, strongly haemolytic *in vitro*, the amount of A blood injected was kept to a minimum. She received approximately 0.7 ml. of A_1 cells (labelled with ^{32}P) together with a solution of haemoglobin prepared from approximately 1 ml. of A_1 red cells (labelled with ^{51}Cr).

The mixture was diluted in saline and injected over a period of 3 minutes. Before the injection was completed the patient's face became flushed. Two minutes later she complained of constricting pain in the chest and pain in the lumbar region. The symptoms had disappeared completely 15 minutes later but during the night the patient vomited and had diarrhoea. She had quite recovered by the following day.

Such symptoms are not observed when similar amounts of haemolysed compatible blood are injected into the circulation; it is therefore safe to assume that they are caused by some substance released by the combination of antigen and antibody.

It seems that symptoms occurring within 2 or 3 minutes of injection are observed only when there is substantial intravascular lysis of the injected cells. The present author has experience of injecting 0.2 to 1.0 ml. of incompatible red cells on some fifty occasions but has only once (in the case referred to above) induced any symptoms in the recipient. Jandl and Tomlinson (1958) gave injections of 10 ml. of washed incompatible A or B red cells and observed transient symptoms (lumbar pain, dyspnoea, hyperperistalsis, flushing of face) in a few subjects. These subjects all had anti-A and anti-B haemolysins in their serum (Dr James H. Jandl, personal communication).

RCPE0002068_0260

In another series of experiments Jandl and Kaplan (1960) also observed an immediate reaction only when there was intravascular red cell destruction. In this series, injections of ^{51}Cr-labelled group B red cells were injected into a group A patient with hypogammaglobulinaemia whose serum contained no detectable anti-B. The B red cells initially underwent only slow red cell destruction corresponding to a $T\frac{1}{2}$Cr of about 10 days. After 5 days various amounts of anti-B were injected, and only when the amount of anti-B was sufficient to produce intravascular lysis were any symptoms noted, namely facial flushing beginning 2 minutes after injection lasting 45 minutes, followed (7 minutes after injection) by pain in the groins, thighs and lower part of the back, lasting 45 minutes.

It appears that it is the release of the stroma of incompatible red cells within the circulation that produces the signs and symptoms because the injection of a stroma-free haemolysate is without effect (Hadnagy and Szabó, 1959).

In subjects whose serum contained anti-D the injection of 10 ml. of washed D-positive cells produced no immediate symptoms but chills and fever developed after about 1 hour. Subjects receiving red cells of incompatible ABO group developed a small rise in body temperature but no chills (Jandl and Kaplan, 1960).

The symptoms provoked by the transfusion of incompatible blood are, of course, abolished by anaesthetics (see Young *et al.*, 1947) and modified by morphia:

> A woman aged 30, grouped as A, was transfused with 1000 ml. of group A blood. After receiving 5 ml. the patient complained of throbbing in the head and back, and the transfusion was stopped. The symptoms were considered to be 'neurotic', however, and omnopon 20 mg. was injected. The transfusion was re-started and 500 ml. were given, without the production of further symptoms, in $1\frac{3}{4}$ hours. At the end of that time a second injection of omnopon was given. The patient slept well, and on waking in the morning had no symptoms. No abnormality of the urine was noted and she did not become jaundiced. The blood urea was estimated on the sixth day after transfusion and found to be normal (30 mg./100 ml).
>
> The patient was first seen by the author 9 days after transfusion, not because any suspicion of incompatibility had been aroused, but merely because a request had been received for a further transfusion. Investigations showed that her blood group was O and not A as had been previously believed. Her cells showed no trace of agglutination with a potent anti-A serum, demonstrating (as might have been expected) the complete elimination of the blood given at the first transfusion. Titration of the recipient's iso-agglutinins showed that, whereas the anti-B agglutinin had a titre of 1000, the anti-A titre was approximately 200,000. Recovery was uneventful (from Mollison, 1943a).

In patients who are anaethetized or are under the influence of drugs during transfusion the only two signs which may call attention to the possibility that incompatible blood has been transfused are, first, hypotension despite apparently adequate replacement of blood, and secondly, abnormal bleeding; this latter syndrome is discussed a little later in this chapter.

Effect on White Cells of the Transfusion of Incompatible Red Cells

It is an old observation that following the transfusion of incompatible blood, erythrophagocytosis may be observed in samples of peripheral blood (Hopkins, 1910; Ottenberg, 1911). More recently it has been found that gross leucopenia may occur. Thus Jandl and Tomlinson (1958) observed that following the injection of 10 ml. of 'ABO incompatible' red cells, the total leucocyte count sometimes fell from 9000 to 4000 per c.mm. in 5 minutes. The biggest effect was on the mature granulocytes and monocytes. In further experiments they showed that the injection of as little as 0.05 ml. of incompatible red cells (5×10^8 cells) caused a significant fall in leucocytes. They calculated that as many as fifteen leucocytes were removed for each incompatible red cell injected. Intravenous injections of the stroma of compatible cells produced no effect but the injection of the stroma of incompatible cells produced leucopenia. Rather surprisingly, the injection of A substance into O subjects produced leucopenia in six out of seven instances.

When 10 ml. of D-positive cells were injected into subjects whose serum contained anti-D, leucopenia developed more slowly, becoming maximal at 1 hour. The injection of 50 ml. of compatible but non-viable red cells (stored at 37° C. for 48 hours) did not produce leucopenia (nor did it produce a chill).

Abnormal Bleeding following the Transfusion of Incompatible Blood

When red cells are lysed, thromboplastic substances are liberated. Gross intravascular lysis is therefore likely to lead to the deposition of fibrin and subsequently to afibrinogenaemia. In dogs it has been shown that the infusion of large amounts of haemolysed blood results in gross prolongation of the clotting time (Fogelman *et al.*, 1952).

In man there are many reports of abnormal bleeding developing during the transfusion of incompatible blood.

In a case reported by Wiener (1954a) a patient was transfused 1 week after an operation for prostatectomy; he had been transfused twice before, once at the time of operation and once a year previously. Following the third transfusion of 250 ml. of blood the patient began to bleed from the

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site of operation; he was given more blood and taken to the operating theatre, where he died. It was found that the blood he had been given was incompatible due to the presence in his serum of anti-c.

In a case reported by Schneider (1956) a group O patient developed a haemorrhagic state following the transfusion of 100 ml. group A blood. A sample of venous blood failed to clot; 5 hours later, a further sample was obtained and treated with thrombin; only a very small clot formed. Two further cases were described by Krevans *et al.* (1957). In the first, severe bleeding and hypertension developed in a group O subject who had been transfused under anaesthesia with 500 ml. of group A blood. Seven hours later the plasma fibrinogen was 32 mg./100 ml. In a second case a group O patient developed backache, breathlessness and dyspnoea shortly after the start of transfusion of group B blood. 500 ml. were transfused and the patient developed a fever. Four hours after transfusion vaginal bleeding was noted and 5 hours after this the plasma fibrinogen was found to be only 42 mg./100 ml. In both these cases there was hypoprothrombinaemia and thrombocytopaenia as well as hypofibrinogenaemia.

In a case described by Moore (1958), 360 ml. of citrated goup A blood were transfused in 2¼ hours to a group O woman following operation. A blood sample taken 1¼ hours later contained no fibrinogen. Intractable bleeding developed from which the patient died.

Renal Failure following the Transfusion of Incompatible Blood

Until comparatively recently it was generally believed that the excretion of haemoglobin by the kidney necessarily caused renal damage. This opinion was based largely on work done by Baker and Dodds (1925) who found that in rabbits given intravenous injections of haemoglobin solutions, renal failure occurred if the urine was acid but could be prevented if the urine was rendered alkaline. However, there are many observations to show that in normal subjects large amounts of haemoglobin can be excreted in the urine without producing any ill effects. For example, Bayliss (1920) injected intravenously into rabbits a volume of tap water equal to the entire plasma volume. There was gross haemoglobinuria but the rabbits did not appear to be harmed.

De Navasquez (1940) showed that even when the urine was deliberately made acid, large quantities of haemoglobin could be excreted by normal animals without harm.

It has been suggested that the renal damage observed by Baker and Dodds may have been related to the diminished urinary output of the animals whose urine was rendered acid and that the apparently contradictory findings of De Navasquez can be explained by the fact that in his animals urinary output was normal.

There is evidence that haemoglobinaemia itself produces transient oliguria. Thus Blackburn *et al.* (1954) induced plasma haemoglobin levels of approximately 140 mg./100 ml. in human subjects by infusing distilled water intravenously at the rate of 4 ml. a minute. Oliguria developed in seven out of ten subjects.

Mueller and Mason (1956) infused sufficient haemolysed dog blood into dogs to produce plasma haemoglobin levels of 2000 mg./100 ml. Although the dogs were fully hydrated, there was an immediate fall in urinary output following the infusions. Normal output was restored in less than 2 hours. They concluded that the extent of reduction in blood flow was insufficient to cause severe renal damage.

Earlier, Yuile *et al.* (1949) had studied the effects of large transfusions of incompatible blood in dogs whose circulation was normal. The amounts of incompatible blood transfused were equivalent to giving 200-1000 ml. to human adults. The dogs developed haemoglobinuria for periods up to 24 hours; the total amount of haemoglobin excreted in the urine was equivalent to 10 to 40 per cent of that in the transfused blood. Nevertheless, there were only transient disturbances of renal function. Usually there was an initial rise in effective renal plasma flow and glomerular filtration rate, followed by a fall to a level slightly below the initial figures. In every case the figures were back to normal by the end of 24 hours.

Yuile *et al.* suggested that renal failure accompanies haemoglobinuria only when there is some degree of previous or concomitant kidney damage. The term 'concomitant kidney damage' here includes any possible ill-effects on the renal circulation caused by severe dehydration, low blood pressure, anoxaemia, etc.

This may not be the whole story, because Mueller and Mason (1956) were able to produce renal damage in previously healthy animals by transfusing blood to which the recipient had been previously immunized. Thus dogs were transfused on two occasions with human group A blood. The first transfusions, of 200-250 ml., were well tolerated. If the second transfusion was given within 10 days of the first the dogs became only mildly ill but if the interval was 3 to 4 weeks, severe collapse developed after as little as 5-10 ml.; 50 ml. was lethal. Dogs which survived developed vomiting and diarrhoea and became cyanosed and bled from the gums. Urinary flow was sometimes arrested for 4 to 8 hours and oliguria persisted for up to 3 weeks in some animals.

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These observations strongly suggest that the transfusion of incompatible blood may itself cause the interference with renal blood flow which, combined with the excretion of haemoglobin, is sufficient to cause severe renal damage. Presumably the only kind of incompatible transfusion which can itself cause an interference with renal circulation is one which produces appreciable intravascular destruction since, as described above, it is only this type which appears to be accompanied by the release of vasoconstrictor substances into the circulation. Thus with very few exceptions only transfusions of blood of the wrong ABO group will be expected to produce an immediate interference with renal blood flow.

Treatment of Anuria following Haemolytic Transfusion Reactions

Anuria is a well-recognized sequel of severe haemolytic transfusion reactions although, as mentioned above, its onset seems to be determined to a considerable extent by the condition of the patient at the time of transfusion. In any case, the management of anuria is the same whether it follows prolonged 'shock' without transfusion or whether it follows the transfusion of incompatible blood.

Treatment may be divided into three phases: the period during the few hours after incompatible blood has been given, the period of oliguria and the period of diuresis.

Immediate Treatment

During the few hours after the transfusion of incompatible blood the patient is likely to be still oligaemic and if so, dextran, plasma or compatible blood should be transfused. In any case, the patient should be given 1 litre of 20 per cent or even 50 per cent glucose. If diuresis occurs, a further quantity, equal to the volume of urine passed, should be given but if not, no more fluid is given for 24 hours.

The Period of Oliguria

Once oliguria is established it is extremely important not to give more water than the patient can excrete. This was emphasized by Lattimer (1945) in reviewing thirty-three cases of urinary suppression. He found that of patients given less than 2000 ml. of fluid a day, none died, whereas of those given 3500 ml. or more, 75 per cent died. Bull, Joekes and Lowe (1949) stated that they had not seen a patient dying within 14 days of the onset of anuria who did not show signs of water overload in life or signs of gross pulmonary and cerebral oedema at autopsy.

The patient with anuria can excrete only about 500 ml. of fluid a day via the lungs, skin and faeces, and the fluid intake should therefore be limited to this amount. In theory, it is desirable to correct abnormalities in chloride and bicarbonate balance, but in practice it is difficult to do this. It has proved to be sufficient simply to exclude electrolytes from the patient's intake during the period of anuria.

It is known that an increase of serum potassium may cause sudden death from cardiac arrest, and it is suspected that other products of protein breakdown are harmful. Therefore, another principle of the treatment of anuria is to provide a diet which is not only free from protein but is also sufficiently rich in calories to depress endogenous metabolism (Borst, 1948).

Thus, what is required is a protein-free mineral-free diet containing an adequate number of calories and having a water content of approximately 500 ml. a day.

One standard conservative regime (Loughridge *et al.*, 1960) is as follows. The patient is given only 20 per cent lactose by mouth (lactose is not as sweet as glucose). The volume given each day is 400 or 500 ml. plus the measured fluid loss from the previous day (e.g. fluid loss by vomiting and by any urine passed).

If there is intractable vomiting (due to uraemia) sterile 40 per cent glucose in the same volume as above is given through an indwelling catheter passed into the superior or inferior vena cava. Soluble insulin is given if the blood sugar exceeds 200 mg./100 ml.

Antibiotics are given only in established infection. Transfusions of packed red cells are given to maintain the haemoglobin concentration above 10 g./100 ml.

Each day the blood urea, plasma potassium and bicarbonate are estimated and an electrocardiogram is obtained. Indications for haemodialysis are as follows:

(*a*) Blood urea more than 400 mg./100 ml.
(*b*) Plasma bicarbonate less than 12 mEq./litre.
(*c*) Plasma potassium more than 7.5 mEq./litre, despite treatment, with cation-exchange resin by mouth. (It was shown by Evans *et al.*, 1954, that approximately 100 mEq. of potassium a day could be removed by giving 100 g. of iron-exchange resin in the sodium cycle.)
(*d*) Electrocardiographic evidence of intraventricular block with widening of QRS complexes from hyperkalaemia.
(*e*) Uraemic stupor, coma, vomiting, hiccough or fits.

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The Period of Diuresis

After diuresis has been established for 48 hours the patient is in danger of becoming grossly deficient in electrolytes, particularly potassium. In this phase the patient should be given sodium and potassium in amounts equal to the urinary loss on the previous day. The diet should otherwise contain only carbohydrate until the blood urea falls below 100 mg./100 ml. (Loughridge *et al.*, 1960).

CHAPTER FOURTEEN

HAEMOLYTIC TRANSFUSION REACTIONS

A haemolytic transfusion reaction may be defined as the occurrence of signs of red cell destruction following transfusion, the most obvious of these signs being haemoglobinuria and jaundice. The previous chapter was concerned mainly with the rate at which incompatible red cells are removed from the circulation and did not deal in any detail with the resulting changes in pigment excretion. The present chapter describes the occurrence of haemoglobinaemia and hyperbilirubinaemia after incompatible transfusions and considers other causes of these signs following transfusion.

It is convenient to recognize two types of red cell destruction, intravascular and extravascular (Fairley, 1940). By intravascular destruction is meant the rupture of red cells within the blood stream with consequent liberation of haemoglobin into the plasma. By extravascular destruction is meant the removal of intact red cells from the blood stream by the cells of the reticulo-endothial system; this type of destruction is characterized by hyperbilirubinaemia, and little or no haemoglobin is liberated into the plasma.

Before dealing with the causes of haemolytic transfusion reactions, an account will be given of the way in which haemoglobin is disposed of after the red cells have been destroyed in various ways.

Intravascular Destruction

Clearance of Haemoglobin from the Plasma

The plasma normally contains a protein, haptoglobin (Polonovski and Jayle, 1940) which can bind up to about 100 mg. of haemoglobin per 100 ml. of plasma (Laurell and Nyman, 1957; Nyman, 1959; Lathem, 1959). Therefore, when amounts of haemoglobin not exceeding this level are injected or liberated into the blood stream the haemoglobin circulates as a complex with haptoglobin. This complex is not excreted by the kidney but is cleared by the reticulo-endothelial system. At very low levels of plasma haemoglobin (a few mg. of haemoglobin per 100 ml. of plasma), haemoglobin is cleared exponentially with a half-time of the order of 20 minutes (Garby and Noyes, 1959) but at higher haemoglobin levels the clearance system is saturated and a constant amount of approximately

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13 mg. haemoglobin per 100 ml. of plasma is cleared per hour (Laurell and Nyman, 1957).

Although the clearance of haemoglobin was linear in the two cases investigated by Laurell and Nyman (1957), it was exponential in some of the cases reported by Lathem (1959).

The fact that the haemoglobin-haptoglobin complex is taken up by the reticulo-endothelial system was demonstrated (in rabbits) by Murray *et al.* (1961), who also showed that in the nephrectomized rabbit, free haemoglobin, that is haemoglobin not combined with haptoglobin, was more rapidly cleared by the liver than the larger molecule of combined haemoglobin-haptoglobin.

When the binding capacity of the plasma is exceeded, free haemoglobin circulates in the plasma and some of this combines with albumin to form methaemalbumin, a pigment originally described by Fairley (1940, 1941). Apart from acute haemolytic incidents, methaemalbumin is found in the plasma only when the amount of haptoglobin has been reduced to a negligible level, that is less than 5 mg. per 100 ml. (Nyman *et al.*, 1959). According to Fairley, the rapid intravenous injection of 14 g. haemoglobin into an adult will lead to the formation of sufficient methaemalbumin to give a positive Schumm's test (see below), although about three times this amount of pigment must be present before it can be detected spectroscopically. Methaemalbumin can be detected about 5 hours after injecting haemoglobin and then remains detectable for 24 hours or more (Fairley, 1940).

When the amount of free haemoglobin in the plasma reaches about 25 mg. per 100 ml., some is excreted in the urine. The clearance of haemoglobin by the kidney is only about 5 per cent of that of water; that is the clearance is equivalent to 6 ml. of plasma per minute per 1.73 m^2 of body surface compared with 100 ml. of plasma per minute for inulin. There appears to be little or no reabsorption of haemoglobin by the renal tubules (Lathem, 1959).

The fact that in most subjects haemoglobinaemia occurs only when the plasma level exceeds about 150 mg. per 100 ml. was of course known long before the role of haptoglobin was appreciated; see Ottenberg and Fox (1938); Gilligan *et al.* (1941); Yuile *et al.* (1949). These earlier investigators also collected useful information about the time taken to clear the plasma of haemoglobin. For example, when the initial plasma haemoglobin concentration was 40-60 mg. per 100 ml., the plasma was cleared in 5 hours; when the initial level was 100-225 mg. per 100 ml., the time was 8 hours; and when the initial level was 280-300 mg. per 100 ml., the plasma was not cleared for 12 hours (Gilligan *et al.*, 1941).

After the injection or liberation of relatively large amounts of haemoglobin into the blood stream, as much as about one-third may be excreted in the urine; for example, Amberson *et al.* (1949) found that in six persons receiving rapid injections of from 12-18 g. of haemoglobin, the average amount excreted was only about 18 per cent of the amount injected. In dogs transfused with incompatible blood in amounts equivalent to the giving of 200 to 1000 ml. to an adult human, Yuile *et al.* (1949) found that the amount of haemoglobin excreted in the urine varied from 10-14 per cent of the amount in the transfused red cells. (In these particular experiments the antibody concerned was readily haemolytic *in vitro*.)

Bilirubinaemia following Intravascular Destruction

Fairley found that the serum bilirubin concentration might rise by 1 mg./100 ml. following the injection of 14-21 g. haemoglobin into an adult; the maximum concentration was reached 3 to 6 hours after injection. Gilligan *et al.* (1941) noted a similar rise in a subject injected with 16 g. haemoglobin (maximum plasma haemoglobin concentration 380 mg./100 ml.).

According to With (1949) each gramme of haemoglobin is converted to 40 mg. of bilirubin. Therefore the katabolism of 16 g. of haemoglobin should yield 640 mg. of bilirubin. If this were liberated into the plasma of an adult who was incapable of excreting bilirubin, the plasma bilirubin concentration would rise by about 10 mg. per 100 ml., assuming that about half the liberated bilirubin diffused rapidly into the extravascular fluid space (Weech *et al.*, 1941). In practice the bilirubin is delivered to the circulation over a period of several hours and excretion almost keeps pace with production.

Pigment Katabolism after Extravascular Destruction of Red Cells

It is possible for red cells to be removed from the blood stream without the liberation of any detectable amount of haemoglobin into the blood stream. Examples are given below in describing the removal of non-viable stored red cells from the blood stream. In other cases of extravascular destruction, for example, red cell destruction brought about by Rh antibodies, some haemoglobin is released into the plasma.

Whether or not there is any haemoglobinaemia, 'sequestration' of the red cells in the liver or spleen is followed within an hour or so by a rise in serum bilirubin which reaches a peak 3 to 5 hours after the onset of red cell destruction. Further details are given later in this chapter in describing cases in which predominantly extravascular destruction is observed.

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Haemolytic Reactions Characterized by Haemoglobinaemia, with or without Haemoglobinuria

Incompatibility due to Antibodies which are readily Lytic in vitro

The commonest cause of true intravascular red cell destruction is the transfusion of incompatible red cells to a subject whose plasma contains an antibody which is readily lytic *in vitro*, e.g. anti-A or anti-B. In such cases, as described in the previous chapter, up to 90 per cent of the cells may be lysed in the blood stream when a very small volume of incompatible red cells is injected and at least 50 per cent of the cells may be lysed in the blood stream even when a relatively large volume of blood is transfused.

Mrs X suffered a severe post-partum haemorrhage. Her past notes recorded that she belonged to group A, and a transfusion of group A blood was begun without any tests. Fifteen minutes later the laboratory reported that a second bottle of group A blood that had been matched against the patient's serum, with a view to continuing the transfusion, was incompatible. The transfusion of the first bottle was stopped, some 400 ml. of citrated blood having been given. After a further 20 minutes, that is to say 40 minutes after the beginning of the transfusion, the patient started to shiver, having previously shown no untoward signs of any kind. A sample of blood was obtained after a further 30 minutes. This showed that the patient was group B and that no group A cells were present; the plasma was very heavily stained with haemoglobin, the concentration being almost 1000 mg./100 ml. It was calculated that this corresponded to about 65 per cent of the concentration expected if all the cells had been haemolysed and none of the haemoglobin had yet disappeared from the plasma. A sample taken 60 minutes later contained approximately 800 mg. haemoglobin per 100 ml. of plasma.

Haemoglobinaemia associated with Red Cell Destruction by Antibodies which are not Lytic in vitro

As already mentioned, some haemoglobin is liberated into the blood stream even when red cell destruction is predominantly extravascular. Careful measurements have been provided by Jandl *et al.* (1957) who showed that when D-positive red cells were injected into subjects whose plasma contained anti-D or when D-positive cells were incubated with antibody *in vitro* and then reinjected so that the injected red cells were removed by the spleen, haemoglobinaemia developed within a few minutes of the injection and increased steadily to reach a peak 2 or 3 hours after the injection. In subjects injected with approximately 10 ml. of red cells the maximum plasma haemoglobin concentration did not as a rule exceed 6 per cent of the amount injected in the red cells but in one

subject injected with a larger amount of red cells (about 20 ml.) the plasma haemoglobin concentration reached the equivalent of 12 per cent of the injected cells. As discussed on page 505, the difference between the proportion of haemoglobin appearing in the plasma after the two doses is probably due simply to saturation, by the larger dose, of the capacity of the R.E. system to clear the haemoglobin-haptoglobin complex from the plasma. In any case, after the extravascular destruction of a relatively large volume of incompatible red cells, substantial haemoglobinaemia must be expected. In fact it has long been recognized that haemoglobinuria may follow the transfusion of incompatible blood even when the antibody concerned is not readily lytic *in vitro*. Several examples associated with Rh incompatibility were described by Vogel, Rosenthal and Levine (1947); examples associated with anti-S (Cutbush and Mollison, 1949), anti-K (Swisher and Young, 1954) and anti-Fy^a (Wiener, 1954b) have also been described.

When haemoglobinaemia occurs in a patient in whom the plasma haptoglobin level is reduced, haemoglobinuria may occur even though the level of plasma haemoglobin is relatively low. In a patient reported by Stewart and Mollison (1959) (see Fig. 81 in the previous chapter) haemoglobinuria occurred after every transfusion although when a test dose of ^{51}Cr-labelled red cells was given most of the cells seemed to be removed extravascularly since the maximum level of radioactivity reached in the plasma was equivalent to 6.7 per cent of the haemoglobin injected as red cells. Subsequent tests on plasma from this patient showed that the haptoglobin concentration was very low.

In another patient whose serum contained anti-Le^a which produced only very slight lysis of Le (a+) red cells *in vitro*, a transfusion of 250 ml. of Le(a+) blood was followed by haemoglobinuria. A subsequent test using only 2 ml. of red cells from the same donor showed that the cells were very rapidly eliminated (half-time 2 minutes) with the liberation of only very small amounts of haemoglobin into the plasma (maximum level of radioactivity equivalent to 2 per cent of the injected dose). Assuming that after a larger dose (250 ml.) of Le(a+) blood the plasma haemoglobin level may have reached the equivalent of 5 per cent of the amount injected, the plasma haemoglobin concentration would have reached at least 50 mg. per 100 ml. Although this would not have produced haemoglobinuria in a normal subject, it might well have done so in a patient with a chronic haemolytic process. The patient was in fact later shown to have a reduced plasma haptoglobin concentration.

Following the transfusion of plasma containing potent anti-A or anti-B

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to a recipient of group A or B, haemoglobinaemia is usually only mild although haemoglobinuria can occur (see pp. 486, 491 and 492).

Red Cells Swollen by Contact with 5 per cent Glucose

True intravascular destruction may be brought about by osmotic lysis of the red cells. This is thought to be the mechanism which brings about the destruction of red cells which have been left in contact with 5 per cent glucose before transfusion.

Ebaugh and Bove (1957) mixed one volume of ACD blood with two volumes of 5 per cent dextrose in 0.225 per cent sodium chloride. After 3 hours red cell volume had increased to about 140 per cent of normal and red cell glucose content had increased to about 1000 per cent of normal. When the red cells were suspended in normal plasma there was further swelling and in many instances marked haemolysis. When the cells were labelled with ^{51}Cr and injected, normal survival was usually observed although in a few instances a large proportion of the injected cells were destroyed within 10 minutes of transfusion.

Haemolytic transfusion reactions have sometimes been observed in patients receiving transfusions of whole blood passed through a bottle containing 5 per cent glucose and 0.225 per cent saline (personal communication from Dr F. G. Ebaugh).

Noble and Abbot (1959) also noted that if a giving set, of the type in which the filter is inserted into the bottle, was transferred from a bottle of blood to a bottle of dextrose, that after about 12 hours there was visible haemolysis. They showed that red cells suspended in excess 5 per cent dextrose or in 4.3 per cent dextrose with 0.18 per cent saline were almost completely lysed after 24 hours at room temperature.

Injection of Water into the Circulation

Bayliss (1920) produced haemoglobinaemia in animals by injecting a volume of tap water equal to the plasma volume over a period of 20 minutes. About one-fifth of the red cells were haemolysed, and there was a temporary reduction in urinary flow, but no other ill effects were observed.

The intravenous injection of 100 ml. of water into a human adult produces only trivial haemoglobinaemia (10-20 mg./100 ml.) but the injection of 300-900 ml., infused over a period of 1 to 4 hours produces plasma haemoglobin levels of 200-400 mg./100 ml. Water may gain entrance into the circulation when the bladder is irrigated during transurethral prostatectomy and it appears that occasionally amounts as great as 500, or even 1000 ml. may get into the blood stream with resulting

haemoglobinuria. In patients who already have renal ischaemia, this may precipitate renal failure (all information in this paragraph from Landsteiner and Finch, 1947).

Although entry of water into the circulation during irrigation of the bladder has nothing to do with transfusion, if the patient is being transfused at the time and haemoglobinaemia is detected, the transfusion may be blamed if the possibility of entry of water through the operation site is not remembered.

Simulated Intravascular Lysis

If enough free haemoglobin is injected into the circulation the recipient may develop haemoglobinuria, and intravascular haemolysis may then be suspected. Appreciable quantities of free haemoglobin may be injected in any of the following circumstances:

Transfusion of over-heated blood. Blood haemolyses if warmed to a temperature of 50° C. or more. Accidents occasionally occur when a bottle of blood is placed in a vessel containing hot water with the intention of warming the blood to body temperature. As a general rule, blood should not be warmed before transfusion; if there is thought to be some special reason for warming it, the temperature of the water into which the bottle is to be placed should be checked with a thermometer and, as an extra precaution, felt with the hand to ensure that it is not above body temperature.

Landois (1875) showed that red cells were irreversibly damaged by being heated to approximately 50° C. Harris *et al.* (1957) heated ^{51}Cr-labelled red cells to 49.6° C. for 15 minutes and re-injected them into the circulation. Surface counting showed a progressive rise over the spleen reaching a plateau at 60 minutes. On the following day only 10 per cent of the injected cells were still present in the circulation. An experiment by the present author is illustrated in Fig. 87; although there was no haemoglobinaemia following the injection of the washed, labelled red cells, there would of course be gross haemoglobinaemia following the transfusion of whole haemolysed blood.

Transfusion of frozen blood. Blood is sometimes stored in refrigerators whose temperature is not correctly regulated; if the temperature falls below about −3° C., the blood may freeze and when it is subsequently thawed it may be severely haemolysed. Such an accident can be prevented by storing blood only in refrigerators provided with a recording thermometer or an alarm signal which rings when the temperature falls outside a

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predetermined range. In any case blood which has undergone appreciable lysis through freezing has a purple appearance which should provide a warning.

Transfusion of infected blood. Blood which is contaminated with certain bacteria becomes grossly haemolysed. The transfusion of such blood may produce haemoglobinuria but this sign is likely to pall into insignificance

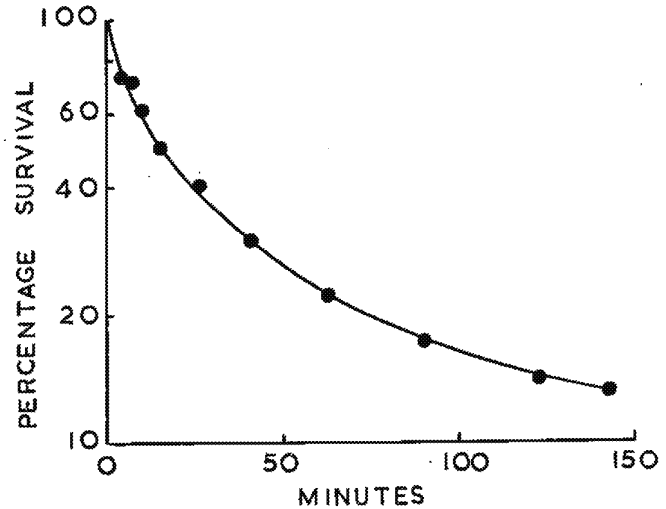

FIGURE 87. Rate of removal from the circulation of red cells damaged by being heated. A sample of red cells was taken from a normal subject and labelled with ^{51}Cr. The suspension was then placed in a water bath at 50° C. for 20 minutes. The red cells were washed three times and the suspension was then re-injected into the donor's circulation. Destruction of the damaged cells appeared to be entirely extravascular (splenic) and the amount of radioactivity in the plasma never exceeded a level corresponding to 0.5 per cent of the total dose injected.

compared with the very toxic effects of bacteria, for a discussion of which see the following chapter.

Blood haemolysed by being injected under pressure. If blood is forced through a needle with sufficient pressure, some of the red cells will be ruptured. Experimentally it is possible to haemolyse blood by injecting it very rapidly even through a relatively wide-bored needle, e.g. gauge (SWG) 19 (unpublished observations). In practice significant haemolysis is likely to be produced only when trying to inject blood through a very fine needle. In one case which the present author helped to investigate,

haemoglobinuria which developed following a transfusion was traced to this cause.

The patient was an infant aged 1 month. Fifty-five millilitres of a partially packed suspension of red cells from 10-day old stored blood was injected through a 24-gauge needle into a scalp vein. Considerable pressure was needed to inject the blood. One hour after the transfusion dark red urine was passed and later found to contain about 850 mg. Hb/100 ml. A sample of plasma taken at about the same time contained approximately 400 mg. Hb/100 ml. By the technique of differential agglutination it was demonstrated that the infant's blood contained only about 30 per cent of the expected number of transfused red cells. No serological cause for the destruction could be found and it was concluded that the blood had been haemolysed by being forced through a fine needle. Experiments showed that when stored blood was injected through a 24-gauge needle at rates exceeding about 0.3 ml. per second the amount of lysis increased very rapidly. When a 22-gauge needle was used there was very little lysis until the rate of injection exceeded about 1.5 ml. per second (Macdonald and Berg, 1959).

As a practical guide, one can take it that no serious degree of haemolysis will be produced unless one has to use really considerable pressure to force the blood through the needle (personal observations).

Haemoglobinaemia following cardiac by-pass. Blood which is passed through pump-oxygenators, otherwise known as 'heart-lung' machines, undergoes slight haemolysis. With the earlier machines, the patient's plasma haemoglobin level sometimes reached about 100 mg./100 ml. (see for example Kay *et al.*, 1956), but with present machines the patient's plasma may remain free of visible haemoglobin (i.e. level less than 30 mg./100 ml.) even after 2 hours' perfusion at 4 l. a minute (personal communication from Dr Denis Melrose).

Haemoglobinaemia following Transfusions of Stored Blood.

Vaughan (1942) studied pigment metabolism after giving transfusions of stored blood to four subjects. She failed to detect haemoglobinaemia or methaemalbuminaemia but observed considerable rises in serum bilirubin; the highest concentrations of bilirubin were attained 5 to 6 hours after transfusion. Mollison and Young (1942) who gave many transfusions of aged blood, stored in poor preservative solutions, very seldom observed haemoglobinaemia, and when noted it may well have been due to the transfusion of blood which had already been haemolysed *in vitro*.

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Like Vaughan, they found that the rise in serum bilirubin was greater 5 hours after transfusion than immediately after transfusion; after 24 hours the bilirubin values had returned to normal.

Jandl and Tomlinson (1958) studied the removal from the blood stream of non-viable red cells labelled with ^{51}Cr and were impressed by the fact that no ^{51}Cr at all was liberated into the plasma. Observations by the present author have confirmed these findings; thus after the injection of 10 ml. red cells stored in trisodium citrate at 4° C. for 2 weeks to render

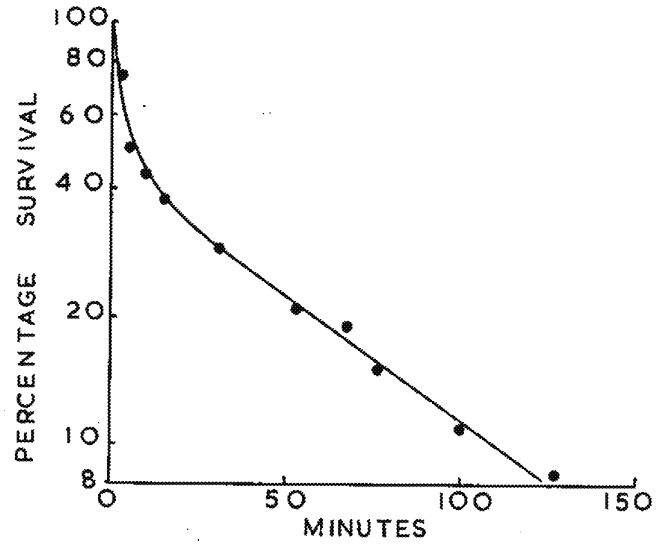

FIGURE 88. Rate of destruction of 'non-viable' stored red cells. A sample of blood was taken from a normal subject and stored at 4° C. with trisodium citrate for 2 weeks. The red cells were then washed, labelled with ^{51}Cr, washed again and injected. As the figure shows, the red cells were virtually all removed from the circulation in 2 hours. The amount of radioactivity in the plasma never exceeded a level corresponding to 0.2 per cent of the total dose injected.

the cells non-viable, 50 per cent of the cells were removed from the circulation within a few minutes of re-injection, but the amount of ^{51}Cr in the plasma was insignificant (less than 0.2 per cent of the dose injected); see Fig. 88.

Cassell and Chaplin (1961) made further observations, using the very sensitive method of estimating plasma haemoglobin referred to later in this chapter. They found that even when large amounts of old stored red cells were transfused there was no evidence of liberation of haemoglobin

into the blood stream. Any free haemoglobin found in the plasma after such transfusions could be accounted for precisely by taking into account the amount of haemoglobin in the plasma of the transfused blood.

Haemolytic Reactions characterized by Hyperbilirubinaemia with or without jaundice

As already explained, hyperbilirubinaemia follows red cell destruction whether this is predominantly intravascular or extravascular. In this section the syndromes to be considered are those in which destruction is predominantly extravascular so that hyperbilirubinaemia is the most important sign of red cell destruction.

Hyperbilirubinaemia after Transfusions of Stored Red Cells

As explained above, 'non-viable' stored red cells undergo little or no intravascular destruction and their removal from the circulation occurs predominantly in the cells of the reticulo-endothelial (R.E.) system.

The increase of plasma bilirubin concentration following the extravascular destruction of red cells is approximately that which would be expected following the katabolism of an equal amount of haemoglobin injected intravenously. For example, when the red cells from 150 ml. of stored blood equivalent to 20 g. of haemoglobin are rapidly removed from the blood stream, the plasma bilirubin concentration rises during the following few hours by about 1 mg./100 ml. (Mollison, 1956, page 376). As mentioned above, a similar rise in plasma bilirubin is produced by the intravenous injection of about 16 g. of haemoglobin. The increase after the transfusion of stored red cells would be expected to be slightly less than that following the injection of an equivalent amount of haemoglobin because the stored red cells are removed over a period of a few hours so that the liberation of bilirubin will be spread out over a longer period.

The destruction of greater quantities of red cells is not expected to produce a proportionally greater rise in plasma bilirubin concentration since the rate of clearance of bilirubin varies approximately as the square of the plasma bilirubin concentration (Weech *et al.*, 1941). In practice, subjects sometimes rapidly dispose of the red cells from 1000 ml. of blood without developing jaundice and it is reasonable to conclude that in such cases the bilirubin concentration has not exceeded a level of 3 or 4 mg. per 100 ml. or has done so only very transitorily.

Nevertheless, any interference with liver function at a time when haemoglobin is being katabolized inevitably leads to jaundice. This pre-

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sumably explains why jaundice is quite commonly observed on the day after operation in patients transfused with stored blood during operation, particularly when the blood has been stored for 2 to 3 weeks (personal observations).

Similarly, jaundice is commonly observed in subjects with severe injuries who receive transfusions of stored blood. Sevitt (1958-9) reported a careful study of sixteen such cases and found hyperbilirubinaemia in all, reaching a maximum about 5 days after the initial transfusion; all the subjects had bile in the urine, indicating some interference with liver function. Sevitt suggested that in these circumstances a transfusion of stored blood might be regarded as a 'bilirubin-loading' test.

Hyperbilirubinaemia following the Extravascular Destruction of Incompatible Red Cells

Some increase in serum bilirubin concentration is inevitable after every episode of red cell destruction. The extent of the increase depends not only upon the amount and the rate of red cell destruction but also upon the efficiency of bilirubin excretion. Some subjects become jaundiced after destroying the red cells from a single bottle of blood.

A woman aged 47 whose last pregnancy had occurred 14 years previously was transfused with 500 ml. of citrated group O, Rh-positive blood for anaemia secondary to menorrhagia. The transfusion was followed by a rigor and on the following day by jaundice, pain in the loins and oliguria. A sample of urine obtained 36 hours after transfusion contained albumin, haemoglobin and bile pigment. The patient's serum was shown to contain anti-Rh (Mollison, 1943a, Case 6).

Some subjects develop no trace of jaundice after the rapid destruction of the red cells from a bottle of blood so that in investigating suspected haemolytic transfusions reactions it is necessary to estimate the serum bilirubin concentration rather than simply examining the patient for obvious jaundice of the skin.

When red cell destruction occurs only a few days after transfusion, it may be followed by transient jaundice. For example, in the patient of group A described in the previous chapter, who was transfused with two bottles of group B blood, the transfused red cells temporarily absorbed all the anti-B; red cell destruction began only on about the fifth day after transfusion, by which time a substantial amount of immune anti-B was being produced, and the patient only then became mildly jaundiced. Other similar cases have been described in Chapter XIII.

Hyperbilirubinaemia following the Transfusion of Incompatible Plasma

Jaundice may occur within a few hours of transfusing incompatible plasma, as described by Gasser (1945). Alternatively it may not reach a peak until about 60 hours after transfusion (Colonel J. H. Akeroyd—personal communication quoted in the second edition of this book).

INVESTIGATION OF HAEMOLYTIC TRANSFUSION REACTIONS

The Need to Search for Signs of Blood Destruction

Since large amounts of red cells or haemoglobin can be rapidly removed from the circulation without causing either haemoglobinuria or jaundice, haemolytic reactions cannot be diagnosed with certainty without laboratory tests. The following case is a striking example:

The patient was an elderly woman with carcinoma of the stomach who was transfused with a bottle of citrated blood (equivalent to 420 ml. of whole blood). By mistake she was given old blood which was awaiting return to the transfusion depot, instead of the fresh blood which had been selected for her. The bottle of blood she received had been stored in a blood bank for 19 days and had then stood at room temperature for a further 4 weeks. The transfusion was completed in 2¾ hours. After transfusion the patient developed a temperature of 101° F. and a pulse rate of 135, and the doctor-in-charge asked for further investigation. A venous sample taken an hour after transfusion showed the plasma haemoglobin level to be 100 mg./100 ml. A sample of urine passed an hour after transfusion contained no free haemoglobin. Differential agglutination tests showed that there were no surviving donor cells. Two hours later the degree of haemoglobinaemia had diminished but the serum bilirubin had risen considerably. The patient did not become jaundiced. Thus this elderly patient eliminated 400 ml. of blood within 4 hours without any specific signs of blood destruction (Mollison, 1943a).

Some Common Difficulties in carrying out the Investigation

In maintaining a high standard of safety in blood transfusion, it is of the greatest importance to investigate the cause of every haemolytic transfusion reaction. The difficulties of doing this are two-fold: firstly, as has just been emphasized, many transfusion reactions which are in fact haemolytic do not produce obvious clinical signs of blood destruction. Sometimes the only hint that a haemolytic reaction has occurred is a small increase in a patient's body temperature. At one time pyrexial reactions were very common after transfusion, presumably due to the presence of pyrogens in citrate and in imperfectly-cleaned apparatus. Now that standards have risen and most transfusions provoke no rise whatever in

RCPE0002068_0270

the patient's temperature, it is very helpful to record the temperature at ½-hourly intervals during the transfusion and for 2 hours after its end and to pay attention to any rise observed. This is perhaps most helpful when a patient is having a series of transfusions; if it is found that as a rule there is no fever following transfusions, then on any particular occasion when a fever develops, there will be a clear indication to search carefully for a cause.

The second difficulty in investigating haemolytic reactions is the absence of important sources of evidence. For example, it is still a common practice to wash out a bottle of blood immediately after it has been given to the patient. If one simply wishes to re-test the reaction of the donor's red cells it is still possible that the hospital laboratory or blood bank will have a sample. But, if one wishes to discover whether the bottle of blood, as it was given to the patient, was haemolysed by being over-heated, it will be impossible to obtain decisive evidence.

Recommendations to Overcome Difficulties

It cannot be too strongly urged that it should be a routine practice to leave a few millilitres of blood in every bottle after transfusion and to re-cap the bottle and place it in a refrigerator for 24 hours. The bottle should not be discarded until the doctor responsible for the transfusion has seen the patient again and come to the conclusion that there is no evidence of a haemolytic reaction.

Almost equally important is the routine taking of a pre-transfusion sample of blood from the patient. This can easily be collected at the beginning of every transfusion. From the standard giving set, the needle, with a small length of rubber tubing, can be detached and inserted separately into the patient's vein. Then, as soon as a blood sample has been collected, the main tube of the giving set is connected to the needle and the transfusion begun. If such a sample is taken as a routine and placed in a refrigerator for 24 hours, the investigation of transfusion reactions is greatly simplified. It might appear that a sample of blood could very well be obtained from the patient only when the suspicion of a haemolytic transfusion reaction had been aroused. However, a sample taken before transfusion may help in the investigation in three ways:

Firstly, the patient's full blood groups can be determined. This may be very important in the identification of a blood-group antibody present in the serum. Thus, for example, if the patient is found to be Fy(a+), he cannot have formed anti-Fy^a. In a sample taken after transfusion there may be large numbers of transfused cells even though the cells are incompatible and it may be difficult or impossible to decide the group of the patient's own cells.

Secondly, the presence of blood-group antibodies can be determined with certainty. After the transfusion of incompatible blood, the antibody may all, or almost all, be adsorbed on to the transfused cells. It is true that this implies that if a further transfusion of incompatible blood is now given the cells will not be rapidly destroyed but in a few days' time the amount of antibody in the circulation will usually increase rapidly and all incompatible cells will then be rapidly eliminated.

Thirdly, it may be useful to compare the colour of the pre-transfusion serum with that of a sample taken shortly after transfusion, to detect any gross increase in bilirubin concentration.

Methods of Investigation

The establishment of the diagnosis of a haemolytic transfusion reaction falls conveniently into two parts: (1) the demonstration of the occurrence of increased blood destruction, and (2) the demonstration of the cause of the increased destruction.

The signs of increased blood destruction have already been described, but an account must be given of some of the simple tests to be applied.

Visual Detection of Haemoglobinaemia

In withdrawing blood samples special precautions must be taken to prevent haemolysis. The commonest cause of haemolysis *in vitro* is the drying of small amounts of blood with subsequent liberation of haemoglobin when the dried blood is mixed with the rest of the sample. Therefore, one of the most reliable ways of getting an unhaemolysed sample is to take the blood into a syringe which has been wetted with sterile saline. Other important points are to use a reasonably wide-bored needle (SWG 20 or larger) and, when the sample has been obtained, to remove the needle from the syringe before ejecting the sample slowly into a suitable container with anticoagulant. The top of the container can now be covered with parafilm and the contents mixed gently. It is again important not to allow any blood to dry on the walls of the container. Thus either the container should be filled almost to the top with blood or the container should be centrifuged immediately before any blood can dry on its walls. It is a good practice to examine the part of the container above the level of the blood before centrifugation and to wipe away, with some wool on a stick, any traces of blood. When these precautions are observed, the amount of free haemoglobin in the plasma should not exceed 2 or 3 mg.

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per 100 ml. as estimated by a method such as Crosby and Furth's modification of the method of Bing and Baker (see below). Further precautions to be observed when using the very sensitive method of Hanks *et al.* (1960) are described later.

A sample of plasma which contains about 20 mg. of haemoglobin per 100 ml. of plasma will appear very faintly pink or light brown if examined in a thickness of about 1 cm. Even smaller amounts of the haemoglobin can be detected with a small spectroscope. Quantities of free haemoglobin of the order of 100 mg./100 ml. make the plasma appear red.

Benzidine Method for Estimating Haemoglobin

According to Crosby and Furth (1956) the original method of Bing and Baker (1931) gives too low a recovery of haem pigment from plasma. Even when Ham's (1952) modification of adding alcohol is used, the recovery is only about 85 per cent. Crosby and Furth claim that with the following method, recovery exceeds 95 per cent:

Reagents (Glassware should be cleaned in acid. Distilled water should be free from sulphate; this can be checked by testing with $BaCl_2$):

1. *Benzidine reagent.* Dissolve 1 g. of benzidine base (Merck 40889) in 90 ml. of glacial acetic acid and make up to 100 ml. with distilled water. Store at 4° C. (the small amount of water prevents solidification at ordinary refrigerator temperatures).

2. *Hydrogen peroxide, 1 per cent.* Prepare fresh every few days; keep in a refrigerator when not in use. (Avoid peroxide stabilized with sulphuric acid.)

3. *Standard haemoglobin solution.* Wash 2 ml. of red cells and lyse by freezing and thawing. Dilute solution to approximately 10 g./100 ml. and determine exact concentration by a method suitable for whole blood samples. Prepare standard by diluting 0.02 ml. of concentrated haemoglobin solution in 10 ml. saline. The standard contains 20 mg. haemoglobin per 100 ml. if the concentrated solution has 10 g. per 100 ml.

4. *Diluent* consists of 10 per cent glacial acetic acid in sulphate-free distilled water.

Procedure:

1. For each 'unknown', place 1 ml. of benzidine solution in a 15 ml. test-tube. Add two extra tubes, one for the standard and one for the reagent blank.

2. Add 0.02 ml. of 'unknown' sample to one tube and 0.02 ml. of standard haemoglobin to another tube. Mix by swirling.

3. Add 1 ml. of peroxide to each tube; mix immediately.

4. When colour change is complete (approximately 20 minutes) add 10 ml. of diluent. Mix by inverting. Allow to stand 10 minutes. Read in a photoelectric colorimeter at 515 mμ.

A more sensitive method. The method described above is very suitable for detecting amounts of free haemoglobin exceeding about 10 mg. per 100 ml. of plasma, but a method is now available for detecting amounts in the range approximately 0.2 to 10 mg. (see Hanks *et al.*, 1960).

Blood is collected through a 15 g. needle connected to a length of PVP tubing; the first 4 ml. are rejected and blood is then allowed to flow into a siliconized container which has been sprayed with a fine mist of heparin. The sample is centrifuged immediately and the plasma separated. The modification of the method consists essentially in using undiluted glacial acetic acid instead of a 10 per cent solution. This means that a larger volume of plasma can be added to the acid without the development of opacity which occurs when 10 per cent acetic acid is diluted with a watery solution. With the method of Hanks *et al.* the normal range of free haemoglobin in the plasma is found to be 0.16–0.58 mg./100 ml.

Detection of Methaemalbuminaemia

When the plasma contains free haemoglobin, detectable by naked eye examination, it is usually pointless to look for methaemalbumin. However, if the sample has been obtained 12 hours or more after transfusion and the plasma contains only traces of free haemoglobin, which might have been produced by careless taking of the sample, it is worth carrying out a spectroscopic examination.

In practice an ordinary hand spectroscope is the most satisfactory instrument. In such an instrument the spectrum is less spread out than it is in the Hartridge reversion spectroscope; absorption bands are narrower and thus more intense, and when small amounts of pigment are present it is easier to detect them. If much methaemalbumin is present, a band will be seen in the red part of the spectrum. In practice this band is seldom seen unless the patient's serum is examined in a layer 2 cm. thick. Moreover, as already mentioned, the pigment is only detectable spectroscopically after fairly large amounts of haemoglobin have been liberated into the blood stream. Fairley (1940) failed to detect it spectroscopically in one subject who received 21 g. haemoglobin intravenously but succeeded in a patient who received 45 g. haemoglobin. If a band in the red is detected, it remains possible that the pigment is methaemoglobin. If the band is sufficiently clear to be identified in the Hartridge reversion spectroscope,

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its position will decide the issue. The alpha band of methaemalbumin lies at 623-624 mμ, whilst that of methaemoglobin lies at 630 mμ.

When methaemalbumin cannot be detected spectroscopically, Schumm's test, which is much more sensitive, should be used.

Schumm's test. A volume of plasma is covered with a layer of ether; then one-tenth volume or slightly more of concentrated ammonium sulphide (Analar) is run in with a pipette and mixed by shaking. The aqueous layer is examined spectroscopically and if methaemalbumin was present in the original sample, a sharply defined band at 558 mμ should now be visible, due to the formation of a haemochromogen. This band is difficult to detect if much oxyhaemoglobin is present in the original sample. The test is thus chiefly valuable in examining samples that do not contain haemoglobin visible to the naked eye. In some cases described by Fairley, including three subjects receiving intravenous injection of 14-21 g. haemoglobin, Schumm's test became positive at about 6 hours after the injection and remained positive for approximately 24 hours.

Estimation of Serum Haptoglobin Level

In theory this examination should be most useful a day or two after a suspected haemolytic reaction. If there has been any appreciable amount of haemoglobin liberated into the plasma, this will all have been cleared but the plasma haptoglobin level will be very low.

The simplest method of estimating haptoglobin is to carry out electrophoresis on paper after adding amounts of haemoglobin varying from 30 to 150 mg. per 100 ml. to aliquots of plasma. The end-point is taken as the amount of haemoglobin which the patient's haptoglobin can bind. Beyond this point, a second band – representing free haemoglobin – will appear. There is a wide normal range of haemoglobin-binding capacity (50-180 mg. per 100 ml. plasma).

If many samples are to be estimated, it is more convenient to use a method such as that of Owen *et al.* (1960) in which a colour is developed in solution depending upon the peroxidase activity of the Hp-Hb complex (see Polonovski and Jayle, 1940). A convenient modification in which samples are read in a spectrophotometer and a quantitative estimate obtained was described by Owen *et al.* (1960).

Detection of Haemoglobinuria

In urine, oxyhaemoglobin may be converted to methaemoglobin. Therefore the urine may appear red or brown, depending upon which

pigment predominates. When there is doubt about the nature of the pigment, the urine should be examined spectroscopically. The bands of oxyhaemoglobin lie at 578 mμ (alpha) and 540 mμ (beta) in the yellow and green parts of the spectrum respectively; the alpha band of methaemoglobin lies in the red at 630 mμ. For the demonstration of this band, the urine must not be alkaline, since the band is not present in alkaline solution.

Haemosiderin in the urine. In patients with *chronic* haemoglobinaemia, haemosiderinuria is a constant feature. According to Crosby and Dameshek (1951) haemosiderinuria is invariably found in adults whose plasma haemoglobin concentration exceeds 25 mg./100 ml. Only microscopical amounts are found when the plasma haemoglobin is below 20 mg./100 ml. but larger amounts are found when the level exceeds 50 mg./100 ml. It must be supposed that patients with chronic haemolytic anaemia whose plasma haemoglobin level is 20-25 mg./100 ml. excrete some Hb in the urine, having presumably a negligible level of Hp in the plasma. This is suggested by the observation of Newman and Whipple (1932) that in dogs no haemosiderin appeared in the epithelium of the renal tubules unless the 'glomerular threshold' for Hb was exceeded.

The author has been unable to discover whether haemosiderin can be detected in the urine after a single episode of intravascular haemolysis but it would seem to be worth looking for it when investigating haemolytic transfusion reactions, particularly when investigations are begun only a few days after the incident. Crosby and Dameshek (1951) recommended the following test: Centrifuge 15 ml. of urine; re-suspend the sediment in 1 ml. of supernatant. Add an equal volume of 5 per cent hydrochloric acid, followed by 0.5 ml. of 10 per cent potassium ferrocyanide in water. When there is gross haemosiderinuria, deep blue particles are now seen. If these are not obvious the mixture is centrifuged; blue granules may now be seen in the tip of the centrifuge tube. If not the sediment is examined microscopically; when the result is positive, blue granules are seen in renal epithelial cells together with a blue amorphous sediment.

Detection of Hyperbilirubinaemia

Often the simple comparison, by naked eye examination, of samples of plasma taken before and after transfusion will provide proof of rapid blood destruction. Slight changes in colour must be ignored but a change from the normal pale yellow to a bright yellow colour is virtually diagnostic.

It must be remembered that the maximum bilirubin concentration is usually not attained for 3 to 6 hours after an episode of blood destruction. Therefore detection is most likely when a sample is obtained during this period. For record purposes the serum bilirubin concentration of this sample and the one taken immediately before transfusion should be estimated.

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Wiener's Biological Test

Wiener, Silverman and Aronson (1942) suggested that in special cases it might be wise to supplement laboratory tests of compatibility by a new form of biological test. This test consists in first taking a sample of blood from the recipient and then injecting 50 ml. of the donor's blood. After an hour a second sample of blood is taken from the recipient and the serum of this sample is then compared with that of the first (pre-injection) sample. If the serum of the second sample shows no trace of free haemoglobin and is no more yellow than the first sample, a full transfusion of the donor's blood is given. Wiener *et al.* emphasized that the rise in icteric index might be small. They advocated that whenever possible, a second injection of 50 ml. should be given at the end of the first hour and a final sample taken for examination at the end of a further hour. However, the need to inject two separate amounts of 50 ml. of blood and to wait 2 hours makes the test very tedious and Wiener himself sometimes relies on the single 1-hour test (Wiener, 1950).

In the author's experience this test is not very sensitive, at least when only a single injection of 50 ml. of donor blood is given. The following case is an example:

The patient was a woman who had formed two immune antibodies, anti-Rh and anti-S; samples of O, Rh-negative, S-negative blood did not react with her serum *in vitro*, even using the indirect antiglobulin test. Nevertheless, Rh-negative, S-negative erythrocytes were rapidly removed from her circulation and on one occasion a transfusion of Rh-negative, S-negative blood was followed by jaundice. On a subsequent occasion it was decided to perform a biological test in the manner advocated by Wiener and his co-workers. Accordingly, after withdrawing a sample of blood from the patient, 50 ml. of O, Rh-negative, S-negative blood was rapidly injected; a second sample was taken 6 minutes later and a third sample after 3 hours. There was no significant difference between the bilirubin concentration before injection (1.2 mg./100 ml.) and 3 hours later (1.3 mg./100 ml.). Nevertheless it was possible to demonstrate, by differential agglutination tests, that approximately half the transfused cells had been eliminated during the 3 hours following the injection, and the patient had an unpleasant reaction. This consisted in a slow rise of temperature, reaching 100.8° F. after 3 hours, a rigor after 4 hours and finally, after 6 hours, a temperature of 103.2° F. Presumably, the rate of destruction of the small number of red cells transfused was insufficient to cause a rise in the bilirubin level.

The counts of unagglutinated cells per c.mm. were as follows:

Before	5000
6 minutes after	48,000
3 hours after	25,000
20 hours after	11,000

A biological test is needed only when there is doubt about the interpretation of compatibility test *in vitro*.

As described in Chapter XIII a delicate biological test is now available since minute quantities of red cells can be labelled with a radioactive isotope and their survival precisely determined. However, in investigating cases in which incompatible blood has been inadvertently transfused the survival of the cells of that particular transfusion can be estimated only by the method of differential agglutination and in the following section the use of this method will be discussed. It may be added that occasionally when a haemolytic reaction has occurred but cannot be satisfactorily explained, it may be very helpful to obtain a fresh sample of blood from the donor and inject 1 ml., labelled with ^{51}Cr. If the red cells survive normally, it will be clear that blood group incompatibility was not the cause of the haemolytic transfusion reaction.

It must of course be remembered that for a few days after a large transfusion of incompatible blood there may be no free antibody in the patient's plasma. If, therefore, incompatible red cells are injected at this time they may survive normally for a day or two although as soon as enough antibody has been produced they will be rapidly eliminated; for an example see Fig. 62 on p. 447.

Differential Agglutination as a Test of Compatibility

Wiener (1941 a and b; 1942 a and b) was the first to insist on the importance of survival tests in demonstrating incompatibility; and he first used the term 'inapparent haemolysis' to describe those cases in which, despite the absence of clinical signs of blood destruction, the red cells leave the circulation at a greatly increased rate. No haemolytic transfusion reaction can be considered to have been properly investigated unless an attempt has been made to demonstrate the survival of the donor cells. Moreover, in patients who are investigated for the first time some days after a suspected haemolytic transfusion reaction, estimation of the survival of the donor cells is often the only means of establishing the diagnosis with certainty.

As has been explained in more detail in Chapter V, two methods are available:

1. The method of indirect differential agglutination, which can be used when the recipient's red cells contain an agglutinogen which is not present in the donor's red cells. Difficulty in obtaining potent agglutinating serum

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usually limits the application of the method to the following circumstances:

(*a*) Differential agglutination using anti-A or anti-B following the transfusion of group O blood to a recipient of group A, B or AB, or the transfusion of A or B blood to an AB recipient.
(*b*) Differential agglutination with anti-M following the transfusion of Group N blood to a recipient of group M or MN (anti-N serum is usually not potent enough).
(*c*) Differential agglutination with anti-Rh (D) following the transfusion of Rh-negative blood to an Rh-positive recipient.
(*d*) Differential agglutination using anti-c, anti-E or other agglutinating antibodies may be practicable if a sufficiently potent antibody is available.

2. The method of direct differential agglutination; this can be applied if the donor's red cells contain an agglutinogen which is not present in the red cells of the recipient. Again, the method is limited in practice by the difficulty of obtaining potent agglutinating anti-sera other than anti-A, anti-B, anti-M and anti-D.

By the application of one or other of these methods it is often possible to obtain decisive evidence for or against the normal survival of the transfused red cells. The indirect method of differential agglutination is the most informative. For instance, the direct method may show some group M cells present in the circulation of a group N recipient; this leaves undecided the question whether the number of cells present is less than expected in view of the number transfused. By contrast, quantitative differential agglutination, e.g. agglutination of the patient's (A) cells, and counting of the donor's (O) cells, may show only 400,000 donor cells per c.mm. despite the transfusion of 1000 ml. of citrated blood. This finding would decisively indicate the destruction of a large proportion of the transfused red cells. In fact, the indirect method can give an accurate estimate of the actual concentration of surviving cells, whereas the direct method can distinguish between three circumstances only: namely, survival of many transfused cells, survival of a few transfused cells, and no survival of transfused cells.

A few examples will serve to illustrate the value and application of the different methods.

Demonstration of normal survival of transfused red cells. The patient was a male of group A who had been severely burnt 2 days previously. He was

transfused with 1000 ml. of group O blood; no compatibility tests were made. After transfusion he was found to have a temperature of 109° F. and he died 10 hours later. The question was raised whether the patient had been given incompatible blood. No sample was available from the bottles of blood used for transfusion, but a sample of blood was taken from the body after death and found to be unhaemolysed; this sample contained a mixture of A and O cells and it was estimated that there were approximately 1,000,000 group O cells per c.mm. This made it clear that the donor red cells had survived normally and could be considered compatible.

Demonstration that transfused red cells have failed to survive. If an antibody (active at 37° C.) can be demonstrated, and shown to react with the donor's red cells, the cause of the haemolytic reaction is no longer obscure and the demonstration that the transfused red cells have failed to survive is little more than an academic exercise. In view of this and in view of the fact that in the preceding chapter numerous examples have been given of the effect upon survival of different forms of incompatibility, only one further example will be given.

The patient was a woman who had been transfused with three bottles of group O blood following a severe post-partum haemorrhage; the foetus was born dead and was grossly oedematous. The patient had been transfused once before, following the birth of a previous child. After the second transfusion she had a rigor and during the following week passed little urine; she finally recovered.

Blood was taken from the patient 11 days after transfusion and found to be A, Rh-negative; anti-Rh was present in the serum. Samples from the donors were not available. However, since the patient was group A and the donors allegedly group O, a test could be made with anti-A. This showed that there were some 400,000 group O cells per c.mm. in the circulation. A test with a potent anti-Rh serum showed that there were no Rh-positive cells present. Since the transfusion of a single bottle of citrated blood produces a concentration of approximately 450,000 cells per c.mm. immediately after transfusion, the finding of 400,000 per c.mm. 11 days after transfusion suggested that the cells of one donor only had survived and this donor was clearly Rh-negative. The report was therefore submitted that if the donors could be traced, it seemed that one would prove to be Rh-negative and the other two Rh-positive. Some 3 weeks later the donors were tested and the forecast confirmed.

As will be gathered, the case occurred before routine Rh grouping had been introduced into normal clinical practice.

Demonstration of partial survival of transfused red cells. In several different circumstances, transfused cells survive less well than normally, but at the same time are usually not all eliminated within a few days of transfusion.

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These circumstances are: following the transfusion of aged stored blood, following transfusions to recipients with acquired haemolytic anaemia and in some types of incompatibility.

If it is known that blood stored for 4 weeks or more was transfused or that the blood transfused had not been properly refrigerated during storage, it will be easy to account for the finding of 'too few' transfused cells, that is, less than the number expected after a transfusion of fresh blood. It may be worth re-emphasizing that just because a bottle of stored blood is unhaemolysed it does not mean that the red cells will survive well. One example was given in Chapter I. A further example is provided by some observations of Gibson, Sack *et al.* (1947). Blood was stored for 3 weeks in ACD with intermittent refrigeration only, the temperature varying between 4° C. and 15° C. or even higher. At the end of the storage period the blood was only very slightly haemolysed (approximately 1 per cent) but within 24 hours of transfusion more than 80 per cent of the red cells were destroyed.

In acquired haemolytic anaemia, the patient's direct antiglobulin test is usually positive and this finding will readily explain a poor survival of transfused cells. A positive direct antiglobulin test in a patient who has recently been transfused may of course also be due to the temporary presence in the circulation of transfused incompatible red cells or to 'coating' of the patient's own red cells with incompatible antibody derived from the plasma of the transfused blood.

Following the transfusion of incompatible blood it is common to find surviving red cells in the recipient's circulation for many days after transfusion. As explained in Chapter XIII, the amount of available antibody may be temporarily exhausted by a large transfusion and in any case the capacity of the R.E. system suffices to remove only about 300 ml. of incompatible red cells a day.

In fact it is only immune anti-A and anti-B, or other antibodies capable of causing severe intravascular lysis which can bring about the removal of most of the transfused red cells within a matter of minutes. In many cases of ABO incompatibility, the recipient's serum does not contain an appreciable amount of immune anti-A or anti-B at the time of transfusion and thus surviving red cells will probably be found for at least a day or two after transfusion.

Mrs D. was an old lady with carcinoma of the lung. Her group was O but she was inadvertently given 600 ml. of group B blood. A sample of urine passed after transfusion was noted to be red but was not tested. The next day when the author was invited to investigate the case, the patient's blood contained a number of group B cells. These were apparent as small clumps visible microscopically in a saline suspension of the patient's blood; the clumps could be increased in size by the addition of anti-B serum. As anticipated the titre of anti-B in the patient's serum on the day following transfusion was very low.

A Routine Procedure for the Investigation of Haemolytic Transfusion Reactions

Secure a Venous Sample from the Patient and a Sample of Donor Blood

As soon as it is suspected that a haemolytic transfusion reaction may have occurred, the transfusion should be stopped and a venous sample should be taken from the opposite arm. Steps should be taken to secure the bottle or bottles of blood that have been given to the patient, as well as any pre-transfusion samples taken from the patient.

Look for Haemoglobinaemia and Bilirubinaemia

Samples of the pre- and post-transfusion blood should be centrifuged so as to obtain evidence of haemoglobinaemia or increased bilirubinaemia, if these have occurred. If more than 6 hours have elapsed between the time of transfusion and the taking of the sample, Schumm's test should be performed (see above).

Re-group Donor and Recipient and do a Direct Antiglobulin Test

The samples of blood from donor and recipient should now be re-grouped. In the first instance it is sufficient to use anti-A, anti-B and anti-D sera. If these tests reveal that A blood has been given to a group B patient or B to a group O patient, etc., no further tests need to be made. If the donor is found to be Rh-positive and the recipient Rh-negative, the patient's serum must be examined for Rh antibody. The indirect antiglobulin test will be found the most reliable single test.

At the same time a direct antiglobulin test should be performed on the post-transfusion sample of blood. The reaction is likely to be positive if more than 100 ml. of incompatible blood have been transfused. The reaction may also be positive if plasma containing immune anti-A or anti-B has been transfused to a group A or B recipient.

If the incompatibility does not seem to be due to anti-A, anti-B or anti-Rh(D), more extensive grouping tests must be made on the recipient's pre-transfusion blood sample. Since the other blood group antibodies most frequently involved are anti-E, anti-c, anti-K, anti-Fy^a, anti-Le^a and anti-S in approximately that order, the patient's red cells should be tested with sera containing these antibodies. Thus if the patient is CcDee, K-

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negative, Fy(a+), Le(a+), S-positive, he can only have formed anti-E or anti-K out of this list.

The donor's red cells should be similarly tested; thus in the present example, if the donor were ccDEe, K-negative, suspicion would centre round anti-E.

When no pre-transfusion sample is available and the patient has been transfused with blood from many donors, it may be difficult to decide which red cells belong to the patient and which are the transfused ones. It is occasionally valuable to test the patient's saliva, particularly in deciding whether or not the patient is 'Lewis positive' (i.e. Le(a+b—) or Le(a—b+)) since only Lewis negative (Le(a—b—)) subjects can form Lewis antibodies (anti-Le^a or anti-Le^b).

Constandoukalis and Kay (1959) tried to make use of density differences between old and young red cells to separate the patient's own red cells from transfused red cells. The object of this was to enable them to determine the blood group of a patient even when many transfused cells were present. In many cases, as expected, the top layer of a spun column of red cells was shown to contain an increased proportion of the patient's own red cells since this layer contained the youngest red cells present. In other cases separation was poor and this was thought to be due to the occasional use of donors with a relatively low MCHC and who thus had red cells which were relatively lighter than those of the recipient.

Repeat Direct Matching Test

A suspension of the donor's red cells should be tested against the pre-transfusion sample of the patient's serum, using the indirect antiglobulin test. Occasionally, the antiglobulin test reveals incompatibility that has been missed by a test in albumin. More often it becomes apparent that any matching test would have revealed the incompatibility and that the test was not done, or that a test was done but the wrong blood samples were tested.

If the donor's cells are not available, differential agglutination tests may be very helpful in providing evidence of the presence of cells of a 'foreign' group. The scope of such tests has already been described and details of technique are to be found in Chapter V.

Identification of Blood-Group Antibodies

As already mentioned, if the recipient's pre-transfusion sample can be tested, the identification of any blood-group antibodies in his serum is greatly simplified. If the recipient is found to be Rh-negative (D-negative) it is highly likely that the antibody in his serum is anti-Rh since this anti-

body occurs so much more commonly than other immune blood-group antibodies.

When it becomes evident that the antibody responsible is not anti-A, anti-B, or anti-Rh, the serum should be tested with a panel of red cells whose reactions with antisera of all the other blood group systems are known. It will normally be quite sufficient to include cells containing the antigens E, c, S, P, K, Le^a and Fy^a; all of these can be involved in haemolytic transfusion reactions. A suitable cell panel is shown as Table XLI in Chapter XII. Suggested steps to be taken in identifying antibodies are described on pp. 432-435.

In an emergency there is often no time to identify a blood-group antibody; compatible blood has to be selected by testing the red cells of available donors against the patient's serum, using the indirect antiglobulin test, and regarding as compatible any samples which fail to react. However, the antibody should be identified at the earliest opportunity because it may then be possible to select donors by using another serum which contains a more potent antibody of the same specificity.

It is particularly useful to identify the antibody when the patient has been transfused with a large amount of incompatible blood and no longer has any free antibody in his serum. Often there is only a small amount of pre-transfusion serum available and this can be saved for direct matching tests, using the cells of donors who have previously been selected (using another serum) as 'probably compatible'.

For example, in the case of Mrs C. (described on p. 448) who had received 14 pints of incompatible blood, no antibody could be detected in the serum for 5 days after the transfusion. Only 0.5 ml. of the pre-transfusion sample of serum was available.

A few hours before her massive haemorrhage, Mrs C. had given birth to an infant whose cells were found to have a negative direct Coombs reaction. The infant's serum was tested and found to contain anti-Fy^a. It was now possible to adopt the working hypothesis that Fy(a—) blood would be compatible with the mother's serum. Several donors of this group were selected and their red cells were matched against the remaining small amount of pre-transfusion serum. Those which were P-negative proved to be compatible. Mrs C.'s serum was then shown to contain anti-P_1 as well as anti-Fy^a.

Titration of Anti-A and Anti-B in Patient's Serum

If blood containing the antigens A or B is given to a person whose serum contains anti-A or anti-B, an immune response follows. When investigations are not begun until a few days after transfusion and the

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patient belongs to group O, and there is a suspicion that group A or B blood was transfused, though the donor cannot immediately be re-tested, then it is useful to titrate the iso-agglutinins. If, for example, the titre of anti-A is very much higher than that of anti-B, it is probable that group A blood was transfused. If the pre-transfusion sample of serum is available, the titre of anti-A and anti-B can be compared with that of a post-transfusion sample.

If the titre of anti-A and anti-B agglutinins is not particularly high a test may be made for immune characteristics; the absence of these characteristics in the serum of a patient transfused within the last 2 or 3 months would virtually exclude the possibility that the blood of the wrong ABO group has been given.

There is another possible advantage in titrating the anti-A and anti-B in the sample of pre-transfusion serum. Occasionally it will provide evidence that the sample which was used for the cross-matching tests did not in fact come from the recipient. Thus in a case investigated by the author, serum alleged to have been taken from a certain group O patient was found to contain anti-B (with a titre of 64), but no anti-A; it was also shown to contain A substance and had evidently come from a group A patient. Group A blood had been cross-matched with this serum and had been transfused to the group O patient whose name was on the label of the serum specimen. The fact that the absence of anti-A from the serum sample used in cross-matching was not due to deterioration on storage was shown by the presence of moderately potent anti-B in the serum and confirmed by demonstrating the presence of A substance.

Tests to Prove Damage by Donor's Plasma

If there is evidence of increased red cell destruction, for example jaundice, following transfusion but it is found that the donor's red cells have survived normally, one should consider the possibility that the recipient's red cells have been damaged by a blood-group antibody in the donor's plasma.

Almost always the only possibility that will have to be considered is of damage to the red cells of a group A (or B) recipient by anti-A (or anti-B) in the plasma of a group O donor.

The signs produced by transfusing plasma containing immune anti-A to a group A recipient have been fully considered in the previous chapter and it will suffice here to summarize the findings.

Haemoglobinaemia is usually slight; there is a slow increase in plasma bilirubin concentration which may not reach its maximum for 2 or 3 days.

The direct antiglobulin test becomes positive during transfusion and, in severe cases, remains positive for several days. The osmotic fragility of the recipient's cells increases, reaching a maximum some hours after transfusion and remaining elevated for some days: during this period the peripheral blood contains numerous microspherocytes. The haemoglobin concentration begins to fall immediately after transfusion and may continue to fall for several days afterwards.

In cases where the transfusion of 400 ml. of group O blood to a group A (A_1) recipient produces signs of considerable red cell destruction, such as jaundice, the donor's serum invariably proves to be strongly haemolytic *in vitro*. Thus, personal observations have shown that when 1 volume of the donor's serum, diluted 1 in 4, mixed with 1 volume of fresh 'neutral' serum (as a source of complement) and 1 volume of a 5 per cent suspension of A_1 cells and incubated at 37° C. for 1 hour, produces only a trace of haemolysis, transfusion of 400 ml. of the blood to a group A_1 recipient produces no detectable red cell destruction although it may produce transient signs such as a small increase in osmotic fragility.

In devising a screening test for group O donors, it would seem reasonable to brand as 'dangerous' only those donors whose serum in a final dilution of one in twelve causes any lysis of a 5 per cent suspension of A_1 cells after 1 hour's incubation at 37° C. Judged by this criterion, 90 per cent of group O donors are safe (Crawford *et al.*, 1952). Gardner and Tovey (1954) devised a simple test which also excluded 10 per cent of group O donors (see p. 400).

Haemolytic reactions due to the presence in the donor's plasma of antibodies outside the ABO system are almost unknown; nevertheless, as described on p. 419, routine testing of the donor's plasma with a 'pool' of enzyme-treated cells, or at least with D-positive red cells, is desirable and should be carried out at the blood collecting centre.

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CHAPTER FIFTEEN

OTHER UNFAVOURABLE EFFECTS OF TRANSFUSION

The mortality from blood transfusion is very difficult to assess. On the one hand there is a tendency not to report mistakes. On the other there is a tendency to make transfusion a scapegoat. Transfusions are frequently given to patients who are already in a precarious state; if the patient dies and any mistake whatever is discovered in the transfusion arrangements, the transfusion may be blamed for the death. In any case it seems that the oft-quoted figure of one death in 1000 to 3000 transfusions is based on figures which are now quite out of date (see Joseph, 1960).

The present chapter is concerned with the various unfavourable effects which transfusion may produce, apart from haemolytic reactions which have been considered in Chapters XIII and XIV. Possibly the commonest ill-effect produced by transfusion is still the febrile reaction.

FEBRILE REACTIONS

A febrile reaction means simply a rise in body temperature following transfusion. Sometimes the rise in temperature is considerable and is preceded by a chill (examples of two severe febrile reactions are shown in Fig. 89); in other cases the rise in temperature is trivial and fleeting and may be overlooked unless the temperature is recorded at least every 30 minutes (for an example see Mollison, 1956, p. 395). Statistics about the incidence of febrile reactions are evidently meaningless unless the term 'febrile reaction' is defined as a certain minimum rise in body temperature, recorded in a particular way at certain definite intervals for a definite period following transfusion. In any case, interest in the overall incidence of febrile reactions in a hospital which was important at a time when pyrogens in transfusion solutions and equipment were the most frequent cause of febrile reactions, has now diminished and attention is chiefly directed to the individual patient who develops severe febrile reactions following the transfusion of incompatible red cells or white cells, or possibly platelets. The various causes of febrile reactions will now be considered in more detail.

Pyrogens

Hort and Penfold (1911) investigated the cause of febrile reactions following the intravenous infusion of saline. They found that the injection of solutions prepared from freshly distilled water did not produce fever, but if the water was allowed to stand after distillation and then injected, febrile reactions occurred. The substance responsible appeared to be a product of bacterial growth but not to be the bacteria themselves. Seibert (1923) succeeded in growing a small gram negative bacillus from pyrogenic tap water and showed that filtrates of cultures of this bacillus were pyrogenic. Co Tui and Schrift (1942) have since shown that many organisms have this property. Pyrogens are now known to be bacterial polysaccharides of high molecular weight (Todd, 1954).

Chemical substances used in the preparation of solutions for clinical use may be contaminated with pyrogens; thus commercially available preparations of citrate, dextrose and sodium chloride may be pyrogenic (see Whittet, 1954).

Febrile Reactions Induced Experimentally by Injecting Pyrogens

Smith (1940) observed that in human volunteers injected with pyrogenic inulin no symptoms developed for the first hour or so; then the blood pressure started to rise and the subject complained of chilliness. Soon there was a more or less severe chill and the onset of fever. At the peak of the reaction the subject might develop nausea, headache and back pain. At about the time of the chill there was a considerable increase in renal blood flow which lasted several hours. All these manifestations, with the exception of the effect on renal blood flow, could be abolished by giving the subject amidopyrin gr. 10, 4-hourly, starting 18 hours before the injection of the pyrogen.

Similarly, Altschule and Freidberg (1945) gave typhoid vaccine intravenously to eight subjects. During the prodrome, lasting from 30 to 90 minutes after the injection, the subject might complain of mild headache or nausea; then, with the onset of the chill phase, the skin became cold, the patient might shiver and the rectal temperature would start to rise. If the patient did not shiver spontaneously in this phase, he could be made to do so by placing a piece of ice in his hand or blowing cold air over him; conversely, heating the patient diminished the severity of the shivering; the chill phase could be abolished by the previous administration of antipyretic drugs. This phase lasted for approximately an hour and a half and was succeeded by the flush phase. In this, the temperature was maintained at a high level and the patient might sweat profusely. Finally, in the

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phase of defervescence, the temperature slowly fell over a period of some hours.

When patients develop febrile reactions following transfusion they also do not start to feel cold for at least 30 minutes after the beginning of the

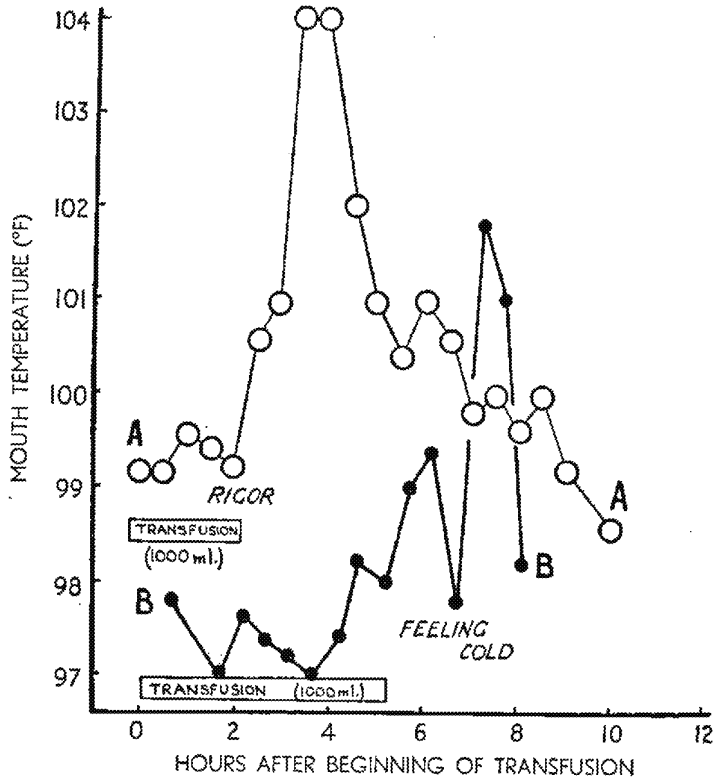

FIGURE 89. Severe febrile reactions following transfusion.
A. Transfusion completed in 2 hours; rigor began before the transfusion was finished.
B. Transfusion of the same amount of blood in 5½ hours. The temperature started to rise at the end of about 5 hours. About 1 hour later the patient felt cold, but did not shiver; the temperature then rose and reached its maximum about 2 hours after the end of the transfusion.

transfusion and often signs do not develop for 60 to 90 minutes (see Fig. 89). Nevertheless in patients transfused with incompatible red cells or white cells other symptoms may develop within a few minutes of beginning the transfusion (see below).

Latency of Pyrogen Fever

Grant and Whalen (1953) observed that the latency of pyrogen fever could be reduced by first incubating the pyrogen with plasma *in vitro*; it appears that bacterial pyrogen interacts with white cells (polymorphs) to form endogenous pyrogen (Snell *et al.*, 1957) and that this latter substance plays a central role in the pathogenesis of many kinds of fever (see Wood, 1958). Grant (1953) showed that if daily injections of pyrogen were given, the latency of fever increased. He concluded that refractoriness to injected pyrogen involved a loss of the capacity to form endogenous pyrogen.

Febrile Reactions in Infants

Infants seem incapable of shivering. Perhaps this explains why so little interest is usually taken in an infant's reaction to transfusion. If an infant's temperature is recorded at intervals after transfusion it is not at all unusual to observe rises in temperature up to 101° F. or thereabouts. Sometimes the rise in temperature is accompanied by a temporary refusal to feed and by diarrhoea.

Although the infant does not shiver in the early phase of the febrile reaction, it does exhibit pallor and its skin feels cold. The following is an example:

Baby R., aged 5 weeks, was recovering from haemolytic disease of the newborn. Its haemoglobin concentration was 10 g./100 ml., but it was decided to give a transfusion as the infant was shortly going on a journey by air and would not be under supervision for a long period thereafter.

Seventy-five millilitres of fresh homologous blood were injected into a scalp vein in 20 minutes. The infant appeared to have stood the transfusion well, but 75 minutes later it was pale and refused to feed. During the next 2 hours it was restless, and 3 hours after the transfusion its pulse rate was 200 per minute. Four hours after transfusion the temperature was 102.4° F. and the pulse rate 180. The temperature remained above 102° F. for a further 3 hours and then fell gradually. Next day the baby appeared perfectly well.

Herpes Simplex

Rarely, herpes labialis develops within a few hours of transfusion. The author has records of two cases. Both were women who received 1000 to 1500 ml. of concentrated red cell suspension in approximately 4 hours; both had moderate febrile reactions (temperature reaching 101° F. at the peak). Both developed herpes labialis within a few hours of transfusion. One of the two stated that she had frequently had attacks of herpes before,

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but had never before had so severe an attack. On the day after transfusion this patient had several blisters on her upper lip; they had almost completely healed 10 days later.

It appears that in susceptible subjects a rise of temperature, from any cause, will provoke an attack of herpes.

Fever following the Transfusion of Incompatible Red Cells

It has been recognized for some time that the transfusion of incompatible blood usually causes a rise in temperature (see cases recorded by the present author (1943a) and by Drummond (1944)). More detailed observations have since been made following the deliberate injection of relatively small amounts of incompatible red cells.

The injection of 10 ml. of washed A or B red cells to subjects whose serum contained the corresponding antibody produced only a slight rise in temperature (0.5° F.) and no chills; but the injection of washed D+ cells to subjects whose serum contained incomplete anti-D caused chills and fever in four out of four cases, suggesting the possibility that the onset of fever was somehow related to splenic sequestration (Jandl and Tomlinson, 1958).

Fever following the Transfusion of Incompatible White Cells

The possibility that white cell antibodies might cause transfusion reactions was mentioned by Goudsmit and van Loghem (1953a) and by Dausset (1954). The first direct evidence in support of this hypothesis was produced by van Loghem *et al.* (1956) and was based on a single case in which a patient with leuco-agglutinins tolerated white cell-poor blood better than whole blood. Very soon after this, abundant evidence in support of the hypothesis was published (Brittingham and Chaplin, 1957; Dausset *et al.*, 1957; Payne, 1957; van Loghem *et al.*, 1958).

The pathogenicity of white cell antibodies was shown decisively by an experiment reported by Brittingham and Chaplin (1957). Fifty millilitres of blood containing a leuco-agglutinin with a titre of 256 was transfused to one of the two authors. Within 45 minutes the recipient developed a chill, severe hypotension with cyanosis, and a respiratory rate of 60 per minute; his white cell count fell from 9000 to 2000 per c.mm., but this was not necessarily due to the leuco-agglutinin because leucopenia is part of the normal 'pyrogen' reaction.

The role of white cells in causing transfusion reactions in patients with white cell antibodies was also shown very clearly by Brittingham and Chaplin. In five patients who had a history of severe febrile reactions

following blood transfusion and whose serum contained white cell agglutinins, the transfusion of an extract of blood containing more than 90 per cent. of the buffy coat produced a severe febrile reaction, but the transfusion from the same bottle of blood of red cells and plasma with less than 10 per cent of buffy coat caused no reaction; see Fig. 90.

The severe reactions were characterized by flushing within 5 minutes of the start of transfusion, with a feeling of warmth. The patient then felt well for 45 minutes; about 60 minutes after the start of transfusion a severe febrile reaction began.

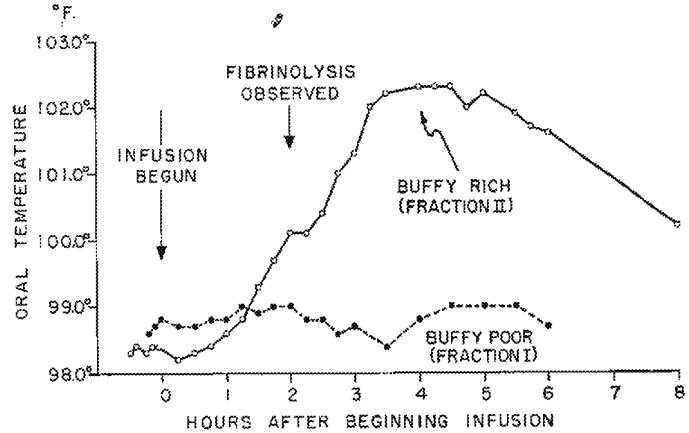

FIGURE 90. Effect of transfusing the 'Buffy' coat to a patient whose serum contained leuco-agglutinins. Two fractions were prepared from fresh blood, fraction 1 containing mainly red cells with very few white cells and platelets, fraction 2 containing a few red cells and most of the plasma, platelets and white cells. As the figure shows, transfusion of this second fraction produced a very severe febrile reaction whereas transfusion of the first 'Buffy-poor' fraction produced no fever (from Brittingham and Chaplin, 1957).

Complementary observations were made by Payne (1957). White cell agglutinins were found in thirty-two out of forty-nine patients with a history of febrile transfusion reactions. Moreover in thirteen out of a series of fifteen patients receiving repeated transfusions white cell agglutinins were first detected about the time when the patient first started developing transfusion reactions. Blood containing less than 200 white cells per c.mm. provoked no reaction in these patients.

The actual number of leucocytes capable of causing a reaction has been considered by several authors. Thus, Dausset *et al.* (1957) concluded that 3×10^8 would produce a reaction whereas 1×10^9 would not. This agrees

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quite well with the findings of Brittingham and Chaplin (1961) in a detailed study of a single subject. 0.4×10^9 leucocytes (the number present in 50 ml. of normal blood) would not produce a reaction, whereas the injection of 1.5×10^9 or more would regularly do so. The reactions produced by 1.5×10^9 leucocytes were very mild when the titre of the leuco-agglutinin was low but severe at a time when the titre was higher.

To be on the safe side it seems wise in patients with leuco-agglutinins to aim to give less than 0.5×10^9 in each 500 ml. of citrated blood. Thus, supposing that citrated blood contains 5000 leucocytes per c.mm. or, in 500 ml., 2.5×10^9, one may conclude that at least 80 per cent of the leucocytes should be removed. Methods of doing this are described below.

As described in Chapter VII white cell antibodies may also develop as a result of iso-immunization in pregnancy, and therefore when transfusion reactions occur in multiparous women the possibility of white cell antibodies should be borne in mind, even when the patient has not received any previous blood transfusions.

Fever following the Transfusion of Incompatible Platelets

According to Marchal *et al.* (1960) platelet iso-agglutinins are often found in the same sera as those which contain leucocyte agglutinins although they are less common than leuco-agglutinins. They are presumably formed as a result of transfusion or pregnancy and are found more commonly in women (70 per cent of total). They were found by Marchal *et al.* in approximately 50 per cent of subjects who had received as many as 100 transfusions.

Because most sera which contain platelet iso-agglutinins also contain leuco-agglutinins it is impossible to assess the role of either antibody separately when a febrile reaction follows transfusion. However, in one case studied by Marchal *et al.* (1960) a patient with a history of severe transfusion reactions whose serum contained both platelet and leucocyte agglutinins was given 100 ml. of platelet-poor plasma without reaction, followed 2 hours later by 50 ml. of plasma containing its normal number of platelets, which produced a severe rigor.

The effect of transfusing plasma containing platelet iso-antibodies to a normal subject was described by Harrington (1954). The donor was a person who had himself previously received many transfusions; potent platelet antibodies were found in his plasma and the test injection of 10 ml. into a normal subject produced profound thrombocytopenia. The

transfusion of plasma from patients with thrombocytopenic purpura to normal subjects also produces profound thrombocytopenia (Harrington *et al.*, 1951).

Role of Transfused Plasma in Febrile Reactions

Dameshek and Neber (1950) published details of some cases in which severe febrile reactions followed the transfusion of whole blood but did not follow the transfusion of well-washed red cells. In two cases they secured suggestive evidence of sensitivity to plasma; febrile reactions were provoked by the intravenous injection of as little as 10–20 ml. of fresh plasma. Precipitins against normal plasma could not be demonstrated in any of the cases.

In retrospect it seems possible that in some of the cases described by Dameshek and Neber the patients were sensitive to white cells rather than to plasma and that well-washed red cells were well tolerated not because they were free from plasma, but because they were free from white cells; but this explanation will not do for the two cases in which the intravenous injection of as little as 10 ml. of plasma provoked febrile reactions nor will it do for the case described by Mendes de Leon and van der Hart (1955) in which the injection of even 2 ml. of serum provoked alarming symptoms followed by a rigor.

The idea that the transfusion of plasma from one individual to another may produce a reaction has recently received experimental support. In dogs transfusion of plasma from one animal to another may produce a fall in blood pressure, development of oedema and haemoglobin concentration, whereas transfusion of the animal's own plasma produces no untoward signs (Remington and Baker, 1959).

In an investigation carried out in human subjects by Hutchison *et al.* (1960), subjects receiving an infusion of their own (stored) plasma showed good plasma volume expansion amounting to 82 per cent of the theoretical maximum but when plasma from another donor was infused, only half the subjects showed good plasma volume expansion and in a few subjects plasma volume actually diminished following the transfusion; on the average, plasma volume increased only by 18 per cent of the expected amount. Poor expansion of plasma volume was associated with urticaria and systemic reactions, such as chills.

In occasional human subjects who have received many transfusions, antibodies are found which *in vitro* react with the serum of other subjects; it is possible that these antibodies are responsible for some post-transfusion reactions; see Allison and Blumberg (1961); see also Chapter VII.

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Role of Non-viable Red Cells and White Cells

There is evidence that the incidence of febrile transfusion reactions is not significantly influenced by the period for which the blood has been stored (DeGowin and Hardin, 1940; Stewart, 1940). Since red cell viability diminishes with the period of storage, it is evident that non-viable red cells do not themselves cause febrile reactions. For example, Jandl and Tomlinson injected 50 ml. of blood which had been incubated at 37° C. for 48 hours to render virtually all the red cells non-viable. Ninety per cent of the cells were removed from the circulation within 20 minutes of injection, but the recipient showed no reaction.

It is not quite so certain that the transfusion of compatible but non-viable white cells produces no reaction. It must be remembered that white cells probably become non-viable after only a very short period of storage so that blood stored for only a few days may contain as many non-viable white cells as blood stored for 2 to 3 weeks. MacQuaide and Mollison (1940) obtained some evidence that transfusions of concentrated suspensions of red cells from which the buffy coat had been removed were followed by fewer febrile reactions than transfusions of whole blood and concluded that since plasma transfusions gave rise to very few reactions, the leucocytes in stored blood might be responsible.

Prophylaxis of Febrile Reactions

Elimination of Pyrogens

The importance of scrupulous care in preparing anticoagulant solutions and in cleaning all equipment that may come into contact with the transfusion fluid cannot be overstressed. There is convincing evidence that the incidence of febrile reactions can be reduced to a very low level by following a planned routine of which these are the essentials:

The distilled water should be prepared in stills fitted with baffles to prevent carry-over of spray into the condenser; all solutions must be prepared from water that has been distilled during the previous few hours and not from water distilled on the previous day; as soon as they have been prepared, the solutions should be autoclaved in their final containers. The still itself and the bottles into which the solutions are to be put must be rinsed with freshly distilled water. Used or new rubber tubing and glassware must be cleaned thoroughly so as to remove all debris and must be finally rinsed in freshly distilled water. If cleaned apparatus is not going to be autoclaved at once, it must be dried rapidly and protected from dust during storage. Throughout, it must be remembered that if pockets of

water are left anywhere for several hours, for example in tubing or in stills, bacteria may multiply and produce pyrogens.

Solutions for intravenous administration may be tested for freedom from pyrogens by injection into rabbits. The importance of the experimental conditions in equating results in rabbits and man has been discussed by Dare and Mogey (1954).

There may be special difficulties in testing citrate solutions for pyrogens since citrate is toxic. To counteract this effect it has been recommended that the citrate should be mixed with apyrogenic calcium chloride before injection (Charonnat and Lechat, 1952). Collier (1954) has mentioned that calcium gluconate is antipyretic in rabbits and has referred to the possibility that in some pyrogen tests pyrogenicity may be masked by an antipyretic substance.

Transfusion sets (i.e. recipient sets) can be tested by injecting into rabbits 10 ml. per kg. body weight of the pooled effluent obtained by passing 40–50 ml. of sterile pyrogen-free normal saline through a number (e.g. 10) of sets at a rate of approximately 10 ml. per minute.

Nowadays, disposable plastic sets for single use are becoming more widely used and the methods of preparing and assembling the components make the presence of pyrogens in the final product unlikely.

Use of White Cell-Poor Blood

Two methods of obtaining white cell-poor blood have been tried: centrifugation of the blood followed by aspiration of the red cells from below the white cell layer, and differential sedimentation of the blood after mixing with dextran, with rejection of the supernatant containing most of the white cells. Dextran sedimentation is considerably more efficient.

Chaplin *et al.* (1959) found that using centrifugation and aspirating the red cells from the bottom of the container they could get rid of only about 90 per cent of the white cells and this at the cost of leaving behind (i.e. wasting) 20 per cent of the red cells. Incidentally they found that attempts at removing the white cells by aspirating them directly were very unsatisfactory so that even when the top 20 per cent of the red cells was removed, 20 per cent of the total number of white cells was still left behind.

By contrast, using dextran sedimentation, 97.5 per cent of the white cells could be rejected with only a small loss of red cells, although it was necessary to carry out two successive sedimentations to achieve this. The method used was as follows:

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Approximately 500 ml. of ACD blood stored for less than 48 hours was transferred to a container of capacity 1100 ml. and 250 ml. of 6 per cent dextran (British Intradex) was added. The volume was then made up to 1100 ml. with saline and the mixture was allowed to stand for 40 minutes, by which time the red cells had sunk to the 350 ml. level. The supernatant was now removed and the volume made up to 575 ml. with saline; then 250 ml. of dextran was added and the volume made up once more to 1100 ml. with saline. After the red cells had been allowed to settle for 30 minutes, the supernatant was discarded and the red cells were re-suspended in saline before transfusion.

It was found that 84 per cent of the original number of white cells were removed by the first aspiration and 97.5 per cent by two aspirations. Chaplin *et al.* concluded that these were the numbers expected to be removed from a knowledge of the amount of plasma removed. They also concluded that the final suspension contained about 50 ml. of dextran. They found that even patients who received infusions of a suspension every 6 to 8 weeks for periods up to 14 months developed no signs of sensitivity to dextran.

Use of well-washed red cells. Chaplin, Crawford *et al.* (1956) noted that following the transfusion of de-glycerolized red cells, that is red cells which had been washed repeatedly in successively more dilute solutions of glycerol, febrile reactions did not occur in thirty out of thirty cases and this was confirmed in a more extensive series by Haynes *et al.* (1960). It seems likely that this freedom from reactions is due to the removal of white cells during washing rather than to the removal of plasma, for reasons discussed above.

The Effect of Drugs in Suppressing Febrile Reactions

As already mentioned, both Smith (1940) and Altschule and Freidberg (1945) have claimed that pyrogenic reactions can be modified by the previous administration of antipyretic drugs. It is undesirable to use amidopyrin, although it has been shown to be effective, since this drug is known to cause agranulocytosis. Fortunately there is evidence that aspirin also suppresses many of the disagreeable phenomena. Thus Dare and Mogey (1954) gave intravenous pyrogens to volunteers and found that if 1 g. of aspirin (15 grains), with a cup of hot tea (!), was administered at the onset of shivering, all symptoms were suppressed within 20 minutes in all except the most severe cases; moreover there was no subsequent rise in temperature.

In a single case investigated by Jandl and Tomlinson (1958), 1 g. aspirin given to the subject 1 hour before the injection of 10 ml. of D-sensitized red cells, followed by three doses of 1 g. at 2-hourly intervals starting 2 hours after the injection, appeared to suppress a febrile reaction. Thus in the treated subject there was no chill and only a slight rise in temperature, whereas in every other subject receiving a similar injection, but without prophylactic aspirin, chills developed. The same authors observed that in two other subjects receiving the same dose of D-sensitized cells after receiving cortisone, 50 mg. orally every 6 hours, for 24 and 48 hours respectively before transfusion, no febrile reaction developed.

It has been claimed that the administration of anti-histamine drugs to patients before transfusion, or the addition of such drugs to the blood to be transfused reduces the incidence of febrile reactions, but in those trials in which the control and test series have been collected at the same time, the results have been negative.

For example, Wilhelm *et al.* (1955) added 50 mg. of benadryl (diphenhydramine hydrochloride) to 500 ml. of blood at the time of collection and found that the patients receiving such blood were as likely to develop febrile reactions as those receiving untreated blood. Hobsley (1958) found that the same was true when the drug used was piriton (chlorprophen-pyridamine maleate), 10 mg. being added to 500 ml. of blood.

Wilhelm *et al.* also found that in rabbits the subcutaneous injection of anti-histamine drugs 15 minutes before the intravenous injection of purified pyrogen did not prevent a febrile reaction; the drugs they tested were the two mentioned above and pyribenzamine (tripelennamine hydrochloride).

Factors which Moderate the Severity of Febrile Reactions

It is probable that the severity of febrile reactions can be mitigated by warming the patient (see the experiment of Altschule and Freidberg referred to above); thus it should be a routine to see that patients receiving transfusions are kept thoroughly warm.

It seems evident that when pyrogenic substances are being infused the rate of infusion will affect the severity of the reactions. This is one reason for using a moderate rate of transfusion, e.g. 500 ml. in 1 hour in non-urgent cases. Many clinical impressions have been recorded to the effect that febrile reactions are more severe when transfusions are given rapidly (see Grant and Reeve, 1951).

RCPE0002068_0284

ALLERGIC REACTIONS

The term 'allergic' is used here to denote the occurrence of manifestations such as urticaria, oedema of the face, bronchial spasm, etc., due to one of two causes:

(*a*) the presence in transfused plasma of some antigen ('atopen') whose corresponding antibody ('reagin') is present in the recipient's plasma.

(*b*) the transfer of unusual antibodies ('reagins') present in the donor's plasma, so that later the ingestion by the recipient of the corresponding antigen produces a reaction.

Evidence that the Abnormality usually Lies in the Recipient

Maunsell (1944) produced convincing evidence that allergic reactions occurring during, or shortly after, transfusion of serum, depend upon an abnormality in the recipient, rather than in the donor. Transfusions of pooled human serum were given to two groups of subjects, namely normal subjects and 'atopic' individuals, i.e. those known to be sensitive to such common atopens as pollen, dust, milk and egg.

Moderate or severe urticarial reactions, that is to say, the occurrence of many large wheals surrounded by areas of erythema, occurred in fourteen out of the seventeen atopic subjects but in none of the normal subjects, although in some of the normal subjects a few solitary small wheals were noticed.

In two of the atopic subjects the reactions were alarming; one boy, with bronchial asthma, developed an asthmatic attack, severe abdominal cramps and a fall in blood pressure in addition to severe urticaria. Another asthmatic patient, a young woman, developed itching and swelling of the eyelids, followed by deep cyanosis, an increase in heart rate and a fall in blood pressure. This was followed by a prolonged attack of asthma. In both these cases adrenalin was given when the signs became alarming and both patients recovered fairly quickly.

It is to be noted that Maunsell divided cases of urticaria into two groups, namely those in which only a few small solitary wheals occurred and those in which there were large wheals on an erythematous background. The first group, which Maunsell considered of no importance, is likely to cause trouble to anyone collecting statistics on transfusion. In the present author's experience the detection of these small solitary wheals depends to a large extent on the state of undress of the patient. Often the wheals do not itch and they are bound to be overlooked unless they occur on the face or on some exposed part of the body. In many series of cases in which the incidence of urticaria is reported, the authors have not discriminated between the few small wheals and the more widespread attacks.

Sensitivity to Nickel. Stoddart (1960) reported two cases in which within 24 hours of receiving infusions of blood or plasma patients developed an urticarial rash (generalized in one case, round the site of infusion in the other) apparently due to sensitivity to nickel. Both patients on questioning gave a history of sensitivity to nickel and it was presumed that the nickel-steel shafts of the needles through which the transfusions were given were responsible.

Desensitization

In the literature, there are many reports of severe reactions following the use of the same donor on successive occasions. These reactions have often been ascribed to the development by the recipient of hypersensitivity to some protein in the donor's plasma. It is thus particularly interesting that in further experiments conducted by Maunsell (1944), the effect of giving successive transfusions of the same batch of serum to a group of recipients, at intervals of about 14 days, was in fact to desensitize them. Her subjects were the same group of atopic individuals mentioned above. A second transfusion was given to fourteen patients, eleven of whom had developed a rash on the first occasion; ten out of these eleven appeared to have become less sensitive. A third transfusion was given to eight subjects, all of whom had produced urticaria when transfused for the first time. Of these, seven showed a decreased sensitivity, five not reacting at all. In three patients who had been transfused repeatedly with serum from one pool, serum from another pool produced no reactions.

Eight non-atopic subjects failed to become sensitized after repeated transfusions. In the face of this evidence one is bound to wonder whether cases previously described, of serious allergic reactions following the repeated use of the same donor, have really been allergic reactions at all.

The second type of allergic reaction to consider is the passive transfer of sensitivity from the donor to the patient; evidently this can occur only when the donor's plasma contains reagins. Many convincing cases have been published and the case of Ramirez (1919) is striking enough to quote again. The patient, who had received a transfusion 2 weeks previously, went for a carriage drive and promptly developed a severe attack of asthma, a thing that had never happened to him before. It was then discovered that the donor of the transfusion had long known that exposure to horses brought on attacks of asthma in him and his skin tests to horse-

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dandruff were strongly positive. The conclusion seems to be that those occasional donors who give a really striking history of hypersensitivity should be rejected.

Incidence of Allergic Reactions

Severe allergic reactions following transfusion are rare. Thus cases in which the patient develops laryngeal oedema or bronchial spasm are encountered only once or twice per thousand transfusions.

The development of wheals is comparatively common; as already mentioned the recorded incidence depends to a large extent on the thoroughness with which the patient is examined. Thus in a series in which even a few wheals were counted the incidence was found to be approximately 3 per cent (Stephen *et al.*, 1955). Other observers have found the incidence to be only 1 per cent (e.g. DeGowin and Hardin, 1940).

Effect of Antihistamines

The addition of 25 mg. of pyribenzamine (tripelennamine hydrochloride) in 1 ml. of distilled water to 500 ml. of blood immediately before transfusion greatly reduces the incidence of allergic reactions (Ferris *et al.*, 1952; Stephen *et al.*, 1955).

Ten milligrammes of piriton (chlorprophenpyridamine maleate) added to blood before transfusion also seems to be highly effective in suppressing allergic reactions (Frankel and Weidner, 1953; Simon and Eckman, 1954).

Although antihistamines have been shown to be effective when given in this way, two points must be made. First, allergic manifestations are not common following transfusion and antihistamines should be given only to those recipients with a history of previous allergic manifestations. Second, when antihistamines are indicated they should not be added to the blood to be transfused but should be given by mouth or, if necessary, by injection. For example, 50 mg. of benadryl may be given by mouth 1 hour before transfusion and a further 50 mg. after the start of the transfusion. If antihistamines have not been given and an allergic reaction develops, 25 mg. or 50 mg. of benadryl or pyribenzamine may be given intramuscularly or even intravenously (Davenport, 1954).

Effect of Steroids

In patients who are known to get severe allergic reactions it may be wise to give steroids prophylactically before transfusion in view of the evidence that prednisolone, for example, given in sufficiently large doses can completely protect mice against anaphylaxis, whereas pyribenzamine is completely ineffective (Fox *et al.*, 1958).

INFECTED BLOOD

Sources of Infection

Introduction of Bacteria at the Time of Blood Collection

There can be no doubt that skin contaminants frequently gain entrance to blood collected by ordinary venepuncture. Spooner (1942) collected the first few millilitres of blood drawn from donors' veins during the routine collection of blood for transfusion. The blood was taken into broth and cultured. Bacteria were obtained from thirty-one out of 601 (5 per cent) of the samples. The incidence of bacterial contamination was significantly lower when cleaning of the skin with ether soap was followed by the application of a chemical disinfectant, than when ether soap alone was used.

Spooner quoted a personal communication from Professor A. A. Miles to the effect that in a large series of blood cultures, where the greatest possible care had been taken to avoid contamination, organisms – apparently skin staphylococci – grew in about 2 per cent of the cultures.

The inference must be drawn that a small proportion of bottles of blood, taken for transfusion, are contaminated with skin organisms at the time of collection; these must usually be killed on storage for Spooner was unable to culture bacteria from any of 114 bottles of whole citrated blood stored for 3 weeks.

Braude *et al.* (1952) examined almost 1700 samples of fresh bank blood and found that 2.2 per cent of the samples were contaminated. These bloods had been stored at 4° C. for only 24 hours; with a longer period of storage most of these contaminants would probably have died since they were mostly staphylococci presumably growing preferentially at 37° C.

When bottles which have been stored for 2 to 3 weeks are cultured, very few bottles are found to be contaminated; thus James (1958, p. 144) found only one infected bottle in the course of testing 700 and Chaplin *et al.* (1955) found none in the course of testing 200 outdated bottles. Both James and Chaplin *et al.* carried out their cultures at 22° C. (as well as at higher temperatures) to provide optimum conditions for the growth of 'psychrophilic' organisms, that is to say, those growing preferentially in the cold. The importance of these organisms has been emphasized in many reports. Thus Wetterlow *et al.* (1954) found that of all contaminants

RCPE0002068_0286

detected in the routine examination of biological products over a 10-year period, 24 per cent failed to grow at 37° C. and were detected only by culturing at 20-25° C.

Pittman (1953) made an extensive study of organisms isolated from bottles of blood that had become infected; in many cases the blood had been transfused with fatal results. The commonest organisms isolated were from the pseudomonas and coli-aerogenes groups.

Similarly, Braude *et al.* (1955) found that the majority of cold-growing contaminants were pseudomonads, coliforms and achromobacters (all gram-negative). These organisms were readily grown from faeces, dirt and soil. Of the bacteria originally isolated at 4-8° C. many strains grew heavily in blood in 1 week and most did so in 2 weeks, reaching a concentration of 10^8 to 10^{10} bacteria per millilitre. At room temperature growth was much more rapid, the log phase of multiplication beginning after only 6 hours. Thus if blood contaminated either with cold-growing, or warm-growing organisms is left at room temperature for 24 hours the number of bacteria may reach a lethal level (Braude *et al.*, 1952).

Gram-positive organisms are occasionally isolated from infected stored blood; of seven cases collected from the literature by Pittman (1953) only one had proved fatal. These organisms did not grow at 5-7° C. but sometimes grew slowly at 10° C. within 7 days.

Pittman pointed out that *Escherichia freundii* differed from *E. coli*, in being able to use citrate as the sole source of carbon; all organisms isolated from stored blood were capable of using citrate. Similarly, Braude *et al.* (1952) found that many strains of organisms isolated from contaminated stored blood produced clotting by consuming citrate. Among bacteria isolated from stored blood at 4-8° C. by Braude *et al.*, 75 per cent produced haemolysis and 15 per cent produced clotting.

Braude *et al.* (1955) investigated the effect of antibiotics on bacteria in stored blood. They found that chlortetracycline (aureomycin), oxytetracycline (terramycin) and polymyxin B in concentrations as low as 10 mg. in 500 ml. of blood (i.e. 20 µg./ml.) would all prevent large inocula of either cold-growing or warm-growing bacteria from multiplying in human blood either at refrigerator temperature or room temperature. Nevertheless, there are several reasons why the routine addition of antibiotics to stored blood cannot be recommended. Firstly, antibiotics cannot be autoclaved and would therefore have to be added later and this addition might itself introduce infection. Secondly, no antibiotics are effective against all organisms. Thirdly, the risk of sensitizing patients to particular antibiotics must be considered.

Bacteria already present in the Apparatus

When several bottles of blood in a bank are found to be contaminated at the same time, the inference may be drawn that there has been a failure to sterilize the apparatus or vessels used for blood collection.

More than one outbreak of this type occurred during the years 1940-42. During that time there were periods when, due to air raids, there were particularly heavy demands for stored blood. There was a tendency to pack autoclaves and ovens to the limit of their capacity and to reduce the period of 'sterilization' to the minimum. Presumably not all the contents were adequately heated, and blood was later drawn into a citrate solution that was already teeming with organisms.

Following the recommendations of Spooner and Turnbull (1942), new standards of sterilization were adopted and, so far as the author can discover, whenever these standards are adhered to, there is no risk of the recurrence of the wartime epidemics of infected blood.

The Properties of Infected Blood

In vitro

Reissman (1942) observed that a sample of stored blood might be grossly contaminated with organisms but show no obvious haemolysis. Dr W. d'A. Maycock (personal communication) states that in an 'outbreak' of contaminated stored blood investigated by him, in which there were some fatalities, the blood was not haemolysed, though 'hanging-drop' preparations of the plasma were teeming with organisms.

Unless blood is heavily contaminated with organisms, microscopical examination is an unreliable method of detecting infection.

Walter *et al.* (1957) added serial dilutions of an inoculum of a coliform organism to blood and found that when 24×10^5 organisms per millilitre were present they could be detected readily but that 24×10^4 could only be detected with difficulty since on the average only one organism was found in every 100 fields examined. On the other hand when 0.3 ml. of the material was cultured for 24 hours, contamination could be detected when the number of organisms was as small as 24 per ml.

In routine work the examination of a stained film of stored blood as a method of excluding bacterial contamination is unsatisfactory because debris is present which is in practice very difficult to distinguish from organisms, so that it is usually impossible after looking at the stained film to be really confident that no organisms are present. It has been claimed that it is more satisfactory to examine an unstained preparation by phase-contrast microscopy (Faria, 1960).

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In vivo

Borden and Hall (1951) reported two cases in which the transfusion of infected blood had proved fatal. The organisms were shown to produce a potent pyrogen which was fatal to rabbits.

Geller and Jawetz (1954) found that blood contaminated with *Escherichia intermedium* or *Paracolobactrum intermedium* was toxic to mice, when injected intraperitoneally, only if it contained 10^8 to 10^9 viable bacteria per millilitre. If the bacteria were first killed by heating, much larger numbers had to be given to kill the mice. The administration of antibiotics within 1 hour of the injection of the contaminated blood had a distinct protective effect.

The observations of Swierczynska (1957) suggest the possibility that the presence of a naturally-occurring antibody to the organism in question may be important. Thus he found that if the red cells of guinea-pigs were incubated with *Bacillus subtilis* and then injected, amongst thirty-seven animals with a naturally-occurring antibody twenty-one died, whereas amongst seventeen animals without antibody only one died.

When human subjects are transfused with heavily infected blood there is usually immediate collapse, as in the following case (Mollison, 1943a):

> The patient was a woman aged 46, with a history of menorrhagia due to uterine fibroids. Her haemoglobin concentration was 7 g./100 ml. and, as a preparation for operation, it was decided to give her a large transfusion.
>
> A concentrated suspension of red cells was prepared from blood previously stored for 3 to 4 days; the suspension was left at room temperature for 4½ hours before being transfused.
>
> The needle was inserted into a vein at the right wrist but after only about 10 ml. of blood had been given the needle was noticed to have come out of the vein. A new vein in the other arm was selected and the transfusion was re-started. The blood was allowed to flow at a rapid drip for between 1 and 2 minutes. At the end of this time the patient complained that her face was feeling hot; a few seconds later she said she was feeling uncomfortable and then that her face felt very hot indeed. She had developed a bright red malar flush. The transfusion was stopped immediately. At this point the patient gave two or three short dry coughs, and said that her breathing 'caught her'. During the next few minutes she gave several more short coughs and complained that her head felt as if it were bursting. About 5 minutes after the transfusion had been stopped she said that her face was not burning so much and she felt a little better. The patient next started to feel very sick and soon afterwards vomited copiously; she also complained of severe pain in the abdomen and had diarrhoea. One hour later she was shivery but after a further hour she felt a little better. Her temperature was 96.2° F., pulse 100, blood pressure 100/40. Soon afterwards her temperature started to rise and eventually reached 105.6° F.; thereafter it fell slowly, the patient became progressively weaker and died 14 hours after the injection of the blood.
>
> Meanwhile investigations had shown that the bottle, from which about 45 ml. of blood had been transfused, had a slight permanganate colour and a faint smell of hydrogen sulphide. Microscopic examination revealed numerous bacteria. Centrifugation of a mixed sample of the blood revealed gross haemolysis. This was a surprise because previously, when the concentrated cell suspension was prepared, the supernatant plasma had appeared clear. Apparently the leucocyte 'mat' in the infected bottle had prevented the diffusion of haemoglobin.
>
> The organism in the blood was found to be an achromogenic, gelatin-liquefying coliform – an organism commonly found in soil and water. It grew vigorously at 20° C. and less well at 37° C. and 4° C. An apparently identical organism was grown from a bottle of blood which caused a second death at about the same time.

Investigations following the suspected Transfusion of Infected Blood

Any blood remaining in the bottle should be cultured at various temperatures, including 4° C. and 20° C. A negative culture excludes the possibility that the blood was seriously infected at the time of transfusion. A positive culture does not enable one to say with certainty whether the blood was contaminated before the transfusion or became contaminated during or after transfusion, although if a heavy growth is obtained, the evidence points strongly to contamination before transfusion. It might be thought that if the blood remaining in a bottle had been left at room temperature for 24 hours it would be useless to culture it, since it would inevitably be infected, but according to James and Stokes (1957) such blood is usually sterile or at most lightly contaminated.

In fatal cases, blood should be collected from the body (e.g. by cardiac puncture) as soon as possible after death and be cultured. Pittman (1953) found that when blood contaminated with a coli-aerogenes bacterium had been transfused, cultures collected after death were usually positive although when pseudomonads were concerned blood collected at autopsy was sterile.

Sterilization of Apparatus for Blood Collection

Standards of autoclaving should be very high to provide a wide margin of safety. It is now considered that apparatus should be steam sterilized at a pressure of 20 lb. per sq. in. for 30 minutes, following the evacuation of all air from the autoclave.

This conclusion is based on the recommendation that dressing drums, with which transfusion apparatus may reasonably be compared, should be exposed for 30 minutes to steam at 20 lb. pressure (≡ 126° C.). The exposure time here means the

RCPE0002068_0288

sum of the penetration time, that is the time taken for the steam to penetrate the load, the holding time, that is the time known to be necessary to kill spores at this temperature, and the safety period, which is usually half the holding time (M.R.C. report, 1959). The M.R.C. report recommended that Brown's tubes, type I, should be incorporated with the material to be sterilized to confirm that the correct temperature has been reached and held.

Autoclaves should be provided with an effective air filter so that air admitted during cooling does not contain organisms. The tops of bottles should be covered with sticky tape of the type which will withstand autoclaving. When the time comes to pierce the closure of the bottle with a needle of a taking set, the top is simply removed to expose a sterile surface through which the needle can be plunged. If the top of the closure is wiped with spirit, it is essential to use spirit which has been autoclaved (James, 1959).

Maintenance of Refrigeration

From the point of view of contamination, in temperate climates it is not essential to refrigerate blood until at least 8 hours after collection, presumably because during this period the blood still contains viable phagocytes (James and Stokes, 1957). The same authors considered the possible advantages of actually incubating the blood for 2 hours at 37° C. immediately after collection as this increases the opportunities for self-sterilization of the blood (at least with certain organisms), but they concluded that the advantages were not definite enough to overcome the slightly deleterious effect on red cell preservation of 2 hours' incubation at 37° C. (see Chapter I). After the first 12 hours or so of storage the maintenance of strict refrigeration at a temperature of 4° C. becomes absolutely vital. When a patient is to be given several bottles of blood only one should be taken to the bedside at a time; the others must remain in the refrigerator until the last possible moment.

Risks of Opening Bottles

Although the bactericidal power of stored blood is less than that of fresh blood 50 per cent of samples of stored blood will suppress the growth of bacteria (Braude *et al.*, 1952). Geller and Jawetz (1954) found that when organisms were added to 3-day old blood there was a big fall in numbers but a few survived. If the blood was now stored in the cold, there was often a lag of at least 1 week before rapid multiplication of the organisms occurred.

The risk of entering a bottle of stored blood, for example to obtain a sample for cross-matching, is that organisms may be introduced which will not be killed subsequently. Although such organisms will probably not multiply rapidly if the bottle is kept at 4° C., they may multiply extremely rapidly if the bottle is allowed to remain for some hours at room temperature. In many countries the 'entering' of bottles before transfusion is specifically prohibited.

Limitation of Storage Period

McEntegart (1956) reported two fatalities from the transfusion of infected stored blood; the bottles had been stored for 15 days and 21 days respectively. The organism was a gram-negative bacillus growing best at 30° C., less well at 4° C. and not at all at 37° C. When blood samples inoculated with the organism were stored at 4° C., there was no growth for 3 or 4 days; thereafter the number of organisms doubled every 8 hours. The author suggested that stored blood does not become dangerous until it has been stored for about 2 weeks.

Precautions in using Suspensions of Red Cells

Red cell suspensions are usually prepared by simply removing most of the supernatant citrate-plasma from a bottle of stored blood that has sedimented. It is convenient to pool the concentrated red cell suspensions from two bottles. Since red cell suspensions are normally transfused for the relief of anaemia, they should be prepared from blood which has been stored for not more than 2 weeks.

Even when the cell suspension is prepared in a 'sterile room', with all reasonable precautions, organisms will occasionally be introduced. If the cell suspension is kept at 4° C. and is used within 6 hours, the introduction of a few bacteria at the time of preparation will be of no account. However, should the cell suspension be exposed to room temperature for periods much exceeding 6 hours, the situation grows dangerous. Insufficient information is available to allow the precise definition of safety limits. It is possible merely to re-affirm the principles: all cell suspensions prepared from stored blood should be regarded as possibly infected. They must be kept in the refrigerator until they are used and they should be used within 24 hours of preparation.

Precautions during Transfusion

When cell suspensions are used for transfusion it must again be remembered that they may contain bacteria. If the cell suspension is taken from the refrigerator shortly before transfusion and the transfusion is completed

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within a few hours, the blood will scarcely have time to reach room temperature and the hazard of bacterial multiplication will be negligible. However, if the blood is warm when the transfusion is begun and the transfusion is continued over a long period of time, the hazard cannot be considered to be negligible. When giving slow transfusions it is unreasonable and unwise to warm the blood. DeGowin *et al.* (1940) found that the incidence of transfusion reactions was not increased by using blood taken straight out of the refrigerator.

When long-continued transfusions are given and the same 'giving set' ('recipient set') is used for a series of bottles, there is a risk of contaminating the infusion, particularly when the set is of the type in which the filter is inserted into the bottle, and of carrying over bacteria from one bottle to another.

Michaels and Ruebner (1953) described the occurrence of pyrexia in two patients receiving intravenous infusions lasting several days and they demonstrated the presence of coliform organisms in the sets, which apparently were not changed during the period of infusion.

This risk can and should be reduced by changing the giving set at the same time as the bottle, in all transfusions which last for more than 8 hours. Another obvious precaution in slow transfusions is to guard the air-entry into the bottle with a small cotton wool filter, and this is now incorporated in most sets.

TRANSMISSION OF DISEASE

Serum Hepatitis (Homologous Serum Jaundice)

This disease is caused by a virus which is present in the blood of a small proportion of the population. The disease is commonly transmitted by transfusion of blood or plasma from a carrier to a susceptible subject; the incubation period is generally between 60 and 160 days (W.H.O. Tech. Report, 1953). The disease may be so mild as to cause only transient liver dysfunction without clinical jaundice or so severe as to cause fatal hepatic necrosis.

Carriers of Hepatitis

The exact incidence of carriers is unknown but is probably of the order of 1 in 200.

This is based on the evidence that approximately 0.5 per cent of subjects transfused with two bottles of blood develop hepatitis (see below), suggesting that after a single bottle transfusion the incidence might be 0.25 per cent. If all subjects were susceptible this would imply a carrier rate of 0.25 per cent but in fact only 50 per cent of subjects are susceptible (Murray, 1955), so that the carrier rate may be double this, or 0.5 per cent.

Assuming that the carrier rate is less than 1 per cent, it is easy to understand that Loutit and Maunsell (1945) failed to demonstrate virus in ninety-nine consecutive samples of serum. They injected each sample into an average of six recipients and observed no case of jaundice in any of the subjects during a 150-day follow-up period.

It is known that a subject may remain a carrier for at least 1 year (Murray *et al.*, 1954) and perhaps for as long as 5 years (Stokes *et al.*, 1954). The importance of following up all blood donors concerned when a recipient develops jaundice 2 to 4 months after transfusion is emphasized by the paper of Heistö and Julsrud (1959) which records ten cases of probable serum hepatitis caused by blood given by two donors who over a period of at least 3 to 4 years remained carriers of serum hepatitis.

The value of liver function tests in detecting carriers is uncertain. Neefe *et al.* (1954) reported that of eight donors, transfusion of whose blood was followed by hepatitis in recipients, seven had abnormal liver function tests. The blood of five out of six of these donors caused hepatitis when injected into volunteers (Murray *et al.*, 1954). Dr R. Murray (personal communication) found that the thymol turbidity test was never positive in 30 per cent of subjects with serum hepatitis and even when it was positive during the illness, was almost always negative after recovery from the illness.

In a study of 3655 presumably healthy blood donors, subjected to a group of hepatic function tests which commonly give abnormal results in viral hepatitis, nearly 30 per cent were classified as abnormal (Fitch *et al.*, 1955). In 17 per cent the results deviated only slightly from normal values but the remaining 12 per cent showed abnormalities similar to those in moderately severe hepatitis. The results of the thymol turbidity test were those which were most frequently abnormal (16 per cent). As Fitch *et al.* point out, the rejection of 30 per cent of donors on the basis of abnormal hepatic function tests would cause acute embarrassment to donor panels and, although some asymptomatic carriers of the virus would be excluded by such an operation, not all carriers would be excluded since it is known that they may not exhibit abnormalities detectable by such tests (Stokes *et al.*, 1954).

There is evidence that newer liver function tests based on estimation of liver enzymes in the serum, namely serum glutamic oxalacetic transaminase (SGOT) or isocitric dehydrogenase (ICD) may give a better

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discrimination. Thus Bang *et al.* (1959) found that among 600 patients who received blood from donors whose SGOT level was below 40 units, the incidence of hepatitis was 1.5 per cent compared with an incidence of 3.5 per cent in 624 patients receiving blood with the level of 41-99 units, and an incidence of 5.5 per cent in 144 patients receiving blood with a level exceeding 100 units. If all donors with a SGOT level of over 40 units had been excluded, the incidence of hepatitis would have been reduced from thirty-nine in 1371 (2.8 per cent) to nine in 600 (1.5 per cent), but at a cost of rejecting 57 per cent of all donors!

The conclusions of Norris (1960), based on the results of a battery of tests, including estimation of the ICD activity, have similar implications. Norris collected thirty-nine donors who were all almost certainly carriers of serum hepatitis since transfusion of their blood alone had been followed within the known incubation period by an attack of hepatitis in the recipient. Of these donors, twenty-four (61.5 per cent) had abnormal liver function tests compared with an incidence of 30 per cent in a control series. Again then, the conclusion is that a very large proportion of donors would have to be rejected to reduce the incidence of serum hepatitis and even then, perhaps one-third of the carriers would pass the screening tests and be accepted as 'safe' donors.

Incidence of Hepatitis in Recipients of Plasma and Blood

The transmission of the virus of hepatitis by the transfusion of pooled plasma at one time threatened to prevent altogether the use of plasma. When it is recalled that in one of the first series to be studied (Morgan and Williamson, 1943) nine out of fifty patients receiving a plasma transfusion later developed an illness lasting 3-12 weeks, the seriousness of the problem can be realized. At first it was not clear that the pooling of large numbers of plasma samples played an important role in the incidence of the disease. Later, when this was realized, small pools were prepared and the incidence of hepatitis was greatly reduced. Thus Lehane *et al.* (1949) found that whereas the incidence of hepatitis in patients transfused with large-pool plasma was 11.9 per cent, in patients transfused with small-pool plasma it was 1.3 per cent. The large-pool plasma was prepared from the blood of at least 300 donors and the small-pool plasma from not more than ten donors.

In a further survey (M.R.C., 1954a) the incidence of hepatitis following the transfusion of small-pool plasma was found to be less than 1 per cent.

The incidence of hepatitis in recipients transfused with blood alone has been found to vary between 0.8 and 0.16 per cent (Lehane *et al.*, 1949; M.R.C., 1954a). The average number of donors for each transfusion was approximately two.

Murray (1955) made an estimate of the minimum volume of plasma which could transmit infective hepatitis. He used a pool of plasma made up as follows: 1 litre of plasma, taken from several different donors in the acute stage of hepatitis, was added to 130 litres of plasma from approximately 400 apparently healthy donors. Dilutions were made from this pool and 1 ml. of each dilution was given to five subjects. The results were as follows: 1 in 1000 dilution, two cases of hepatitis out of five; 1 in 10,000 dilution, one case out of five; 1 in 100,000 to 1 in 10,000,000, no cases out of fifteen. He concluded that the injection of as little as 0.000,001 ml. of known infected plasma was probably capable of inducing an attack of hepatitis.

Attempts to Reduce Infectivity of Plasma

The virus of hepatitis can be killed by exposure to ultra-violet light (Oliphant, 1944). Unfortunately it is difficult in practice to sterilize the plasma without damaging the plasma proteins and the method is thus of doubtful value. In the Medical Research Council (1954a) survey the incidence of hepatitis in patients transfused with irradiated plasma (from pools of sixty to eighty donors) was approximately 4 per cent. Murray, Oliphant *et al.* (1955), using three different ultra-violet irradiations to treat known icterogenic plasma, failed to sterilize it although the irradiated plasma appeared to cause a milder form of hepatitis with longer incubation periods than the unirradiated plasma.

Of the many chemicals tested, β-propiolactone was found by Hartman *et al.* (1954) to be the most satisfactory. When pooled plasma is treated with this compound and then submitted to ultra-violet irradiation, it appears to be no longer infective but the conditions of treatment of the plasma appear to be fairly critical (LoGrippo, 1960).

Storage at room temperature seems to result in attenuation of the virus (Allen, J. G. *et al.*, 1950; 1954). In experiments in volunteers, Murray *et al.* (1954) induced hepatitis in only one out of nineteen subjects receiving known infected plasma previously stored at room temperature for 6 months. By contrast the incidence of hepatitis in volunteers receiving unstored infected plasma from the same pool was approximately 50 per cent (Murray *et al.*, 1954). Of five volunteers who received plasma stored at room temperature for only 3 months, three developed hepatitis.

It appears that storage of liquid plasma at 31.6° C. (89° F.) for 6 months makes it almost completely free from the risk of transmitting serum

RCPE0002068_0291

hepatitis (Sayman *et al.*, 1958); the same authors found that storage at 22.4° C. (72° F.) for 7 months was ineffective.

Storage at 31° C. does not seem to be a completely satisfactory answer to the problem of providing safe and satisfactory liquid plasma first because as many as 20 per cent of bottles become infected and have to be discarded (Allen, J. G., *et al.*, 1959) and second because the extent of protein denaturation occurring at this temperature is unknown and the transfusion of denatured protein is potentially hazardous.

Incidence of Hepatitis in Recipients of Plasma Fractions

Human albumin prepared by the alcohol fractionation method of Cohn appears to be free from hepatitis virus (Paine and Janeway, 1952). This albumin is normally heated for 10 hours at 60° C. but it has been shown that it is not the heating alone which is responsible for the freedom from infectivity, since unheated albumin produced from known infected plasma is not infective (Murray, Diefenbach *et al.*, 1955). Heating infected plasma to 60° C. for 10 hours does in fact kill the virus although heating for 4 hours does not (Murray and Diefenbach, 1953). Unfortunately, whole plasma subjected to 60° C. even for 4 hours is not fit for use as a transfusion fluid, owing to the denaturation of globulin (Murray, Diefenbach *et al.*, 1955).

Gamma globulin prepared by the Cohn fractionation process, which has been used extensively in the United States, appears to be free from virus (Murray and Ratner, 1953). In Britain gamma globulin is prepared by the ether-fractionation procedure (Kekwick and Mackay, 1954); this material is also very safe. Only a single case of hepatitis has been related to the injection of this material and in that case the gamma globulin had been prepared from a pool of plasma subsequently found to be infected (Cockburn *et al.*, 1951). As far as is known, no other cases of hepatitis after the injection of gamma globulin have been recorded.

Human thrombin may carry virus and induce hepatitis after local application (Porter *et al.*, 1953).

It is not known whether fibrinogen, prepared from human plasma, carries the virus of serum hepatitis and it would be difficult to find out because almost all patients who receive fibrinogen also receive blood or plasma or both (Anderson and Gibson, 1957).

Failure of Gamma Globulin to Protect against Serum Hepatitis

Gamma globulin injected at the same time as infected whole blood or serum and again 40 days later, failed to protect volunteers (Drake *et al.*, 1953). The gamma globulin used was prepared from the plasma of subjects convalescent from serum hepatitis.

Other Methods of Transmitting Serum Hepatitis

The risks of transmitting serum hepatitis by using imperfectly sterilized syringes are now well recognized. The danger exists not only when injections are given but also when samples are withdrawn. When blood is being withdrawn into a syringe it is virtually impossible to avoid some reflux of the contents of the syringe into the subject's vein at the moment when traction on the barrel of the syringe ceases (Mendelssohn and Witts, 1945).

If many blood samples have to be taken and there are insufficient sterile syringes, blood should be collected with a needle alone.

Serum hepatitis can be transmitted by minute volumes of plasma injected intradermally (Bradley *et al.*, 1944) and by the use of unclean tattooing needles (Smith, B. F., 1950; Hobson *et al.*, 1952). It can also be acquired by handling infected blood; the risk probably exists only when the hands are cut or pricked. Thus in one case the patient was a female blood bank worker who 'often' pricked her hands and fingers with needles withdrawn from donors' arms. Compensation was awarded (Leibowitz *et al.*, 1949).

Kahn *et al.* (1952) reported that in an 8-year period they had encountered three cases of virus hepatitis amongst their washroom workers but none elsewhere. The washers stated that they frequently cut their hands on broken glass and spilt blood on their hands. Trumbull and Greiner (1951) collected sixteen cases of hepatitis amongst workers in four blood banks over a 3-year period. According to Madsen (1952) hepatitis is ten times more common in nurses employed in laboratories than in those employed elsewhere. It is obviously a duty to warn laboratory workers of this danger and to encourage them to avoid contaminating their hands with blood.

It is thought that serum hepatitis can be transmitted by a mother to her foetus across the placenta. Thus Stokes *et al.* (1954) investigated a case in which an infant developed jaundice at the age of 4 months and died of hepatic cirrhosis at 18 months, and found that the mother was a carrier of hepatitis. Dible *et al.* (1954) reported a series of cases of foetal and neonatal hepatitis; in some instances successive siblings were affected, suggesting that the mother might be a carrier of hepatitis virus.

Syphilis

From a review of the literature Hartmann and Schøne (1942) concluded that the incubation period in transfusion syphilis varies from 4 weeks to

RCPE0002068_0292

4½ months, averaging 9–10 weeks, and that the recipient usually exhibits a typical secondary eruption. They also concluded that even donors with latent syphilis could transmit the infection.

Transfusion syphilis was once a serious problem; for example, before 1939 as many as twelve cases had been recognized at a single hospital (Ravitch *et al.*, 1949). At the same hospital not a single case was recognized during the following 10 years and the authors concluded that this was mainly due to two factors: firstly, adoption of the routine of testing all donors serologically; secondly, the storage of blood in the refrigerator before administration.

Routine serological testing is not by itself an adequate safeguard. Turner and Diseker (1941) were able to collect from the literature twenty cases of transfusion syphilis in which routine serological examination had failed to disclose the infection. This is not surprising when it is remembered that some 35 per cent of individuals with primary syphilis are serologically negative.

Storage of blood at 4 to 6° C. is one of the most important factors in the decline of transfusion syphilis. The work of Bloch (1941), Turner and Diseker (1941) and Kolmer (1942) is in essential agreement in demonstrating that sphirochaetes are unlikely to survive for more than 72 hours in citrated blood. Selbie (1943) found slightly longer survival, namely 6 days. In all these experiments, citrated blood was heavily inoculated with spirochaetes and sometimes with tissue containing spirochaetes. It is probable that the conditions obtaining in practical blood transfusion work would be less favourable to the survival of spirochaetes. It may be concluded that blood stored for 4 days or more is virtually safe from this point of view and that blood stored for even as little as 24 hours is probably much safer than fresh blood. Spirochaetes do not survive for more than 24 to 48 hours in plasma kept at −20° C. (Ravitch and Chambers, 1942) nor do they survive drying. Ravitch *et al.* (1949) pointed out that a further safety factor which had been introduced was the routine administration of penicillin to a large proportion of patients ill enough to require transfusion.

Syphilitic reagin can be transferred and thus, if present in the donor's plasma, can cause temporary positive serological tests in the recipient. In some experiments Ravitch *et al.* found that the test never remained positive for more than 20 days after transfusion.

Malaria

According to Hutton and Shute (1939) all known species of malaria parasites are able to survive for days and even weeks in blood stored at

4° C. For example, quartan malaria (*P. malariae*) has been accidentally transmitted in blood previously stored for 5 days (McClure and Lam, 1945) and malignant tertian malaria (*P. falciparum*) has been transmitted by blood stored for 14 days (Grant *et al.*, 1960).

The problem of preventing the accidental transmission of malaria cannot then be solved simply by using stored blood. Neither can it be solved by examining blood films from potential donors. In the case reported by Nabarro and Edward (1940) in which an infant died following the accidental transmission of malaria by transfusion, several hours' search of thick films was required to find two parasites (Hutton and Shute, 1939). Similarly, in the case reported by Grant *et al.* and referred to above, an initial prolonged search of films failed to disclose any parasites although a single one was found later.

The conclusion must be that ideally in non-malarious areas one should avoid using as a blood donor any person who has previously resided in an area in which malaria is endemic; if such donors have to be used, the best step seems to be to give a single dose of 600 mg. (base) Chloroquine phosphate to the donor a few hours before taking his blood. According to a W.H.O. report (1955) this renders the peripheral blood of malaria carriers free of parasites. If blood from an untreated suspected carrier is transfused the administration of anti-malarial drugs to the recipient will prevent the development of infection.

Officer (1945) injected into each of five healthy volunteers blood containing some 300 million malaria parasites. All the volunteers were given anti-malarial treatment after transfusion and none developed malaria. Similarly, Thoroughman (1940), who worked in an area of China where malaria was endemic, observed no attacks of malaria in thirty-four recipients given quinine for 3 days after transfusion. In a control group receiving no quinine, approximately half the recipients developed an attack of malaria within 20 days of transfusion. Presumably nowadays a short course of chloroquine would be given instead of quinine.

In connection with the therapeutic injection of malarious blood, it is of interest that when incompatible blood is used, the incubation period is significantly longer than when the blood is compatible. It was first shown by Wendelberger (1927) that the delayed onset of induced malaria was associated with incompatibility of the injected red cells. Patients in whom malaria was induced within 8 days, despite incompatibility, were found to have a low iso-agglutinin titre (Obermayer and Wendelberger, 1928).

In a more systematic study Polayes and Derby (1934) found the incubation period to average 4.3 days when compatible blood was used and 8.2

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days when the blood was incompatible; this difference was shown to be highly significant. Presumably, in the compatible red cells the malaria parasites have time to develop normally; by contrast, when the red cells are incompatible, the parasites are either set free in the blood stream and have to find new red cells to enter, or are engulfed within the remains of the destroyed red cells and taken up by the cells of the reticulo-endothelial system.

Brucellosis

In a case described by Wood (1955) a donor felt unwell immediately after giving blood; undulant fever was diagnosed 4 weeks later. The recipient felt unwell 13 weeks after the transfusion and developed fever and a rash; specific agglutinins appeared soon afterwards. Wood gives a reference to two other cases in which brucellosis was clearly transmitted by blood transfusion (Meno Brito, 1948).

TOXIC FACTORS IN PLASMA, INCLUDING ANTICOAGULANTS

Toxicity of Citrate

The plasma normally contains approximately 1 mg. of citrate per 100 ml., expressed as citric acid (Howland *et al.*, 1957). During the infusion of citrate the plasma level rises and may reach approximately 100 mg. per 100 ml. (see below). At such levels, serious toxic effects may be observed. In man these levels have been reached either during exchange transfusion to newborn infants or during rapid transfusions to adults with impaired liver function.

Newborn infants treated by exchange transfusion may receive as much as about 600 mg. of citrate per kilogramme in 1 hour and their plasma citrate level may reach 100 mg. per 100 ml. (Wexler *et al.*, 1949). There is no doubt that signs of citrate toxicity may develop during exchange transfusion. Thus in one series of infants receiving 500 ml. of citrated blood in 100 minutes, muscle tremors and changes in the electrocardiogram, namely a marked prolongation of the ST segment, were observed. The changes were rapidly reversed by the administration of 4-8 ml. of 10 per cent calcium gluconate (Furman *et al.*, 1951). Similarly Gustafson (1951) exchanged 500 ml. in 75 minutes and found gross skeletal muscle tremors after 100-150 ml. of blood; these could be rapidly abolished by the injection of 2 ml. of calcium gluconate. The corresponding rate of administration of citrate can be calculated to be approximately 0.03 m-mole/kg./minute (assuming that approximately 2 g. of disodium citrate was injected in 80 minutes into an infant weighing 3 kg.).

Experiments in animals suggest that such a rate is only just safe. Thus, according to Adams *et al.* (1944) the administration of 0.06 m-mole/kg./minute for 20 minutes is lethal, although 0.04 m-mole/kg./minute is safe. Cumulative effects must be considered as well as rate for Nakasone *et al.* (1954) concluded that there was ' ... an inverse ratio between the rate of intravenous infusion of ACD solution and the cumulative amount of citrate required to produce equivalent ECG changes'. Nevertheless by keeping the rate of administration at 0.02 m-mole/kg./minute they were able to inject a total dose of approximately 1.8 m-moles of citrate per kilogramme (in 90 minutes). This corresponds to the administration of almost 7 litres of citrated blood in an adult man.

Observations in adults are in general agreement with those in infants and in experimental animals. For example, Howland *et al.* (1957) gave infusions of 40 ml. of ACD per minute for 20-30 minutes to three anaesthetized patients, a rate corresponding to 6-8 mg. citric acid per kilogramme per minute, that is almost 0.04 m-mole/kg./minute. The plasma citrate level rose to about 80 mg. per 100 ml. and there was a prolongation in the QT segment of the electrocardiogram.

Nakasone *et al.* (1954) found that in dogs the toxic changes observed were: a prolongation of QT, pulsus alternans, depression of P and T waves, and development of muscle tremor. With higher doses the arterial pressure declined to zero and the animal died unless calcium was injected promptly.

Although the injection of calcium reverses the toxic effects of citrate it appears that the signs observed are not due solely to the absence of ionized calcium, but are in part due to other effects of citrate. Thus the administration of blood rendered hypocalcaemic by passage across a cation exchange resin does not produce circulatory derangements as severe as those caused by citrated blood (Nakasone *et al.*, 1954).

Firt and Hejhal (1957) recommended that when citrated blood was going to be given very rapidly, that is at rates up to 500 ml. a minute, 10 ml. of 10 per cent calcium gluconate should be given immediately before transfusion and a further 15 ml. given intravenously (into another vein) after the first 100 ml. and a further 10 ml. after each subsequent 500 ml.

It should be pointed out that when calcium gluconate is used the dose of the material must be almost four times greater than when calcium chloride is used. Thus the molecular weight of calcium gluconate is 448

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and that of calcium chloride 111; each of the molecules contains one atom of calcium (atomic weight 40) so that the amount of calcium in calcium gluconate is 9 per cent by weight but in calcium chloride 37 per cent.

The view is sometimes expressed that large citrate infusions may interfere with blood clotting. It seems that this risk is of small importance compared with the risk of producing cardiac failure. Nevertheless, according to Soulier (1958), the blood is sometimes found to be incoagulable following massive citrate infusions, and the coagulability can be restored *in vitro* by the addition of calcium.

From data described above it may be concluded that in an adult weighing 60 kg. as much as 2 l. of citrated blood can safely be given in 20 minutes provided that liver function is not impaired. If more than this has to be given, it would seem wise to inject calcium; an appropriate dosage would be 10 ml. of 10 per cent calcium gluconate for every litre of citrated blood.

Impaired liver function. In patients with liver disease or with mechanical obstruction to the hepatic circulation, the rapid administration of citrate is much more likely to produce toxic effects. Thus Bunker *et al.* (1955) found that when citrated blood was infused at a rate of 500 ml. in 15 minutes the plasma-citrate concentration rose above 0.5 m-mole/litre in almost all patients with liver disease, but in only 50 per cent of normal subjects. In five patients with severe liver disease or with mechanical obstruction to the hepatic circulation, the calculated concentration of ionized calcium was 0.6 m-mole/litre or less, compared with the normal value of 1.0 mM./litre. A concentration of 0.6 mM. must be regarded as very dangerous in view of the fact that at a concentration of 0.5 mM. the isolated frog's heart stops beating (McLean and Hastings, 1934) and the clotting-time of blood is prolonged (Stefanini, 1950).

Bunker *et al.* (1955) found that in patients with citric acid intoxication it was difficult to predict the appropriate dose of calcium; they recommended that if transfusions had to be given to patients likely to develop this syndrome, that is to say patients with liver disease or with mechanical obstruction to the hepatic circulation, packed cells rather than whole citrated blood should be used.

Ludbrook and Wynn (1958) found a considerable rise in plasma citrate in four patients undergoing operations and they concluded that the administration of citrate was particularly dangerous in patients in whom the liver circulation was impaired as, for example, any operations in which hepatic circulation was temporarily interfered with. They also considered that hypothermia increased the risk of citrate intoxication.

Toxicity of EDTA

When EDTA is added as an anticoagulant to whole blood, the amount used is normally just sufficient to chelate the available calcium. Thus when the blood is transfused, EDTA is for practical purposes given in the form of its calcium chelate, sometimes written CaH_2 EDTA.

In animals the LD_{50} of CaH_2 EDTA was found to vary from 500 to 7000 mg./kg., depending on species and route of administration. Repeated administration of smaller doses produced lesions in the renal tubules which were reversible; for example, such lesions were produced in 50 per cent of animals treated daily for 16 days with 200 mg. of the calcium chelate per kg. per day (Foreman *et al.*, 1956). It may be useful to compare this toxic amount with the amount that would be administered to a newborn infant if EDTA blood were used for an exchange transfusion. Thus, if an infant weighing 6½ lb. (3 kg.) were given an exchange transfusion of 500 ml. of blood mixed with 400 mg. EDTA, the dose would be approximately 135 mg. EDTA per kg., that is distinctly less than the amount which, if administered daily for 16 days, would produce reversible lesions in the renal tubules. Moreover, the figure of 135 mg. per kilogramme takes no account of the EDTA which would be removed during exchange transfusion.

When 0.4 g. of the disodium salt of EDTA (Na_2H_2 EDTA) is used as an anticoagulant for 500 ml. of blood, only about 0.3 g. is needed to chelate the available calcium so there may be about 0.1 g. of sodium EDTA in the final mixture. The administration of many such bottles would seem to be very safe in view of the experience of Bechtel *et al.* (1956) that the infusion of 3 g. of Na_2H_2 EDTA in 20 minutes to normal subjects produced relatively mild effects, namely: cramping pains in the vein at the site of infusion, fine muscle tremors and mild ECG changes. All these signs disappeared within 10 minutes of the end of the infusion.

Potassium Toxicity

When red cells are stored in the cold the potassium content of the red cells decreases and that of the plasma increases. Normally red cells contain approximately 100 m.Eq. of potassium per litre (of packed cells) and plasma contains 4-5 m.Eq./litre. After 10 days' storage in acid citrate dextrose the amount in the supernatant plasma-citrate is about 15 m.Eq./litre; after 14 days it is about 20 m.Eq./litre and after 28 days' storage about 30 mEq./litre (Loutit *et al.*, 1943).

In small-pool plasma prepared from blood stored for 21 days or more the potassium concentration is between 9 and 17 mEq./litre (Dr W. d'A.

RCPE0002068_0295

Maycock, personal communication). This unexpectedly low figure may possibly be due to the fact that in some cases the plasma is aspirated from settled red cells without first mixing the bottles.

In blood stored with 'Edglugate-Mg' (see Chapter I) the plasma potassium rises more rapidly than in blood stored with ACD and may reach a level of 18 mEq./litre after 3 days.

As with citrate toxicity, it is to be expected that if dangerous effects occur from potassium they will be most easily observed in infants undergoing exchange transfusion. Miller, McCoord *et al.* (1954) found that in infants transfused with blood stored for less than 1 week the serum potassium concentration did not change significantly. However, when blood stored for 9-21 days was used (with a concentration in the citrate-plasma of approximately 15 m.Eq./litre) the infants' serum potassium concentration rose to 8 m.Eq./litre in two cases; no ECG changes were observed.

Campbell (1955) measured changes in plasma potassium concentration during exchange transfusion in thirty-six infants, using donor blood with a plasma potassium concentration of approximately 30 mg./100 ml. (7.5 m.Eq./litre). There was a tendency for the concentration to increase during exchange transfusion, mean values being 4.0-4.5 m.Eq./litre at the beginning and 5.5-6.0 m.Eq./litre after the exchange of 500 ml. A few samples, mostly from infants who showed signs of collapse or who actually died shortly after the samples were taken, had concentrations of 8 or 9 mEq./litre.

It is known that in man marked electrocardiographic changes occur when the plasma potassium concentration reaches about 8 mEq./litre and that in conditions in which the plasma potassium level is thought to be the immediate cause of death levels of approximately 10 mEq./litre are found (Hoff *et al.*, 1941; Stewart *et al.*, 1948). When the plasma potassium concentration is found to be raised after collapse has occurred it seems possible that the rise in potassium is a consequence rather than a cause of the collapse; for example, in dogs immediately after respiratory arrest there is a rapid agonal rise in serum potassium (Winkler and Hoff, 1947).

In subjects treated with citrated blood it is artificial to consider the toxic effects of potassium and citrate separately because it is known that they reinforce one another. This was demonstrated by Baker *et al.* (1957) on the isolated heart and has more recently been demonstrated during exchange transfusions in rabbits (Taylor *et al.*, 1961).

In the experiments of Taylor *et al.*, 10 ml. amounts of blood were withdrawn and replaced within 1 minute to a total of 60 or even 100 ml./kg. The blood used had a slightly higher citrate concentration

than citrated blood used in normal blood transfusion practice. Fresh blood was used but in some experiments potassium was added to bring the plasma potassium concentration to about 25 mEq./litre (equivalent to 3- or 4-week-old stored blood). When citrated blood without added potassium was exchanged, there was only one death among ten rabbits but when blood with added potassium was used fifteen out of nineteen rabbits died. The figures for heparinized blood were: without added potassium, none out of thirteen deaths and with added potassium, two out of fifteen. The common mode of death was ventricular fibrillation. These figures show how dangerous is the combination of citrate and a raised potassium concentration and they also show that a raised potassium concentration alone is relatively harmless. It should be emphasized that the rate of exchange transfusion in these experiments was considerably greater than the rate employed in exchange transfusion in infants; that the amounts of citrate and, even more, of potassium that were injected were considerably greater than the amounts involved in exchange transfusion in infants; and that rabbits are more susceptible to citrate toxicity than are humans.

Melrose and Wilson (1953) have suggested that the raised potassium concentration in the plasma of stored blood may prove dangerous in intra-arterial transfusion. They give references to work showing that when blood is given intra-arterially in subjects with hypotension it may enter the coronary arteries directly, that is before being adequately mixed with the subject's own blood. Nevertheless it is uncertain how important this is in practice; Nakasone *et al.* (1954) found that when rapid infusions of citrated blood were given into the ascending aorta at the level of the aortic valves the effects on the electrocardiogram were no different from those produced by intravenous injection. Although the observations dealt with citrate toxicity they probably apply with equal force to potassium toxicity.

In subjects who are being perfused with blood from a pump-oxygenator, rises in plasma potassium may be observed. Thus when blood with a plasma potassium concentration of 17 m.Eq./litre is used, the recipient's plasma potassium may reach 8-9 m.Eq./litre in the first 5 minutes, returning to normal levels within a further few minutes (Dr Ivan W. Brown, personal communication).

Apart from the special circumstances in which large amounts of blood are rapidly infused, potassium toxicity need be considered only when transfusing patients whose plasma potassium is already raised; for example, anuric patients with extensive wounds involving muscle. Moore (1955)

RCPE0002068_0296

suggested that in severely wounded patients a useful rule might be not to transfuse more than 10 pints of 10-day-old stored blood in less than 10 hours.

Ammonia in Stored Blood

Fresh blood contains only about 100 μg./100 ml. of ammonia but this may increase in blood stored for 3 weeks to about 900 μg. per 100 ml.; it has been suggested that this amount of ammonia may make it undesirable to give large amounts of stored blood to patients with liver failure (Spear *et al.*, 1956).

Vaso-Active Substances in Shed Blood

It is known that shed blood contains vaso-active substances but little is known about their clinical significance.

If fresh, defibrinated blood is injected into animals there is often a severe fall in blood pressure, an effect to which Freund (1920) gave the name 'Frühgift'. The fall in blood pressure is due to the presence of adenosine (Bennet and Drury, 1931). This substance is rapidly inactivated in shed blood by a deaminase which is present mainly in the red cells (Drury *et al.*, 1938; Conway and Cooke, 1939).

In blood stored for more than a few hours, vasoconstrictor substances predominate. Their chief source appears to be the platelets (LeSourd and Pagniez, 1914; Reid and Bick, 1942). The vasoconstrictor substance in shed blood was shown by O'Connor (1912) not to be adrenaline; and the observations of Shimizu *et al.* (1960) suggest that it is not serotonin. It is generally believed to be a polypeptide produced by the action of an enzyme on a plasma globulin.

It has been claimed that vasoconstrictor substances in the plasma of stored blood are responsible for vasoconstriction affecting the vessel into which the transfusion is running and that the local injection of 2-5 ml. of a 0.5 per cent solution of procaine into the transfusion tubing overcomes this effect (Shimizu *et al.*, 1960, supplemented by a personal communication from Dr H. Tohyama).

TRANSFUSION HAEMOSIDEROSIS

One litre of blood contains about 500 mg. of iron whereas the daily excretion of iron from the body is only about 1 mg. Thus if numerous transfusions are given to a patient who is not bleeding, the amount of iron in the body is much increased.

In transfusion haemosiderosis the serum iron concentration is greatly

increased and the iron-binding capacity of the serum is fully saturated (Rath and Finch, 1949). The diagnosis can be confirmed by demonstrating an increased amount of iron in the bone marrow.

The occasional association of multiple blood transfusions with haemochromatosis was first recognized by Kark (1937) whose patient had received more than 290 transfusions over a period of 9 years. Since then many cases have been described in which patients have developed some but not all of the features of haemochromatosis following a much smaller number of transfusions. Schwartz and Blumenthal (1948), who described several such cases, suggested that the syndrome should be called 'exogenous' haemochromatosis to distinguish it from 'endogenous' haemochromatosis, which occurs in subjects who have never been transfused. Endogenous haemochromatosis is typically a disease of males over 45; by contrast, exogenous haemochromatosis occurs equally frequently in females and often affects much younger subjects.

According to Sherlock (1955, p. 336) 'probably over one hundred transfusions of blood must be administered before the liver becomes affected by iron deposits and fibrous tissue develops'. In cases in which haemosiderosis and fibrosis of the liver are recognized in patients who have received as few as twenty transfusions, it has been suggested that the iron-overload may have been caused partly by earlier administration of iron by mouth, as treatment for anaemia (Schwartz and Blumenthal, 1948).

Despite the implication that the iron-overload is itself the cause of the hepatic cirrhosis, experimental evidence is strongly against this view. Thus Brown, Dubach *et al.* (1957) injected dogs with 0.5-1.0 g. per kilogramme over a period of 4 to 7 years (equivalent to the transfusion of more than 100 units of blood). Heavy iron deposits were found in the tissues of sacrificed animals, but there was no evidence of any fibrous tissue reaction. Cappel *et al.* (1957) described five patients who had received multiple blood transfusions, one having received 415 litres, the equivalent of 200 gm. of iron, but who showed no hepatic cirrhosis at autopsy. The conclusion then is simply that although heavy deposits of iron in the tissues must necessarily follow multiple transfusions in patients who are not bleeding, tissue damage is not an inevitable concomitant.

When hepatic cirrhosis is found in association with transfusion haemosiderosis, there is always the possibility that the liver damage is a consequence of serum hepatitis for such patients are fairly likely to have had an attack.

If a patient with iron-overload has recovered from the condition for which he required transfusion it may be practicable to remove the extra iron by bleeding.

RCPE0002068_0297

In a patient described by Crosby (1958) 55 litres of blood were withdrawn from a patient in 11 months; this sufficed to render the patient slightly iron-deficient and it appeared to produce some improvement in the diabetes from which he was suffering.

Success has also been obtained by the administration of chelating compounds. Thus Drs Clement A. Finch and Frans Cleton (personal communication) have given intravenous injections of, for example, orthohydroxyphenyl acetic acid in amounts of up to 2 or 3 g. and this removed up to 100 mg. of iron over a period of 24 or 48 hours in an individual with iron overload following transfusion. It would be very satisfactory if a chelating compound could be administered during a long course of transfusions to prevent the development of iron overload but the possibility of producing renal damage at present renders this unwise.

THROMBOPHLEBITIS

According to McNair and Dudley (1959) 'if an infusion is run into a peripheral vein by way of a needle, metal canula or short length of plastic tubing, sooner or later a painful inflammatory condition will develop, spreading proximally from the tip of the needle; the vein progressively thromboses ...'

Duration of Infusion

Various factors are now known to influence the incidence of thrombophlebitis following intravenous infusions. One of these is the duration of the infusions. Thus intravenous infusions which last for more than 8 hours are much more likely to be followed by thrombophlebitis than are infusions of shorter duration (Carter, 1951). This conclusion was supplemented by data obtained in a trial of two types of tubing carried out by a committee of the Medical Research Council (M.R.C. Report, 1957). There were no cases of thrombophlebitis following infusions which lasted for 12 hours or less, either when the infusion was given through rubber tubing or through plastic tubing but, as Table XLVI shows, thrombophlebitis was common following infusions lasting more than 12 hours and its incidence was clearly related to the duration of the infusion.

Influence of Tubing

Table XLVI also shows that the incidence of thrombophlebitis was almost twice as high when rubber tubing was used as when plastic tubing was used. The fact that the use of some types of rubber tubing was associated with a greatly increased incidence of thrombophlebitis had previously been shown by Handfield-Jones and Lewis (1952), who found

the highest incidence with a particular batch of natural red rubber tubing and the lowest with latex rubber tubing.

It has been shown that there is a relation between the toxicity of the various types of rubbers and plastics, as revealed by their effects on tissue cultures and their liability to cause thrombophlebitis when used for infusions (Cruickshank *et al.*, 1960).

A very unsatisfactory feature of the present position is that manufacturers are not compelled to reveal the composition of the materials they use so that even when material from a given manufacturer has been shown to be satisfactory there is no guarantee that further material will be the same. It is known that various substances added either to rubber or plastic or brought into contact with them during manufacture may be highly

TABLE XLVI

INCIDENCE OF THROMBOPHLEBITIS (ALL GRADES) FOLLOWING INFUSIONS THROUGH PLASTIC AND RUBBER TUBING RESPECTIVELY
(From M.R.C. Report, 1957)

Type of tubing	*Incidence of thrombophlebitis*		
	Period of infusion		
	13–23 hours	*24–47 hours*	*48 or more hours*
Plastic	13%	36%	48%
Rubber	27%	55%	82%

toxic; an example is given by Gillis (1958). A batch of polythene was found to produce inflammatory reactions when implanted into tissues and this was traced to contamination with dicetyl phosphate used as a stripping agent during manufacture.

Effect of Glucose Solutions

Autoclaved glucose solutions are strongly acid and have for some time been suspected of causing thrombophlebitis. McNair and Dudley (1959) tested Seitz-filtered versus autoclaved dextrose directly in the same subject by giving infusions of each, lasting 20 to 30 hours, into separate veins. They found a similar incidence of thrombophlebitis but it now seems likely that their series was too small to be significant. Thus Vere *et al.* (1960) found that with autoclaved glucose (pH 3-4) thrombophlebitis

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occurred after twenty out of thirty-one infusions, but with Seitz-filtered glucose (pH 6) the incidence was nine out of twenty-eight.

Vere *et al.* (1960) also obtained suggestive evidence that the practice of giving glucose through a giving set which had previously been used for blood leads to an increased incidence of thrombophlebitis, presumably because this practice leads to the haemolysis of blood on contact with dextrose solutions (see Chapter XIV).

Prevention of Thrombophlebitis

The addition of heparin (200 i.u. per 100 ml. of infused fluid) is apparently ineffective (McNair and Dudley, 1959).

The most effective prophylactic measure is to change the site of infusion every 8 hours (Carter, 1951). When this is impracticable it is clearly important to avoid the use of certain types of tubing (particularly red rubber) and to avoid the use of acid glucose solutions. If a highly irritating solution such as 50 per cent glucose has to be infused it should be given through a catheter whose tip lies in a large vessel such as the vena cava, where the injected fluid will be rapidly diluted. McNair and Dudley found that the incidence of serious complications (mainly septic thrombosis) in a series of 130 caval catheterizations was 4 per cent.

AIR EMBOLISM

Healthy animals tolerate relatively large amounts of air, injected intravenously at a rapid rate. Thus the minimal lethal dose in rabbits is 5.5–7.5 ml./kg. (Larson, 1951). The work of Durant *et al.* (1954) has shown that air injected into a vein rapidly finds its way into the pulmonary artery and that this is associated with a rapid fall in systolic pressure. The mortality rate in dogs injected with 7.5 ml./kg. can be halved by placing the animals on their left side at the time of the injection. The air is then displaced away from the outflow tract of the right ventricle.

Although animal work suggests that a normal adult should tolerate air embolism of the order of 200 ml. or so, there is no doubt that smaller amounts can cause alarming signs; for example in Chapter I an instance was quoted of a blood donor who became severely ill after receiving 60–80 ml. of air intravenously.

In persons not in good health even smaller amounts may be fatal; thus Durant *et al.* (1954) considered that 40 ml. might be fatal. Simpson (1942) contended that in a person already gravely ill, less than 10 ml. might be fatal.

The signs produced by air embolism were described by Nicholson and Crehan (1956). Venous air embolism produces a marked rise in venous pressure and cyanosis; a millwheel murmur can be heard over the precordium. There is then a fall in blood pressure, the pulse becomes rapid and thready and there is syncope due to cerebral ischaemia. Death occurs from primary respiratory failure. Treatment consists in putting the patient in a head-down, feet-up position on his left side. In arterial air embolism the patient feels dizzy and faint, loses consciousness and may have a convulsion. The pathognomonic signs are: air in the retinal vessels, marbling of the skin and 'air bleeding', i.e. the issue of air from cut blood vessels.

There are several ways in which air embolism can be produced during transfusion. The simplest case to consider is that in which blood is being driven into the patient under pressure, by blowing air into the bottle with some sort of pump (e.g. 'Higginson's' syringe). Such a practice may be necessary when no other pump is available and when the patient urgently needs rapid transfusion. Whenever this method of applying pressure is used a responsible person must be given the job of watching the bottle constantly and of observing the drip chamber. It must be remembered, that when the outlet needle of the recipient set projects some distance into the bottle air may start to pass into the set while there is still some blood left in the bottle. A special danger associated with the use of a filter inside the bottle was illustrated in the previous edition of this book (Mollison, 1956, p. 427).

It is easy to introduce air into the patient's veins at the beginning of a transfusion or when changing from one bottle to another. This can be avoided, firstly by making sure that the tubing of the apparatus is full of blood before beginning the transfusion; and secondly by leaving a little blood in one bottle before changing to the next, so that the transfusion tubing is left full of blood. If nevertheless the first bottle is allowed to run right out, air can be removed by disconnecting the adapter near the needle and running through some blood from the second bottle before reconnecting the adapter.

Air can be sucked into the tubing or the drip chamber through small leaks if the clip controlling the rate of flow is placed above the drip chamber. Therefore, as a routine, the clip should be placed as low down as possible. Even then, air may be sucked into the rubber tubing through a leaking adapter, close to the needle, if there is a negative pressure in the vein that is being used for transfusion. This might be the case if the internal saphenous vein were being used and the patient were in the Trendelenburg position.

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Finally, when veins are exposed for cannulation, there is a risk of the continual entry of small amounts of air unless the vein has been tied off. Simpson (1942) has reported two fatal cases. In one a ligature had been passed through the vein instead of around it. Some operators prefer not to tie off the vein when they have exposed it, hoping thus to save the vein. Nevertheless once having exposed a vein it seems better to tie it off distally as a routine, rather than run the risk of air embolism.

INJECTION OF PARTICULATE MATTER, INCLUDING SKIN

Particulate Matter in Stored Blood

It is customary to incorporate a filter in transfusion sets, although the object of this has never been clearly defined. The filter will of course serve to prevent the passage of clots which might otherwise obstruct the 'giving' needle, and a filter will also hold back small particulate matter which is found in stored blood. The problem is to know how fine the filter should be in view of the fact that the finer the mesh, the greater is the amount of particulate matter removed. Thus Maycock and Mollison (1960) found that a filter with a pore size of 0.5 mm. (i.e. each pore 0.5 mm. × 0.5 mm.) would remove 36 mg. of solid matter (as dry weight) from 500 ml. of a particular batch of stored blood; a filter of pore size 0.2-0.3 mm. would remove about 46 mg. and filters with a pore size of 0.14 mm., 64 mg. It seems certain that all transfusion filters at present in use let through a certain amount of particulate matter which must be retained in the pulmonary capillaries. Presumably this is relatively harmless, but it ought to be taken into account in considering the pros and cons of transfusing into the arterial side of the circulation.

Glass Particles

Solutions stored in glass bottles may contain large numbers of minute particles of glass. Brewer and Dunning (1947) found that the average size of these particles was 20μ. Repeated injections of deposits from glass ampoules given to over 1000 mice produced no 'acute episodes'. In rabbits daily injections for a month of an amount corresponding to 0.5 g. daily in a human adult produced no obvious ill effects although at autopsy glass particles were found in the lungs. In one animal which received 4 g. over the course of a year, widespread 'silicosis' was found at autopsy.

Similarly, Dr G. H. Tovey and his collaborators (personal communication) found that the injection into rats of deposits from glass bottles produced no demonstrable effects.

A fifty-fold concentrate was prepared of deposits formed in isotonic dextrose and saline solutions which had been standing in glass bottles at room temperature for 2 years.

Intravenous injection of 2 ml. of these concentrates into rats (approximately 200 g. weight) in 5 seconds, to a total of 6 ml. in three divided doses, was without adverse effect.

In similar experiments, 15 ml. of the pooled concentrates were injected into rabbits in 60 seconds without any immediate reaction. No abnormality was detected on histological examination when the rabbits were killed 6 weeks later.

Assuming that the blood volume of a rat approximates 1/500th that of a human adult, a patient would have to be infused with 150 litres of fluid to receive an equivalent quantity of deposit.

Despite this solid body of evidence which certainly suggests that the amount of glass injected in the ordinary course of intravenous therapy should be harmless, conflicting evidence has been obtained by Dr P. H. Køster and his colleagues.

Dr P. H. Køster (personal communication) says that he and his colleagues found that if the deposit (7-8 mg.) in a 500 ml. bottle was centrifuged and taken up in 5 ml. and injected into rabbits, it was lethal. No explanation has yet been found for the discrepancy between these results and those quoted above.

Fragments of Skin

Small fragments of skin (visible to the naked eye) are commonly taken into the bore of the needle when the skin is punctured; thus with needles of S.W.G. eighteen to twenty-four, fragments are found after about two-thirds of punctures (Gibson and Norris, 1958). Fragments are not found when using needles with a side opening, or when using needles with a well-fitting stilette. If such fragments are injected intravenously into animals they can sometimes be found in pulmonary vessels and if injected subcutaneously dermoid cysts may result. Nevertheless no ill-effects from this cause have yet been reported in man.

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CHAPTER XVI

SOME NORMAL BLOOD FINDINGS IN NEWBORN INFANTS: INDICATIONS FOR SIMPLE TRANSFUSION

An understanding of the normal changes in haemoglobin concentration which occur in newborn infants is essential in the diagnosis and treatment of haemolytic disease of the newborn and this is the chief reason for including a brief account in this book.

Sources of Variability in Haemoglobin Concentration in Newborn Infants

Rapid Changes after Birth

The haemoglobin values of normal infants change rapidly after birth, particularly during the hour or two following delivery. This is due partly to the large 'transfusion' of blood that the normal infant receives from the placenta, so long as its cord is left untied for a few minutes after delivery. As a result of this transfer, the infant gains on the average about 100 ml. of blood in a period of 5 minutes (Budin, 1875; Haselhorst and Allmeling, 1930; DeMarsh *et al.*, 1942). The infant readjusts its plasma volume very rapidly so that there is only a small gain in total blood volume. Therefore, the haemoglobin concentration of the blood increases and in some infants the increase may be as great as 6 g./100 ml. or even more.

The mechanism of the normal transfer is presumably as follows: within a minute or so after birth and when the infant first cries, the umbilical arteries constrict and thereafter very little blood flows from the infant to the placenta. Meanwhile the vein remains dilated and blood passes to the infant from the placenta. This unidirectional flow depends partly upon the position of the infant for Gunther (1957) found that if the infant was raised above the placenta, blood might actually flow from the infant to the placenta.

There is evidence that a second factor causes a rise in haemoglobin concentration after birth. Gairdner *et al.* (1958) found that even when the cord was clamped 'as soon as conveniently possible' haemoglobin rose from 16.6 g./100 ml. in cord blood to 19.1 g. in venous samples taken 1-8 hours later. The rise was just as great in infants born by Caesarean section as in others and was just as great in a small series tested a few minutes instead of a few hours after birth. Plasma protein concentration rose from

6.5 to 7.0 g./100 ml. and MCHC from 32.3 to 33.6 (in both cases comparing cord blood with venous blood taken 1 to 8 hours later). The authors concluded that it was mainly whole plasma with a little protein-free fluid that was lost from the circulation. They also suggested that the scatter in the cord blood values was at least in part due to variations in plasma volume.

Although there is a rise in haemoglobin concentration in the first few hours of life, irrespective of the placental transfer of blood, haemoglobin values at 24 hours of life are distinctly greater in infants who have received a substantial amount of placental blood. In a recent survey of this old problem, Lanzowsky (1960) found that 13-24 hours after birth in infants whose cords had been clamped early, the mean haemoglobin concentration was 18.2 g./100 ml., and amongst those whose cords had been tied late (after expulsion of the placenta), the mean haemoglobin concentration was 19.86 g./100 ml.

Difference between Skin-prick and Venous Samples

In newborn infants, skin-prick samples from the heel or toe usually have a higher haemoglobin concentration than samples taken simultaneously from veins (Vahlquist, 1941; Oettinger and Mills, 1949; personal observations). The difference is greater on the first day of life. Thus Vahlquist (1941) found that immediately after birth the haemoglobin concentration of heel-prick samples was 10 per cent higher than that of samples taken simultaneously form the femoral vein; and Oettinger and Mills (1949) found that, within an hour of birth, the haemoglobin concentration of samples from the toe was 22 per cent higher than that of samples taken simultaneously from the internal jugular vein. The present author has found that, a few hours after birth, samples taken from the heel have a haemoglobin concentration about 5 per cent higher than that of samples from the external jugular vein; this difference is found even when the heel has been warmed by immersion in hot water and the first two or three drops are discarded before taking the skin-prick sample. Vahlquist found that the difference between heel-prick samples and femoral vein samples was still 5 per cent at 24 hours but that by the sixth day it was only 2½ per cent, and by the fourteenth day was nil. In thirteen infants tested by the present author between 2 and 3 days after birth, samples from the heel were on the average 1.6 per cent higher than samples from the external jugular, femoral or scalp veins.

Considerably greater differences have been reported by some other authors. Thus, DeMarsh *et al.* (1948) found that, on the third day of life,

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the haemoglobin concentration of heel-prick blood was 15 per cent higher than that of blood taken simultaneously from the sagittal sinus; and Oettinger and Mills (1949) found that the haemoglobin concentration of toe-prick blood was 12 per cent higher than that of samples taken simultaneously from the internal jugular vein; the same authors found that even at the age of 21 days there was still a difference of 7 per cent.

The fact that some authors have found much greater differences than others between skin-prick and venous blood samples may depend partly upon the choice of vein but seems more likely to be due to the technique of obtaining blood from skin-prick. It is well known that in adults the first drop of blood obtained by pricking the lobe of the ear has a considerably higher haemoglobin concentration than that of venous blood; the haemoglobin concentration falls in subsequent drops, but even when the first few drops are discarded ear-prick blood has a specific gravity at least 5 per cent higher than that of venous blood (e.g. Kitano and Morita, 1960).

The conclusions are that in estimating the haemoglobin concentration of the blood of newborn infants during the first week of life, venous blood should be obtained whenever possible; the particular vein used seems to be unimportant since samples from the external jugular, internal jugular and femoral vein gave the same results (Gairdner *et al.*, 1958). For routine use scalp veins or arm veins are best.

After the first week of life, heel-prick blood may safely be used, provided that the same precautions are taken as in adults; that is to say, the skin must be warm and the first two or three drops that flow must not be used for the estimate.

Haemoglobin Standards

Solutions of cyanmethaemoglobin have been widely adopted as standards; when made up in 50 per cent glycerol (v/v) they remain clear and are stable for at least 6 months (Dr I. D. P. Wootton, personal communication). In England, standardized cyanmethaemoglobin solutions may be obtained from Keeler Ltd, Wigmore Street, London, W.1.

For laboratories without ready access to standard solutions of cyanmethaemoglobin it is convenient to use a grey glass screen of known optical density as a standard. If the photo-electric colorimeter in use has rectangular cells of known optical thickness and a standard filter is used, the haemoglobin equivalent of the sample can be discovered from data published by Donaldson *et al.* (1951). The exact haemoglobin value corresponding to a particular density reading should if possible be checked,

at least occasionally, by testing a 'standard' cyanmethaemoglobin solution.

Normal Values

Having emphasized that the source of the blood sample and the time of sampling have a great effect on the haemoglobin values of the blood of newborn infants, it remains to present some figures which take account of these factors.

In view of the fact that agreement about haemoglobin standardization has only been reached comparatively recently and that results obtained before about 1950 are not in close agreement with one another, only figures obtained since that time will be given.

Cord Blood

The normal haemoglobin concentration of cord blood was found to be 16.6 g./100 ml. (S.D. 1.5 g.) in a series of 133 normal infants tested by Mollison and Cutbush (1951); this agrees well with the mean of 16.9 g./100 ml. (S.D. 1.6 g.) in the series of 221 normal infants tested by Marks *et al.* (1955).

The mean packed cell volume of cord blood in thirty-four normal infants was found to be 53.1 per cent (Mollison, 1951, p. 352); Marks *et al.* (1955) tested cord blood from 106 normal infants and also found a mean of 53.1 per cent (S.D. 2.9).

The normal mean corpuscular haemoglobin concentration is slightly lower in infants than in adults; thus: 32 per cent (Veall and Mollison, 1950); 32.3 per cent (Marks *et al.*, 1955).

The mean normal reticulocyte count of cord blood was found to be 2.94 per cent (range 0 to 8.3 per cent) by Marks *et al.* (1955). Seip (1955) found that all of thirteen infants tested on the first day of life had values between 4.2 and 6.3 per cent and claimed that the wide scatter in many series was due to technical errors but the figures of most authors agree with those of Marks *et al.* The mean number of normoblasts in cord blood was found by Marks *et al.* (1955) to be 590 per c.mm. (range 0 to 5400 per c.mm.).

Difference between umbilical artery and vein. When the blood vessels in the cord are distended the umbilical vein is larger than the two umbilical arteries. However, with a syringe and needle it is easy to obtain blood from an artery if one wishes to; it may therefore be worth emphasizing that samples from the artery tend to have a slightly higher haemoglobin concentration. Thus Dr H. Chaplin, Jr.

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(working in the author's laboratory), found that in fifteen out of nineteen cases blood from the umbilical artery had a higher haemoglobin concentration than blood from the umbilical vein. The mean haemoglobin values of the nineteen cases were 17.0 g./100 ml. and 16.5 g./100 ml. respectively. Similarly the mean packed cell volumes were 56.4 per cent and 54.2 per cent.

Effect of Foetal Maturity

Walker and Turnbull (1953) concluded that the mean haemoglobin concentration in foetal blood rises during the last 2 weeks of pregnancy and continues to rise from then onwards due to progressive oxygen lack. Thus they found the mean haemoglobin concentration at 38 weeks to be 15.2 g./100 ml., at 40 weeks to be 16.5 g./100 ml. and at 42 weeks to be 18 g./100 ml. These findings have not been confirmed by other authors. For example, Marks *et al.* (1955) found no definite trend of haemoglobin values in a series of more than 200 infants with gestational ages varying from 30-44 weeks. Rooth and Sjöstedt (1957a) failed to find any rise in haemoglobin concentration in post-mature infants compared with mature infants, infants born at 43 weeks or more having a cord haemoglobin of 16.92 g./100 ml. compared with 16.98 g./100 ml. in infants born at term. However, they did obtain evidence that haemoglobin rises in the last 2 weeks of pregnancy; thus the average cord haemoglobin concentration at the thirty-eighth week was 15.5 g./100 ml.

In the previous edition of this book the author referred to a small unpublished series of comparisons between infants born at the thirty-sixth week and infants born at term. The cases could be regarded as strictly comparable in other respects (Mollison, 1956, p. 434). In nineteen infants born at term, mean cord haemoglobin was 17.6 g./100 ml. and only one had a haemoglobin concentration below 14.8 g. In sixteen infants born at 36 weeks the mean cord haemoglobin concentration was 16.3 g./100 ml. and five had a haemoglobin concentration below 14.8 g.

There is then a suggestion that at the thirty-sixth to thirty-eighth week of pregnancy the mean cord blood haemoglobin concentration may be 1 g./100 ml. less than in infants born at term, although this difference may prove to be spurious when larger numbers are collected.

Few observations have been made on early foetuses. At 11-15 weeks Wintrobe and Shumacker (1936) found the packed cell volume to be between 23 and 33 per cent, agreeing reasonably with the figure of 30-33 per cent for three 11-week foetuses tested by Turnbull and Walker (1955). The present author has tested five foetuses with a crown-rump length of 110-140 cm., corresponding to an age of 16-18 weeks; the mean packed cell volume was 42 per cent (35-53 per cent), the

mean haemoglobin value 12.3 g./100 ml. (10.6-16.4 g./100 ml.) and the mean reticulocyte count 26.2 per cent (13-43 per cent).

Haemoglobin Values on the First Day of Life

From what has already been said about the placental transfer of blood, it is evident that local obstetric practice will have some influence on the range of values encountered after birth. On the whole though, the tendency is not to tie the cord very soon after birth, and if tying is delayed for a few minutes the bulk of the placental transfer will be complete. Thus it is probable that the normal range given by Mollison and Cutbush (1949a) for venous samples taken from forty-three infants on the first day of life will be found to be fairly typical; mean 18.5 g./100 ml. S.D. 2.0 g. (i.e. approximately 95 per cent of values falling within the range 14.5 to 22.5 g./100 ml.).

As already explained, for haemoglobin estimation during the first few days of life, venous samples should be used whenever possible. Nevertheless it is sometimes difficult to secure a venous sample from a newborn infant, and a knowledge of the normal range of the haemoglobin values of skin-prick samples is thus of some use. Mollison and Cutbush (1949a) found the normal range (mean ± 2 S.D.) of the haemoglobin content of skin-prick samples on the first day of life to be 15.4-22.8 g./100 ml. (thirty-three infants).

Changes in Haemoglobin Concentration during the First Two Weeks of Life

All observers who have worked with venous samples are agreed that the fall in haemoglobin concentration is slow during the first week of life. Vahlquist (1941) found that the average value on the first day was 18.4 g./100 ml., on the sixth day was 19.4 g./100 ml., and on the fourteenth day 17.5 g./100 ml. (observations not all on the same infants). Gairdner, Marks and Roscoe (1952) found that the mean value fell from 20.0 g./100 ml. on the first day to 14.4 g./100 ml. on the twenty-ninth day.

Vahlquist's range (mean ± 2 S.D.) on the fourteenth day was 13.1 to 21.9 g./100 ml. Thus, haemoglobin values below 13 g./100 ml. during the first 2 weeks of life should suggest anaemia.

Haemoglobin Values in the First Three Months of Life

After the first day or two of life there is a steady decline in haemoglobin values which continues for almost 2 months. Guest (1938) found that the mean value of eighty-eight samples of blood taken at 1 to 3 months was 12.1 g./100 ml.; 95 per cent of the values fell within the range 9.5 to 15.0

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g./100 ml.; as many as seven out of the eighty-eight infants had values below 10 g./100 ml. These haemoglobin values are probably slightly too high, by the present accepted haemoglobin standard, since Guest found the mean haemoglobin concentration in cord blood to be 17.9 g./100 ml. However, this reinforces the conclusion that haemoglobin values of about 9.5 g./100 ml. in infants aged 3 months cannot be considered abnormal (Guest and Brown (1957) reported that they now found the mean cord haemoglobin concentration to be 17.1 g./100 ml.).

Marks *et al.* (1955) found that the lowest values were reached at the age of 7 weeks, some infants having haemoglobin concentrations of 9.1 g./100 ml. The same workers drew particular attention to the low erythropoietic activity of the marrow found between about the eighth and thirty-third days of life (Gairdner *et al.*, 1952).

It is generally believed that the fall in haemoglobin concentration which occurs after birth as a normal event is due partly to the rise in the level of oxygen saturation of the blood; and in newborn rats it has been shown that the normal fall in haemoglobin can be greatly slowed by keeping the animal in a lowered concentration (15 per cent) of oxygen (Rooth and Sjöstedt, 1957b).

Summary of Criteria of Anaemia in Newborn (Mature) Infants

An infant born at full term should be regarded as anaemic only if its haemoglobin concentration falls below the limits summarized in Table XLVII.

TABLE XLVII

SUGGESTED LOWER LIMITS OF NORMAL HAEMOGLOBIN CONCENTRATION IN FIRST 3 MONTHS OF LIFE

Age of infant	*Haemoglobin concentration (venous samples)*	
	g./100 ml.	*'per cent Haldane'**
Cord blood	13.6	92
First day of life	14.5	98
2 weeks	13.0	88
2-3 months	9.0	61

* 100 per cent = 14.8 g./100 ml.

Haemoglobin Levels in Premature Infants

As already mentioned, the cord blood haemoglobin concentration of premature infants is either the same or at most about only 1 g./100 ml.

lower than that of infants born at term. The rapid rate of fall of haemoglobin concentration during the first month of life is due to the rapid growth of the infant and sluggish response to the stimulus of anaemia (Gairdner *et al.*, 1955). On the average, haemoglobin concentration reaches about 9.5 g./100 ml. at 60 days, although in infants treated with iron from the third week of life onwards the mean haemoglobin concentration does not fall below about 10 g./100 ml. (Gairdner *et al.*, 1955). In premature infants with a birth weight of less than 3½ lb. (1.7 kg.), the haemoglobin concentration falls to 8-9 g./100 ml. at the age of 2 months whereas in premature infants weighing more than 3½ lb. the value falls only to 10-11 g./100 ml. (Arthurton *et al.*, 1954).

Plasma Bilirubin Levels in Newborn Infants

Standardization of Bilirubin Estimations

It is generally accepted that there is a relationship between the concentration of bilirubin in the plasma and the risk of the development of kernicterus (see Chapter XVII). It is therefore most important that methods of estimating bilirubin should be standardized.

Up to the present time there has been one special difficulty. The 'standard' used in most methods is an artificial one made from a coloured solution; for example, in the method of King and Coxon (1950) the standard is prepared from methyl red. In testing an 'unknown', serum bilirubin is diazotized and the resulting colour approximately matches that of the methyl red solution. However, the precise colour developed by the diazotized bilirubin is affected by pH and the relative light absorption of the two solutions at various wavelengths will vary with the pH. The importance of this in practice was shown by a trial carried out some years ago in collaboration with Drs W. Walker and I. D. P. Wootton. Five samples of plasma containing relatively large amounts of bilirubin (of the order of 20-40 mg.) were tested (*a*) by the method of King and Coxon (1950) and (*b*) by a modification of the Malloy and Evelyn method. In both cases the artificial methyl red standard referred to above was used. When results with the same method were compared with one another, results on the same samples were in reasonably close agreement (maximum differences of the order of 10 per cent) but when results obtained by the two different methods were compared, the Malloy and Evelyn method gave results which were higher by 30-40 per cent. On the other hand, in an exchange of samples carried out with Dr P. Sturgeon, results obtained by him with the Ducci and Watson (1945) modification of the Malloy and Evelyn (1937) method agreed almost precisely with those obtained in the author's laboratory using the King and Coxon method.

The problem of standardization could be overcome by adopting the method of Sims and Horn (1958) in which pure bilirubin dissolved in

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plasma is used as a standard. It appears that bilirubin samples kept in the dark at −20° C. are stable indefinitely, although if exposed to light at room temperature the rate of fading is approximately 7 per cent in 1 hour (Sims and Horn, 1958).

Values in Cord Blood

The mean plasma bilirubin concentration in cord blood is distinctly higher than that in the blood of normal adults; there is some disagreement amongst different authors about the actual mean normal value, evidently due to a lack of standardization of methods. Thus Mollison and Cutbush (1951) found the mean value to be 1.5 mg./100 ml. (range 0.7–3.1) whereas Rosenfield and Ohno (1955) found a mean value of 2.1 mg. per 100 ml.

As in adults, the distribution of the normal bilirubin values is not symmetrical round the mean. The normal range thus cannot be defined in terms of a mean and standard deviation but must be defined as a range which includes either all normal values or some proportion of normal values (this point is discussed by Wootton *et al.*, 1951).

Changes in Plasma Bilirubin Concentration following Birth

In newborn infants the serum bilirubin concentration rises during the first few days of life (Yllpø, 1913; Davidson *et al.*, 1941). It is now known that this is due mainly to the deficiency at birth of a liver enzyme, glucuronyl transferase, which conjugates bilirubin with glucuronic acid, to form a water-soluble compound, bilirubin diglucuronide. It appears that this enzyme is developed more slowly in the premature infant than in the mature infant so that neonatal hyperbilirubinaemia is even more pronounced in premature infants.

Effect of prematurity. The relationship between prematurity and post-natal hyperbilirubinaemia is well shown in the series published by Hsia *et al.* (1953) and Billing *et al.* (1954). Thus Hsia *et al.* found that in twelve full-term infants, the peak bilirubin concentration was on the average 7.1 mg./100 ml. and was in all cases less than 13.5 mg./100 ml. By contrast in fifteen premature infants (with birth weights below 2250 g.) the average bilirubin concentration at the peak was 11.2 mg./100 ml. and in five infants exceeded 15 mg./100 ml.

Billing *et al.* subdivided their cases by birth weight and found the maximum post-natal bilirubin concentration rose steadily as birth weight fell. Thus in infants weighing 8½–9½ lb. the maximum bilirubin concentration was, on the average, only 3.0 mg./100 ml., whereas in infants

with a birth weight of 2½–3½ lb. the average maximum was 14.0 mg./100 ml.

Both these groups of authors emphasized that the maximum bilirubin concentration was reached much later after birth in premature infants than in mature infants. Thus in the series of Hsia *et al.* the maximum was reached on the fourth day of life in premature infants and on the second in mature infants. In the series of Billing *et al.* the maximum was reached as late as the sixth or seventh day in infants weighing 2½–3½ lb. at birth and as early as 1½ days in infants weighing 8½–9½ lb.

Billing *et al.* calculated that the liver of a small newborn infant can probably excrete bilirubin at a rate equivalent to only 1 or 2 per cent of that of the normal adult liver.

INDICATIONS FOR TRANSFUSION IN INFANTS

The commonest indication for transfusion in a newborn infant is haemolytic disease; this subject is dealt with in the following chapter. Newborn infants occasionally suffer from a sufficiently severe degree of blood loss to require transfusion; the circumstances may be considered under the following headings.

Haemorrhage before Birth

Foetal Blood in Retroplacental Haematomata

Using foetal haemaglobin as a 'marker', the amount of foetal blood in retroplacental haematomata was estimated by Bromberg *et al.* (1957) to be about 1 per cent of the total but Oehlert *et al.* (1960) found distinctly more (average 6.8 per cent). In eleven patients with relatively large amounts of foetal blood in the haematoma, foetal red cells were demonstrated in the mother's circulation (Oehlert *et al.*, 1960).

Passage of Red Cells from Foetus to Mother

In at least one in ten pregnancies, and possibly in a far higher proportion, small amounts of foetal red cells gain entry into the mother's circulation; see Chapter IX. In rare cases the amount lost by the foetus is substantial and the newborn infant may be severely anaemic. The first really convincing case of this kind was described by Chown (1954). (A case described by Wiener (1948b) was a little different because the blood loss suffered by the infant was apparently related to placenta praevia and this case is described under a separate heading below.)

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plasma is used as a standard. It appears that bilirubin samples kept in the dark at −20° C. are stable indefinitely, although if exposed to light at room temperature the rate of fading is approximately 7 per cent in 1 hour (Sims and Horn, 1958).

Values in Cord Blood

The mean plasma bilirubin concentration in cord blood is distinctly higher than that in the blood of normal adults; there is some disagreement amongst different authors about the actual mean normal value, evidently due to a lack of standardization of methods. Thus Mollison and Cutbush (1951) found the mean value to be 1.5 mg./100 ml. (range 0.7–3.1) whereas Rosenfield and Ohno (1955) found a mean value of 2.1 mg. per 100 ml.

As in adults, the distribution of the normal bilirubin values is not symmetrical round the mean. The normal range thus cannot be defined in terms of a mean and standard deviation but must be defined as a range which includes either all normal values or some proportion of normal values (this point is discussed by Wootton *et al.*, 1951).

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In Chown's case a newborn infant was found to have a haemoglobin concentration of 7.8 g./100 ml. There was good evidence that this anaemia was not the result of haemolysis since the infant's direct Coombs test was negative and both infant and mother belonged to group B. On the other hand there was good evidence that the infant's anaemia was due to bleeding into the maternal circulation. The infant's Rh genotype was C^wDe/cde and the mother's *cde/cde*; immediately after delivery it was found that there was a small proportion (5–10 per cent) of Rh-positive cells in the mother's circulation. This was demonstrated in two ways: firstly, in testing the mother's blood by the capillary tube method, small agglutinates were seen both with anti-C^w and with anti-D. Secondly in the indirect Coombs test on the mother's cells, using an anti-D serum, there was 'gross partial clumping'.

Twenty days after delivery the number of Rh-positive cells in the mother's circulation was reduced by one-half or more; a faint trace of Rh antibody was now detectable in the mother's serum. Forty-eight days after delivery no Rh-positive cells were present and anti-Rh was readily demonstrable.

Chown concluded that the infant had probably lost a total of 200–300 ml. of whole blood into the mother's circulation; presumably this loss occurred gradually, over a period of some weeks.

Gunson (1957a) described the case of an infant whose haemoglobin concentration 4 hours after birth was only 9.5 g./100 ml. The infant was O, Rh-positive and the mother A, Rh-negative but the mother had no Rh antibody and the infant's direct Coombs was negative. Moreover it could be shown that there were Rh-positive red cells circulating in the mother's blood stream and by differential agglutination with anti-A it was estimated that 3.6 per cent of the cells in the mother's circulation were group O. The Rh-positive red cells continued to be demonstrable in the mother's circulation for many weeks after delivery but by the end of 2–3 months they had disappeared and the mother had produced anti-D.

In two further cases described by Pearson and Diamond (1959) infants were found to be severely anaemic a few hours after birth (haemoglobin 8.5 g. and 5.4 g./100 ml. respectively). Foetal red cells were demonstrable in the mother's circulation by the technique devised by Jones and Silver (1958) for demonstrating the presence of a 'minor population' (see index) and also by estimating the proportion of foetal haemoglobin in the mother's circulation. The results by the two methods agreed and it was estimated that the proportion of foetal cells in the mother's circulation was 5 and 10 per cent respectively in the two cases.

A case reported by Weisert and Marstrander (1960) is of special interest in that the infant was very severely anaemic and displayed many of the features of hydrops foetalis. Thus at birth the infant was extremely pale, had a weak cry and gasping respiration. When the cord was cut across a few hours after birth, blood gushed violently from the umbilical vein and the venous pressure was found to be +12 cm. The haemoglobin concentration at this time was 2.8 g./100 ml. Exchange transfusion, rather than simple transfusion, was carried out because of the raised venous pressure, but the infant died at 26 hours. There was no evidence of haemolytic disease of the newborn but there was definite evidence of severe bleeding into the maternal circulation before birth, about one-twentieth of the red cells in the mother's circulation being of the infant's type.

Accidental Damage to Foetus in utero

The author has encountered a case in which the infant suffered a severe haemorrhage *in utero*, following damage by a needle. The case seems rare enough to be worth a brief description:

In 1950 an attempt was being made to assess the value of premature induction of labour in the treatment of haemolytic disease of the newborn. In connection with this investigation liquor amnii was being examined to see if evidence could be obtained of the severity of the haemolytic process in individual cases.

In one patient, whose serum contained anti-Rh, considerable difficulty was encountered in obtaining liquor by puncture through the abdominal wall. After one attempt in which the needle was passed well into the uterine cavity, 4 ml. of blood were withdrawn. For at least 8 hours afterwards the mother felt no foetal movement and the foetal heart could not be heard. However, foetal movements then began again and on the following day the foetal heart was heard.

Eight days later the membranes were ruptured artificially and the liquor was observed to be stained with blood and meconium. Three hours later a pale infant was delivered. Examination of cord blood showed that the direct antiglobulin test was only very weakly positive. The haemoglobin concentration was 11 g./100 ml., the packed cell volume was 34 per cent and the serum bilirubin concentration 1.4 mg./100 ml. On the following morning blood examination showed haemoglobin concentration to be 12.4 g./100 ml., packed cell volume 39 per cent and serum bilirubin concentration 1.5 mg./100 ml. A stained film contained approximately 80 nucleated red cells per 100 white cells; the reticulocyte count was 15.4 per cent. The infant was a little pale but was not jaundiced; the serum bilirubin concentration was estimated at the age of 2 days, 3 days and 5 days and was never above 1.8 mg./100 ml. At the age of 3 days the direct antiglobulin test was only doubtfully positive. Neither the liver nor the spleen were palpable.

These findings did not suggest that the anaemia was due to haemolytic disease of

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the newborn. When the direct antiglobulin test is very weakly positive there are usually no signs of red cell destruction; moreover the low serum bilirubin concentration throughout the first 5 days was much against the diagnosis of a haemolytic anaemia. The findings could be best explained by supposing that, 8 days before delivery, the infant had had a severe haemorrhage to which it had responded with a greatly increased output of red cells.

Foetal Haemorrhage during Parturition

Possible foetal bleeding in placental praevia. Wiener (1948b) has described a case in which an infant was delivered by caesarean section because of a central placental praevia. At birth the infant was found to be pale with weak cord pulsations; an immediate blood examination revealed a haemoglobin concentration of only 8.7 g./100 ml. After a large blood transfusion the infant recovered. Haemolytic disease of the newborn appeared to be excluded by the serological findings and Wiener suggested that the infant had lost blood through the placenta. It is of course possible that the infant had bled into the mother's circulation as in the cases described above but this was not investigated.

Haemorrhage from the Placenta during Caesarean Section

It is now well recognized that the infant may lose a dangerous amount of blood if the placenta is cut through during caesarean section. A case was described in the first edition of this book in which the infant's venous pressure, measured 20 minutes after birth, was very low; a catheter was passed up the umbilical vein and 60 ml. of blood rapidly injected; the infant made a good recovery.

Siddall and West (1952) observed seven infants with abnormally low haemoglobin concentrations on the first day of life and thought that in six the cause was blood loss due to placental incision during caesarean section.

Neligan and Russell (1954) emphasized the frequency with which placental tissue is found directly beneath the uterine incision during lower segment caesarean sections and made various recommendations to mitigate the consequences. They suggested that a transverse incision no longer than 1 inch should be made in the lower uterine segment and extended either with the finger or blunt-pointed scissors. The placenta should then be separated from the uterine wall, the membranes opened, the head delivered and the cord clamped.

They suggested that an accurate record should be made of the time elapsing between encountering the placenta and clamping of the cord; and

that the placenta and membranes should be examined from the foetal side for evidence of damage to the main umbilical vessels or other parts of the foetal circulation.

Neligan and Russell suggested that if more than half a minute elapses between encountering the placenta and clamping the cord or if there is visible damage to the circulation, the infant probably requires transfusion.

Haemorrhage from Vasa Praevia

In a review of this complication Rucker and Tureman (1945) stated that approximately 60 per cent of the infants are stillborn but that occasionally an infant is born alive suffering from acute haemorrhage from ruptured umbilical vessels. The fact that the blood has come from the foetus rather than the mother can be demonstrated by showing that it contains a high proportion of foetal haemoglobin.

Bleeding from one Uniovular Twin into the Other

If, following the birth of the first of uniovular twins, the cord is left untied, this twin may gain blood at the expense of the second, as yet unborn, twin. A case of this kind, in which the second twin was very pale at birth and relatively anaemic, was described by Bergstedt (1957).

Haemorrhage after Birth

Severe haemorrhages from the umbilical cord during the first few hours of life do occur occasionally although usually, even if the cord is left untied, it does not bleed. The loss of 30 ml. of blood is sufficient to make a newborn infant unusually quiet and pale.

The author has heard of a case in which fatal haemorrhage from the cord occurred following the accidental removal of a plastic catheter; the catheter had been used for a first exchange transfusion and left *in situ* in case a second exchange should be required.

Although haemorrhage from the cord is occasionally seen in apparently normal infants its occurrence should suggest the possibility of some abnormality of the coagulation mechanism. In the following case it was due to inadvertent heparinization of the infant:

Infant S. was given a small transfusion via the umbilical vein at the age of 5 hours. Forty-five millilitres of blood from a normal donor was taken into a syringe containing 5000 units of heparin and then directly injected into the infant. The operator had not considered the possibility that the use of an excess of heparin for mixing with the donor's blood might result in 'heparinization' of the infant. However, it was observed that a sample taken from the infant after the transfusion

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did not clot and instructions were left that the infant was to be watched but not disturbed. A single tight ligature was tied round the stump of the cord. Seven hours later a nurse noticed that the baby's binder was soaked in blood. A fresh tight binder was applied. The author saw the infant after a further half hour; it was pale, cold and apparently slightly cyanosed; its alae nasae were working. Eighty millilitres of blood were injected and the infant made a good recovery. It was subsequently estimated, by extracting haemoglobin from the stained garments, that the blood loss amounted to about 40 ml.

Haemorrhagic disease of the newborn has been referred to briefly in Chapter I.

Technique of Transfusion

The Use of the Umbilical Vein

During the first 12 hours of life the umbilical vein is very easily entered and should be used in preference to all other veins. The technique of passing a polythene catheter up the vein is described in the following chapter.

When the infant is more than about 12 hours old the umbilical vein is often partially occluded by clots which may have to be withdrawn before a free flow of blood is obtained. However, even when the infant is 3 or 4 days old it is often possible to use this route successfully, after finding the opening of the vein in the shrivelled umbilical stump. When the umbilical vein cannot easily be entered, scalp veins should be used for simple transfusion in newborn infants.

Scalp Vein Transfusion

In practice, skin incisions in infants, made for the purpose of finding a vein, rather frequently become infected. This means that if an infant has to have a drip transfusion it must be kept under supervision during the following few days. Moreover, if an infant has to have a series of transfusions it is very undesirable to tie off all the more convenient veins in its body. For these reasons, transfusions which can be given without cutting the skin or tying off a vein, have much to commend them.

In most newborn infants the scalp veins can be entered without great difficulty and scalp vein transfusions have an important place in the therapy of the newborn. It was formerly the custom to use scalp veins for relatively rapid transfusions rather than for drip transfusions. However, this imposes limitations as to quantity. Most full-term infants will tolerate amounts as great as 10 ml./lb. body weight given in 15 minutes, but such amounts are too great to be given safely to premature infants. Personal

experience suggests that in infants weighing less than 5½ lb. (2.5 kg.) the amount should not exceed 6 ml. per lb.

Infant D. was one of twins, born 6 weeks before term. The other twin died of haemolytic disease of the newborn 3 days after birth. The present twin was moderately severely affected and received several transfusions. The following notes refer to a scalp vein transfusion given when the infant was 1 month old and weighed just under 4 lb.

Twenty-five millilitres of group B, Rh-negative citrated blood were injected in 10–15 minutes. Immediately after the transfusion the infant became white and silent. The jugular veins and scalp veins were noted to be full even when the infant was placed in a sitting position. The breathing was shallow. After 30 minutes the infant appeared to be normal again. (This reaction was not related to incompatibility for the transfused cells were shown to survive normally.)

This infant had similar but less severe reactions on two other occasions following scalp vein transfusion.

The usual practice nowadays is to give drip transfusions in the scalp veins using a special needle. The special needle consists simply of the shaft (i.e. without the hub) of a needle of approximately 24-gauge (SWG) on to which is fitted a length of fine plastic tubing which, in turn, is connected to the tubing of the transfusion set.

The Use of the Internal Saphenous Vein

The operation of exposing the internal saphenous vein for canulation is rendered extremely difficult if instruments designed for adults are used. It is of great advantage to use instead a scalpel with a small blade, a fine pair of forceps of the 'mosquito' type and a small aneurysm needle. For the delicate task of partially dividing the vein, a small pair of sharp, pointed scissors is a help.

The infant should be taken from its cot and placed upon a flat table of convenient height, with a good light directed upon the ankle. One leg must then be fixed in an extended position to a suitable splint.

Procain (1 per cent) is then injected under the skin just anterior and proximal to the internal malleolus. If the operator is experienced an incision is made in the line of the leg, just tangential to the anterior border of the malleolus. If the operator is not experienced he may have difficulty in finding the vein through a longitudinal incision and should therefore use a transverse incision. The incision should be quite bold so that the subcutaneous tissues are divided. Once the vein is located, after blunt dissection with the mosquito forceps, it is freed by the passage under it of

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the aneurysm needle, and is then tied as far distally as possible. A second ligature is passed under the vein proximally, but not tied.

At this point, the bottle of blood is removed from its water bath, the transfusion set is assembled and a little blood is run through the apparatus to remove bubbles of air. A suitable fine canula is fitted to the distal end of the giving set in place of the usual giving needle and blood is run through this so that it is all ready for insertion into the vein. Preliminary warming of the blood is advised because the injection of very cold blood may produce sufficient venespasm in the fine veins of infants to stop the flow of blood entirely. In warming blood for transfusion the temperature of the water bath must never exceed 40° C.

The vein is now opened with fine scissors; the canula, attached to the blood-giving set, is inserted into the vein and the proximal ligature is then tied around it. The Bateman needle (really a fine blunt-ended canula within a second slightly wider canula) is very suitable for this purpose. If no special canula is available, an ordinary intravenous needle of small size can be used, after cutting off the point and, if possible, smoothing the cut edges on a stone. Alternatively a small piece of polythene tubing may be used; if so, the opening into the vein should first be dilated with a needle. It should then be possible to introduce tubing with an external diameter of approximately 1.1 mm. Once the catheter is in the vein it can be passed upwards for an inch or two, and the transfusion is then more likely to run smoothly than if a needle is used.

When amounts of blood exceeding 100 ml. are to be given, a slow drip rate is essential. A reasonable rate is 30 ml. an hour; that is to say, if the standard giving set is used, approximately eight drops per minute. Such slow rates of flow demand constant adjustment and supervision.

If the ordinary blood bottle is used, it will be almost impossible to gauge the dose accurately and there is a risk of gross over-dosage. The best plan is to introduce a funnel into the circuit. After filling this to a mark, the tubing from the blood bottle is clipped off and the blood in the funnel allowed to drip into the infant. The funnel can be refilled as often as necessary. A set incorporating a funnel of 30 ml. is supplied by some transfusion centres.

As in giving slow drip transfusions to adults, the possibility of coagulation occurring in the canula due to reflux of the patient's blood can be lessened by adding a little heparin to the transfused blood. Two hundred and fifty units of heparin added to each 100 ml. of blood in the funnel are sufficient to render clotting in the canula distinctly less likely (a total dose

of 500 units of heparin in 4 hours or so will scarcely affect the infant's clotting time).

Calculation of Dosage of Blood for Simple Transfusion

In adults, as in infants, a haemoglobin concentration of 15 g./100 ml. corresponds to a red cell volume of 30 ml./kg. body weight. This is convenient for the purpose of calculation, for it implies that a transfusion of 2 ml. of red cells per kilogramme of body weight will raise the haemoglobin concentration by 1 g./100 ml. Citrated blood, as supplied by most transfusion services, contains approximately one-third its volume of red cells. Thus 6 ml. (3×2 ml.) of citrated blood per kilogramme of body weight are required to raise the haemoglobin concentration by 1 g./100 ml. For those who prefer to think in terms of pounds for body weight and per cent for haemoglobin (100 per cent haemoglobin = 14.8 g./100 ml.), the formula is: 4 ml. citrated blood per pound body weight are required to raise the haemoglobin concentration by approximately 10 per cent.

In using stored blood it is wise to make a small allowance for non-viable red cells and to increase the amount to 4.5 ml./lb. For example: an infant weighs 8 lb. and its haemoglobin is 50 per cent (7.4 g./100 ml.). The intention is to raise its haemoglobin concentration to 80 per cent (11.8 g./100 ml.), i.e. an increase of 30 per cent (4.4 g./100 ml.). The amount of citrated stored blood required is calculated as follows:

4.5 ml. per lb. raises the haemoglobin 10 per cent;
(4.5×8) or 36 ml. raises the haemoglobin concentration of an 8 lb. infant by 10 per cent;
(36×3) or 108 ml. raises the haemoglobin by 30 per cent;
Amount required: 108 ml. of citrated (stored) blood.

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CHAPTER XVII

HAEMOLYTIC DISEASE OF THE NEWBORN

TRANSFER OF ANTIBODIES FROM MOTHER TO FOETUS

In some mammals (e.g. rabbits) the main route of transfer of antibodies from mother to foetus is via the amniotic fluid and yolk sac (Brambell *et al.*, 1949); in others (e.g. bovines) the colostrum is the main vehicle. In humans, on the other hand, there seems to be no doubt that antibody transfer from mother to foetus occurs almost entirely via the placenta.

Placental Transfer of Antibodies

As described in Chapter VII, blood group antibodies are composed either of 7*S* gamma globulin with a molecular weight of approximately 160,000 or of 19*S* gamma globulin with a molecular weight of the order of 1,000,000. Cord plasma contains only about one-tenth to one-twentieth of the adult level of 19*S* globulin (Franklin and Kunkel, 1958; Polley *et al.*, 1961) and it is thought that little or none of this protein passes the placenta. On the other hand 7*S* gamma globulin is transferred across the placenta quite rapidly at term.

DuPan *et al.* (1959) injected ^{131}I-labelled gamma globulin intravenously into pregnant women 3 to 48 hours before delivery. When the interval between injection and delivery was 24 hours the level of labelled globulin in the infant's serum was about 50 per cent of that of the mother's serum. When the interval was 48 hours the level in the infant's serum was 80 per cent of that in the mother's.

It is therefore to be expected that when the mother's serum contains 7*S* blood group antibodies their titre in the infant's serum will be as great as in the mother's serum, providing that the infant's red cells lack the corresponding antigen.

There is little quantitative evidence about the passage of gamma globulin in the early stages of pregnancy. Moore *et al.* (1949) found that the gamma globulin concentration was very low in foetuses of 3 to 4 months and increased steadily towards term. Martin (1954b) found virtually no gamma globulin in the foetal plasma at or before the twenty-sixth week. Vahlquist *et al.* (1950) estimated the titre of two anti-bacterial antibodies, anti-streptolysin and anti-staphylolysin which pass the placenta and are presumably 7*S* globulin. They found that these antibodies were

present in much smaller amounts in foetuses of 16 to 22 weeks, although by 32 to 38 weeks their titre was as high in cord serum as in maternal serum.

The implications for some of the more important blood group antibodies can now be considered.

Anti-A and Anti-B

As explained in Chapter VII, anti-A and anti-B iso-agglutinins are most commonly composed of 19*S* gamma globulin but may be composed partly of 7*S* gamma globulin. When the mother's serum contains only 19*S* iso-agglutinins, none will pass to the infant. On the other hand, when the mother's serum contains a mixture of 19*S* and 7*S* iso-agglutinins, the 7*S* component is readily transported across the placenta and the titre of 7*S* isoagglutinins is similar in mother and infant (Kochwa *et al.*, 1961). It has been shown that cord blood iso-agglutinins are found only in the fraction of serum which contains 7*S* gamma globulin (Kochwa *et al.*, 1961). The titre of iso-agglutinins in the infant's serum may sometimes be as high as 64 (Tovey, 1945) or even 256 (Jungwirth, 1955). The agglutinin titre is apparently much affected by the technique adopted; Munk-Andersen (1956b) found that, provided that the mixture of serum and cells was not centrifuged, the titre of anti-A and anti-B was seldom higher than 2, although if the mixture was centrifuged the titre might be much higher. As Munk-Andersen (1959) concluded, incomplete anti-A is 'less incomplete' than incomplete anti-Rh.

Haemolytic anti-A and anti-B may be either 19*S* or 7*S* γ globulin (Rawson and Abelson, 1960a). Jungwirth (1955) found that when haemolytic anti-A or anti-B were present in the mother's serum they were usually present in the infant's serum but his material included only group O mothers.

It appears that 7*S* anti-A and anti-B are found more commonly in group O mothers than in A or B mothers (Rawson and Abelson, 1960a; Kochwa *et al.*, 1961) and this explains why iso-agglutinins are found more commonly in the sera of infants born to group O mothers. Thus Rosenfield and Ohno (1955) found iso-agglutinins in 90 per cent of infants of group O mothers but in only 35 per cent of the infants of group A or B mothers. Dr R. E. Rosenfield (personal communication) also found that when the mother was group O and her infant group A or B, 'incompatible' iso-agglutinins were present in 30 per cent of cases; for example, if the infant was group A, then anti-A could often be found by testing the infant's serum against adult A_1 cells; see also Zuelzer and Kaplan (1954b).

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Anti-Rh

Anti-Rh saline agglutinins are composed of 19*S* gamma globulin but sera containing 19*S* anti-Rh almost always contain some incomplete, that is 7*S*, anti-Rh as well and this antibody does pass the placenta. Thus when the mother's serum contains agglutinating anti-Rh and she gives birth to an Rh-negative infant the infant's serum will not agglutinate Rh-positive cells suspended in saline, but it is likely to contain some incomplete anti-Rh (Wiener, 1948a; Broman, 1949).

When a mother whose serum contains predominantly incomplete anti-Rh gives birth to an Rh-negative infant, the titre of anti-Rh in her infant's serum is frequently the same as in her own (Wiener, 1948a; Mollison, 1951, p. 370). There is some evidence that anti-Rh sera which behave *in vitro* as purely incomplete antibodies may be composed partly of 19*S* globulin (Hunter and Mollison, 1961; Kekwick and Mollison, 1961) and if this is confirmed it would be expected that there would be some discrepancy between the amount of anti-Rh in the mother's serum and the amount in the infant's serum.

In view of the fact that virtually all anti-Rh sera contain some 7*S* anti-Rh, it is to be expected that Rh-positive infants born to mothers whose serum contains anti-Rh will almost always have a positive anti-globulin test. As reported previously (Mollison, 1956, p. 455) in the course of testing the blood of 300 Rh-positive infants born to mothers whose serum contained anti-Rh a negative direct Coombs test was encountered only three times. In each of these three exceptional cases the mother's serum contained only a trace of antibody.

Anti-Rh can cross the placenta at a very early stage of pregnancy. Positive direct antiglobulin tests have been observed in two foetuses with crown-rump lengths of 30 mm. and 110 mm. respectively, corresponding to approximately 10 and 16 weeks' gestation (see Mollison, 1951, p. 370). In both these cases pregnancy was terminated because the mother had anti-Rh in her serum, had lost a previous infant, and had a homozygous husband.

Other Blood Group Antibodies

The 'naturally-occurring' antibodies anti-M, anti-N, anti-P_1, anti-Le^a and anti-Le^b do not usually pass the placenta, being as a rule composed of 19*S* globulin (see Chapter VII).

A few cases have been recorded in which anti-M capable of agglutinating cells suspended in saline has been present in the serum of a newborn infant, as well as in its mother's serum. For example, in one case the mother's serum agglutinated group M cells to a titre of 16 at room temperature and her infant's cord serum had a titre of 8. The antibody was inactive at 37° C. (Jakobowicz and Bryce, 1951). In the case of haemolytic disease of the newborn due to anti-M referred to in Chapter X the infant's red cells gave a weakly positive direct anti-globulin test and free anti-M could be detected in the serum. When agglutinating antibodies derived from the mother are found in the serum of an infant it must be supposed that the antibody consists at least partly of 7*S* globulin.

Although Lewis antibodies are normally composed of 19*S* gamma globulin, one example consisting of a mixture of 7*S* and 19*S* gamma globulin has been encountered (Kekwick and Mollison, 1961), and it is therefore to be expected that occasionally Lewis antibodies will pass to the foetus. Nevertheless, in view of the very weak development of the Le^a antigen at birth it seems doubtful if anti-Le^a can cause haemolytic disease of the newborn (see Chapter VIII).

It is known that anti-T agglutinins do not pass the placenta (Lind and McArthur, 1947). Normal cold agglutinins and the normal incomplete cold antibody are, however, found in cord blood (see Mollison, 1956, pp. 252 and 254), a fact which at the moment appears rather mysterious.

Most immune blood group antibodies, with the exception of occasional antibodies behaving as saline agglutinins, consist of 7*S* globulin and are therefore expected to pass the placenta freely; examples are incomplete antibodies of the Rh, K, Fy, Jk, Di and Js systems.

Transfer of Antibodies into the Amniotic Fluid

Human amniotic fluid contains about one-tenth to one-twentieth of the concentration of gamma globulin that is present in adult serum (personal communication from Drs D. R. Bangham and D. E. H. Tee) and when Rh antibody is present in the mother's serum it may also be detectable in the amniotic fluid. Thus van Bolhuis (1950) found anti-Rh (titre 16) in amniotic fluid in two cases in which he looked for it and Wild (1960) reported that he had found Rh antibody in amniotic fluid in thirty instances and that it was almost always present when the mother's serum had an anti-Rh titre of 128 or more.

It is very doubtful whether the human foetus absorbs any appreciable amount of antibody from the amniotic fluid. Reasons for this belief have been summarized by Bangham (1960) who also carried out experiments in monkeys. He showed that if ^{131}I-labelled gamma globulin was injected

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into the amniotic fluid of a foetal monkey near term no significant quantity of antibody appeared in the foetal serum although labelled antibody could be found throughout the length of the foetal gut.

Transfer of Antibodies via the Milk

Human milk contains antibodies, including blood group antibodies, but there is extensive evidence that the amount of antibody absorbed via the gut in newborn infants is negligible and certainly does not exceed 0.1 per cent of the administered dose (see review by Vahlquist, 1958). Boorman *et al.* (1958) fed large amounts of human serum containing Rh antibody to normal Rh-negative newborn infants but failed to detect any antibody in the infant's serum even though in some cases they would have been able to detect the absorption of as little as 1/100th or even 1/200th of the injected dose.

Persistence of Passively Acquired Antibodies

In newborn infants, antibodies acquired by placental transfer can be demonstrated for periods up to 24 weeks after birth. In the case of anti-A and anti-B the evidence is not clear-cut since the infant may begin to form its own antibodies within 2 or 3 months of birth (Yliruokanen, 1948). However, when Rh antibody is acquired by an Rh-negative infant, for example as a result of the previous immunization of its mother by a transfusion of Rh-positive blood, there is a perfect opportunity for studying the persistence of the acquired antibody. Wiener (1948a) made serial observations in one case, supplemented by a few observations in two others, and concluded that the antibody persisted for approximately 4 months. In nine cases studied in the author's laboratory, it was found that when the infant had a large amount of antibody at birth (titres 128 to 1000), antibody could be detected for some 20 weeks after birth; when the infant had initially only a small amount of antibody, no trace was left after about 4 weeks.

If the amount of antibody is plotted on a logarithmic scale, against time, the slope of elimination is linear (see Fig. 91). Similar findings have been reported for other antibodies received by the newborn infant from its mother (see Burnet and Fenner, 1949, for references).

The length of time for which the direct antiglobulin test remains positive in an affected infant also affords evidence of the time for which Rh antibody persists after birth. In mildly affected infants who receive no treatment, the test may remain positive for as long as 2 months, though in

the mildest cases of all the test may be positive for only 2 or 3 weeks. In severely affected infants (treated by simple transfusion of Rh-positive blood), the test may remain positive for more than 90 days (Mollison and Cutbush, 1949a).

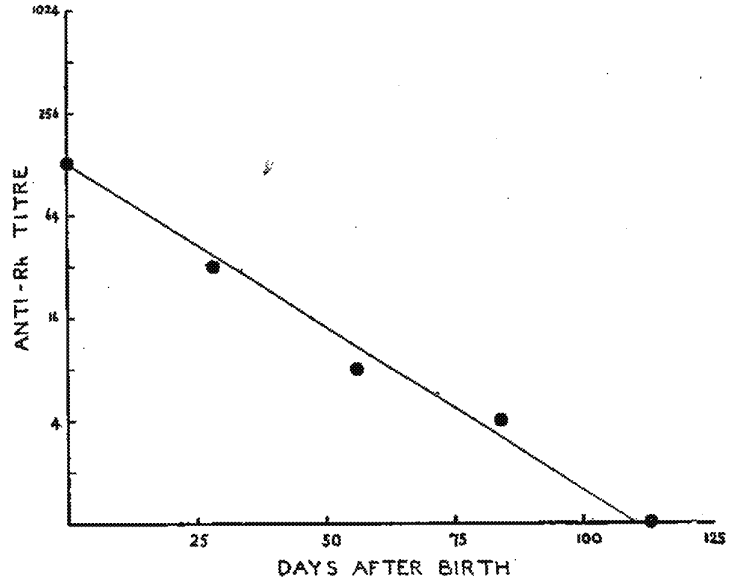

FIGURE 91. Rate of disappearance of anti-Rh (acquired passively by placental transfer), from the plasma of an Rh-negative infant. The titre of anti-Rh is plotted on a logarithmic scale.

From the slope of the line in Fig. 91 the time taken for the amount of antibody to halve can be determined. In the case illustrated it is approximately 15 days; making some allowance for the effect of growth, a better estimate might be 18 days. This rate of disappearance is a little greater than that observed in adults, following the injection of ^{131}I-labelled 7*S* gamma globulin (see p. 43).

Damage to Foetal Leucocytes or Platelets by Maternal Antibodies

Isoimmune Neonatal Neutropenia

Several cases have now been described in which infants (often several in one family) have been affected with severe neutropenia in the first few weeks of life, associated with poor resistance to infection, and it has been

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shown that the mother's serum contains a leuco-agglutinin capable of reacting, *in vitro*, with the infant's leucocytes (Hitzig and Gitzelmann, 1959; Lalezari *et al.*, 1960; Jensen, 1960; Braun *et al.*, 1960). Nevertheless the passage of leuco-agglutinins across the placenta does not necessarily cause leucopenia or produce any other signs of damage to the infant (Payne, 1960).

Isoimmune Neonatal Thrombocytopenia

Thrombocytopenia sometimes occurs in infants whose mothers are affected with idiopathic thrombocytopenic purpura, a fact in keeping with the idea that the disease is due to an auto-antibody. Thrombocytopenia may also occur in the infants of healthy mothers; in one such case it was clearly demonstrated by the mixed antiglobulin technique that the mother's serum contained an iso-antibody against platelets. The mother had had two previous pregnancies and two previous transfusions (Garrett *et al.*, 1960). Presumably if the infant had needed a platelet transfusion, it would have been best to have tested the platelets of potential donors against the mother's serum so as to find compatible platelets. Such tests will presumably one day form part of the normal routine of a transfusion laboratory.

The Incidence of Haemolytic Disease of the Newborn

When blood group antibodies pass the placenta from the mother to the foetus and when the red cells of the foetus contain the corresponding antigen, haemolytic disease develops unless the antibody is very weak or unless the relevant antigen in the infant's red cells is very poorly developed.

Anti-Rh is by far the commonest cause of serious haemolytic disease of the newborn. If intra-uterine deaths due to the disease are included, the incidence in Britain is about 1 in 180 of all pregnancies (Walker and Murray, 1954). Approximately 12 per cent of these pregnancies end in intra-uterine death so that the incidence of live-born cases due to anti-Rh is approximately 1 in 200. About two-thirds of the live-born infants require exchange transfusion so that the incidence of cases requiring treatment is about 1 in 300 of all pregnancies.

Haemolytic disease of the newborn due to ABO incompatibility is usually much milder and for this reason, and because it differs considerably in its serological features, it is described separately later in this chapter.

Of the cases due to anti-Rh, almost all are due to the antibody anti-D. Thus van Loghem, Bakx and Klomp-Magnée (1953) reported the results

of examining the sera of 2274 mothers whose infants were affected with haemolytic disease of the newborn: 2250 (99 per cent) of the samples contained anti-D, with or without anti-C or anti-E. Of the remaining twenty-four samples, ten contained anti-E alone, eight contained anti-c alone, three anti-c plus anti-E, two anti-C^w and one anti-C. (For a further discussion, see p. 324.) Haemolytic disease of the newborn due to anti-c tends to be milder than in the cases due to anti-D (Dr Bruce Chown, personal communication).

Antibodies of other blood group systems cause haemolytic disease of the newborn only with great rarity. Cases due to anti-K are the least rare of these.

Before beginning an account of haemolytic disease of the newborn, it is proper to emphasize that the recognition of its serological cause was the outcome of the work of Levine and his colleagues. The reader will find a most satisfying account of some of the early work in the paper by Levine, Burnham, Katzin and Vogel (1941).

HAEMOLYTIC DISEASE OF THE NEWBORN DUE TO ANTI-Rh

The Predominant Role of Red Cell Destruction in the Disease

It is now accepted that the only direct damage caused by anti-Rh is red cell destruction and that all the other features of haemolytic disease of the newborn stem from this. In the most severe cases the infant dies *in utero* from anaemia which precipitates cardiac failure and the foetus is found to be grossly oedematous with effusions into serous cavities (*hydrops foetalis*). Infants with *hydrops foetalis* may be born alive, although they usually live for only a few minutes. Their cord blood has a haemoglobin concentration in the range of 3-9 g. per 100 ml. (usually less than 5 g.) and a blood film shows numerous nucleated red cells (up to 200,000 per c.mm.); the reticulocyte count may be as high as 60 per cent.

Infants with a slightly less severe haemolytic process may survive, particularly if treated promptly (see below). Those that do survive face a different hazard: the rapid destruction of red cells produces a large amount of bilirubin which the infant cannot excrete from the blood stream. (As described in the previous chapter, production of the enzyme which conjugates bilirubin to form the excretable glucuronide is deficient at birth.) Accordingly in infants with severe haemolytic disease of the newborn the bilirubin can rise rapidly after birth and unless the infant is treated by exchange transfusion the serum bilirubin concentration may reach a level

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of 20-30 mg. per 100 ml. or more after 48 or 72 hours (a condition formerly known as *icterus gravis neonatorum*) and the infant is then in great danger of developing kernicterus, a condition thought to be due to the direct toxic effect of unconjugated bilirubin on brain cells.

If the untreated infant survives this stage of the disease, either because the haemolytic process is mild, or because by chance it has a better than average capacity to excrete bilirubin, it faces a further hazard at any time from about the seventh day of life onwards, for it may then develop very severe anaemia. The mechanism of this anaemia is not really obvious because there is no reason to believe that the rate of red cell destruction increases after birth; rather the reverse. This anaemia is then almost certainly due to a failure of production. In normal infants the reticulocyte count falls to a very low level by the end of the first week of life and it is generally supposed that this is due to the sudden increase in the percentage oxygen saturation of the blood which occurs at the time of birth. This may be the mechanism of the failure of production sometimes seen in haemolytic disease of the newborn, but it also seems possible that at this stage of life there is a sudden fall in the level of some red cell stimulating factor.

Foetal haemoglobin in haemolytic disease of the newborn. Jonxis (1948) noted that the proportion of foetal haemoglobin was lower in infants with haemolytic disease of the newborn than in normal infants and suggested that there was a selective destruction of cells containing foetal haemoglobin. Schulman and Smith (1954a) showed that there was an absolute increase in the amount of adult haemoglobin and this was confirmed by Oppé and Fraser (1961) who also found that the lower the total haemoglobin concentration in haemolytic disease of the newborn the lower the proportion of foetal haemoglobin. As they pointed out, their findings are most simply explained by supposing not that there is a selective destruction of cells containing foetal haemoglobin but rather that near term the infant is synthesizing chiefly adult haemoglobin. Therefore, when an increased number of red cells is needed, there will be a quicker change to adult haemoglobin than in the normal infant in whom foetal haemoglobin disappears only slowly from the circulation.

Antenatal Assessment of Severity

Two main guides are available: the fate of previous infants born to the mother, and the titre of Rh antibody in the mother's serum.

Pattern of the Disease in Successive Siblings

In the first affected infant the disease tends to be less severe than in subsequent siblings; due to the fact that the formation of anti-Rh begins relatively late in pregnancy, the foetus is exposed to anti-Rh for a relatively

short period. Dr H. R. Nevanlinna (personal communication) compared the first two affected infants in sixty-three families and found that the second of the two was more severely affected in twenty-five cases, equally severely affected in thirty-two cases and less severely affected in only six. Although the first affected infant is often mildly affected, in a small proportion of cases it is stillborn. Thus in Walker and Murray's (1956) series amongst first-affected foetuses, 6 per cent were stillborn. Hartmann and Brendemoen (1953) in a study of haemolytic disease in first pregnancies, in women not previously transfused, found that in three out of twenty-three cases the infant was stillborn.

As many observers have pointed out, if one sibling in a family has mild haemolytic disease of the newborn, subsequent siblings are also likely to be mildly affected (Denton, 1950; Davies *et al.*, 1953; Walker and Murray, 1956). Once a mother has given birth to a mildly affected infant her chance of a stillbirth in a subsequent pregnancy is very low (Walker and Murray, 1956).

Very severely affected infants and stillborn infants are likely to be followed by stillbirths; moderately and severely affected infants are on the average followed by somewhat more severely affected infants, so that one in five of succeeding pregnancies ends in a stillbirth.

If first-affected infants are excluded, the rate of intra-uterine death amongst Rh-positive foetuses carried by mothers whose serum contains anti-Rh is about 29 per cent.

Occasionally, mild disease may follow stillbirths due to haemolytic disease, a fact which should make one cautious in interpreting occasional apparent successes in the treatment of pregnant women with a 'bad history'.

Thus, Mrs T., a woman whose serum contained Rh antibody and whose husband was of the probable genotype *CDe/cDE*, had the following obstetric history:

1. Normal infant.
2. Normal infant, apart from slight jaundice.
3. Stillbirth.
4. Hydrops foetalis.
5. Moderately severe haemolytic disease; recovered.
6. Stillbirth.
7. Mild haemolytic disease (cord haemoglobin concentration 14.4 g./100 ml.); recovered.
8. Mild haemolytic disease (cord Hb concn. 17 g./100 ml.). Direct antiglobulin test strongly positive. The infant was not treated; the serum bilirubin concentration never rose above 10 mg./100 ml. and recovery was uneventful.

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The tendency of the severity of the disease to repeat itself, and the existence of a proportion of exceptions is well illustrated by some figures from Dr H. R. Nevanlinna (personal communication). The severity of the disease in two consecutive Rh-positive infants was compared, excluding first affected infants (see above). Among seventy-nine pairs of children in the same families, the later child was equally severely affected in forty-two cases, more severely affected in twenty and less severely affected in seventeen.

The Effect of the Infant's Rh genotype

The potency of the D antigen varies in red cells of different Rh genotypes; for example, *in vitro*, R_2r red cells react more strongly with anti-D than do R_1r red cells. Murray (1957) concluded that R_2r infants were more likely than R_1r infants to immunize their mothers and that on the whole the severity of haemolytic disease of the newborn was greater in R_2r infants. Moreover the risk of stillbirth in first-affected infants was distinctly greater than when the infant was R_2r. These conclusions could not be confirmed by Kirk *et al.* (1959).

Significance of a previous Transfusion to the Mother of Rh-positive blood

Levine and Waller (1946) pointed out that severe haemolytic disease due to anti-Rh in a first-born infant was frequently associated with a history that the mother had been immunized by an earlier transfusion of Rh-positive blood. Similarly, Levine *et al.* (1953) found that the stillbirth rate among the first Rh-positive infants born to immunized Rh-negative women was twice as high when the mother had been transfused with Rh-positive blood. Again, Mollison and Cutbush (1954) found that among first-affected infants the mortality was almost twice as high when the mother gave a history of a previous transfusion of Rh-positive blood.

It appears that this greater severity of the disease amongst offspring born to previously transfused mothers is confined to the first-affected infant and is probably to be explained by the fact that in women not immunized by a transfusion the first infant tends to be more mildly affected than subsequent affected infants (see above). Walker and Murray (1956) found no increase in the stillbirth rate in women immunized by a transfusion; Nevanlinna (1953) also concluded that the prognosis for infants affected with haemolytic disease of the newborn born to mothers previously transfused with Rh-positive blood was not essentially different from that of other affected infants.

The Titre of Anti-Rh in the Mother's Serum as a Guide to Severity

It can be shown experimentally that there is a relationship between the degree to which Rh-+ red cells are coated with Rh antibody and the rate at which they are removed from the circulation (see Chapter XIII). It might be expected then that there would be a close relationship between the titre of anti-Rh in the mother's serum and the severity of haemolytic disease in her infant. In practice the correlation is not very close and it must therefore be presumed that some of the following variables are important: First, even when the mother's serum contains predominantly incomplete Rh antibody it is possible that not all of this can pass the placenta freely; in one case out of about twenty investigated in the author's laboratory the activity of an incomplete Rh antibody was reduced by treatment with SH compounds, suggesting that in this instance the incomplete anti-Rh was composed partly of 19*S* globulin (Hunter and Mollison, 1961). Secondly, it is not known how much different Rh antibodies vary in their avidity and destructive power. It is possible that two antibodies with the same indirect antiglobulin titre have different effects on red cells *in vivo*. Indeed Kirk *et al.* (1958) have endeavoured to show that the degree to which Rh antibodies combine with the infant's red cells is correlated with the severity of the haemolytic process. Thirdly, variations between different infants have to be considered; apart from qualitative and quantitative variations in Rh antigens there are such factors as the capacity of the reticulo-endothelial system in different infants, which must affect to some extent the number of coated red cells removed from the circulation each day and so affect the degree of anaemia; the response of the bone marrow in different infants must also be taken into account. Finally, after birth a number of other factors come into play. As already mentioned the infant's chance of developing kernicterus must depend to a considerable extent on the rate at which it develops the ability to excrete bilirubin and this presumably varies from one infant to another quite independently of the factors causing haemolytic disease of the newborn. For all these reasons it is after all not surprising that the severity of the haemolytic disease of the newborn cannot be accurately predicted from a knowledge of the mother's anti-Rh titre. All the same, the relationship between severity and titre is close enough to be of real clinical value.

Although a definite relationship between the mother's anti-Rh titre and the severity of haemolytic disease of the newborn was demonstrated by titrating the mother's serum in albumin (Davidsohn and Stern, 1948; Mollison and Cutbush, 1949a; and Wiener, Nappi and Gordon, 1952), a better correlation is found if the anti-Rh titre is assessed by the indirect

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antiglobulin test and only results obtained with this method will be discussed.

Watson and Vos (1952) found that in seven women who had had stillbirths due to anti-Rh, the indirect antiglobulin titre just before delivery was between 1024 and 4096. All but one of these women had titres of 512 or more at the sixth month of pregnancy. By contrast, in only one out of twenty-three mothers who gave birth to a live infant was the titre as high as 512 at the sixth month of pregnancy.

Watson and Vos found that in seven patients with mildly affected infants the titre tended to be lower and steady throughout pregnancy and to be between 16 and 64 at term.

In a further paper Kelsall *et al.* (1957) found that when the titre at term was 128 or less the infant's chance of survival was 97 per cent; when the titre was 256–1024 the chance of survival was 79 per cent; and when the

TABLE XLVIII

RELATION BETWEEN INDIRECT ANTIGLOBULIN TITRE IN MOTHER'S SERUM AND FOETAL MORTALITY IN A SMALL SELECTED SERIES

Indirect antiglobulin titre	*Total no. of cases*	*No. of stillbirths*
1–8	3	0 (—)
16–32	8	1 (13%)
64–512	15	4 (27%)
1000–2000	3	2 (67%)

titre was 2048 or greater the chance was only 35 per cent. Subsequently Kelsall *et al.* (1958) recommended that if the antiglobulin titre at term was 64 or less the infant should not be treated. If it was 128 and the cord haemoglobin high the infant should also be left untreated because when the mother's indirect antiglobulin titre exceeded 128 exchange transfusion was indicated. They recommended that the anti-Rh titre should be determined every week from the 26th week of pregnancy onwards and that labour should be induced if by the 35th or 36th week the mother's anti-Rh titre had reached 512 or more.

Although the Australian experience suggests that the indirect antiglobulin test is an indispensable investigation in the management of haemolytic disease of the newborn, it must be emphasized that the correlation between the antibody titre and severity is not perfect. In an investigation carried out in the author's laboratory, several samples were

collected during pregnancy from a selected series of women, many of whom had had a previous stillbirth due to haemolytic disease of the newborn. All tests were carried out against red cells from a particular donor using a technique which was as carefully standardized as possible. As Table XLVIII shows there was one case in which although the mother's titre was only 16 the infant was stillborn. This infant had obvious *hydrops foetalis* and the mother had had a previous similarly affected infant. This case makes the point that the history is sometimes an invaluable guide. The value of taking both the history and titre into account is discussed again later.

TABLE XLIX

RELATION BETWEEN INDIRECT ANTIGLOBULIN TITRE OF ANTI-RH IN MOTHER'S SERUM AND SEVERITY OF HAEMOLYTIC DISEASE IN 200 'FIRST-AFFECTED' INFANTS; FROM TOVEY AND VALAES (1959)

	Indirect antiglobulin titre		
	Less than 10	10–20	40 *or more*
Total	59	84	57
Stillbirth	0	1	14
Neonatal death	0	2	4
No treatment: recovered	50	24	1
Treated by exchange transfusion: recovered	9	57	38

Tovey and Valaes (1959) emphasized that the indirect antiglobulin titre was very valuable in predicting stillbirths. The particular technique they used gave lower figures than those obtained by Watson and Vos and by most other workers, and this emphasizes that the indirect antiglobulin titre has no absolute significance in indicating the amount of antibody present, although provided that as many variables as possible are standardized, close comparisons are possible within a series.

Tovey and Valaes determined the titre of Rh antibody using the indirect antiglobulin test in 200 women who had not previously given birth to an infant affected with haemolytic disease of the newborn and their results are summarized in Table XLIX. Among fifty-nine cases in which the titre was less than 10 there were no stillbirths; among eighty-four cases with a titre of 10–20, there was only one stillbirth but among fifty-seven with a titre of 40 or more, there were thirteen stillbirths.

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Tovey and Valaes stressed particularly the importance of those cases in which the mother had a stillbirth during the first pregnancy in which Rh antibody could be detected. In their series 7 per cent of all 'first affected' pregnancies ended in a stillbirth, a figure which incidentally is very close to that of other published series, e.g. Walker and Murray (1956). Tovey and Valaes suggested that it was in these cases that estimation of Rh antibody titre by the indirect antiglobulin method was particularly likely to be valuable in providing an indication for premature induction of labour which might result in the birth of a living, treatable infant. Tovey and Valaes found a less satisfactory relationship between titre and severity in women who had formed Rh antibody in a previous pregnancy, but the numbers on which they based their conclusions were small.

Effect of Interval between Pregnancies

Kelsall *et al.* (1959) suggested that when the titre of antibody in the mother's serum was high, time should be allowed for it to fall between pregnancies. They considered that anti-Rh was not necessarily stimulated during every pregnancy so that if the titre was allowed to fall and there was no further 'episode of immunization' then the outcome of a subsequent pregnancy might be more favourable. In one case following a pregnancy in which the titre was 1 in 1024 and the outcome a macerated foetus, the titre fell during the subsequent 2 years to 16; the patient then became pregnant and during this pregnancy the titre rose only to 128 and premature induction of labour was carried out resulting in a liveborn infant.

Alterations in Amniotic Fluid as a Guide to Severity

The development of excess amniotic fluid — 'hydramnios' in a woman whose serum contains Rh antibody should always raise the suspicion that her infant has hydrops foetalis and if the foetus is found to be still alive the question of premature induction of labour must be considered immediately.

It is sometimes worth while to obtain a sample of amniotic fluid by puncture through the abdominal wall and to examine its colour, since there is some relationship between this and the severity of the infant's disease. Table L shows some findings from a series investigated in collaboration with Professor J. McC. Browne; samples were obtained from forty-four pregnant women a few days before the birth of their infants and compared with the severity of the haemolytic process as judged by the cord haemoglobin concentration.

When the amniotic fluid was colourless, the infant's cord haemoglobin concentration was always above 11 g./100 ml. and when the fluid was deep yellow the haemoglobin concentration was always below 11 g./100 ml. Unfortunately in most cases the amniotic fluid was pale yellow or yellowish brown and in these the severity of the disease was very variable.

TABLE L

COLOUR OF AMNIOTIC FLUID IN RELATION TO SEVERITY OF HAEMOLYTIC DISEASE IN FOETUS (FROM MOLLISON AND CUTBUSH, 1954)

	Rh Positive		
Amniotic fluid	*Cord haemoglobin concentration* <11 *g./100 ml.*	*Cord haemoglobin concentration* >11 *g./100 ml.*	*Rh Negative*
Colourless	0	12	5
Pale yellow or yellow brown	3	17	3
Deep yellow	4	0	0

Mother's Serum Bilirubin Concentration as a Guide

Nevanlinna and Eklund (1959) calculated that the absolute number of red cells destroyed each day by an infant with severe haemolytic disease of the newborn was greater than the number destroyed each day by its mother. Therefore, assuming that the bilirubin produced by the infant all passed into the mother's circulation via the placenta, the mother's serum bilirubin level might well be expected to be raised. In a series of women pregnant with infants suffering from haemolytic disease, they found that two-thirds had serum bilirubin values above 0.55 mg./100 ml. and half had values above 0.7 mg./100 ml., whereas in a control series of normal pregnant women all values were below 0.5 mg./100 ml.

Assessment of Severity from Cord Blood Findings

About one-third of infants born with a positive direct antiglobulin test require no treatment, whereas others if untreated die within a few hours of cardiac failure or become rapidly jaundiced and may develop kernicterus at any time after the age of about 36 hours. It is desirable to select at the earliest possible moment cases requiring treatment, since it is reasonable to suppose that exchange transfusion is far more effective in preventing the development of high bilirubin levels when it is carried out early. The examination of cord blood therefore plays an essential role in the management of haemolytic disease of the newborn.

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The Haemoglobin Concentration of Cord Blood as an Index of Severity

Fig. 92 shows the relationship between cord haemoglobin concentration and the chance of survival for infants with haemolytic disease treated conservatively; that is to say, not by exchange transfusion. The chance of survival increases with the cord haemoglobin concentration but even when the haemoglobin concentration is within the normal range the chance of survival is not 100 per cent. For any given cord haemoglobin concentration the mortality in immature males is much higher than in immature females or in mature infants of either sex.

It has been pointed out elsewhere that the data are consistent with the assumption that there is a linear relationship between the probit of the survival rate and the

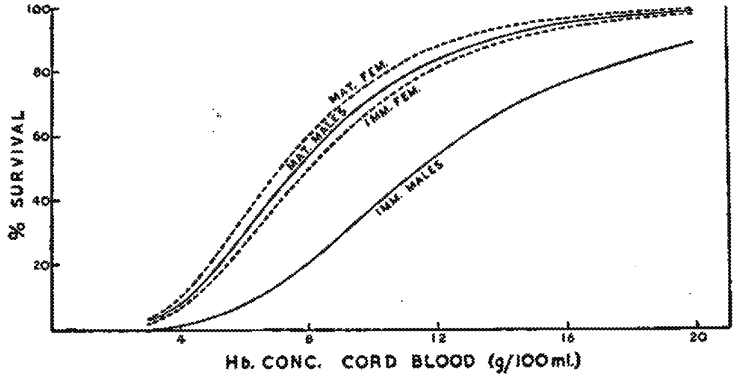

FIGURE 92. Curves relating the haemoglobin concentration of cord blood to the chance of survival in male and female infants not treated by exchange transfusion (anaemic infants were treated by simple transfusion).
'Mat.' = mature, that is, born within 10 days of term.
'Imm.' = immature, that is, born 35–18 days before term.
(From Armitage and Mollison, 1953.)

logarithm of the haemoglobin concentration (Mollison and Cutbush, 1951; Armitage and Mollison, 1953). The curve thus resembles that of a dose/mortality curve observed in toxicity experiments in animals.

These findings can be explained by relatively simple hypotheses. As already mentioned, the most anaemic infants die of cardiac failure. Infants with lesser degrees of anaemia may become deeply jaundiced during the first few days of life and may then develop kernicterus. If the development of deep jaundice depended solely on the rate of red cell destruction it would be expected to be more or less confined to infants with a considerable increase in red cell destruction as indicated by a

haemoglobin concentration below the normal range. The fact that deep jaundice sometimes occurs in infants whose haemoglobin concentration in the cord blood is within the normal range suggests that another factor is operating. The factor is presumably a normal variation between infants in the efficiency of bilirubin excretion.

It has been suggested that kernicterus is virtually independent of the degree of anaemia (Diamond *et al.*, 1950), but this conclusion appears to be refuted by data published by Armitage and Mollison (1953). Thus the overall incidence of kernicterus in 222 infants not treated by exchange transfusion was assessed by including as cases of kernicterus not only fatal cases but also infants surviving with signs of cerebral damage, as judged by examination at 6 months; as Table LI shows the incidence of kernicterus was nine times higher in infants with cord Hb concentrations below

TABLE LI

INCIDENCE OF KERNICTERUS (FATAL AND NON-FATAL) IN INFANTS NOT TREATED BY EXCHANGE TRANSFUSION (FROM ARMITAGE AND MOLLISON, 1953)

Cord Hb. concentration g./100 ml.	*Incidence of kernicterus*	
	K/total	*Per cent*
3.2– 9.9	6/13	46
10.0–15.8	13/92	14
>15.9	5/98	5

10 g./100 ml. than in those with cord Hb concentrations of 16 g./100 ml. or more.

The finding that five out of ninety-eight infants with cord haemoglobin concentrations of 16 g./100 ml. or more developed kernicterus compares fairly well with Walker and Neligan's (1955) figure of five out of ninety-five for infants with cord haemoglobin concentrations above 14.8 g./100 ml.

Distribution of Cases by Cord Haemoglobin Concentration

Approximately half of all infants with haemolytic disease of the newborn have haemoglobin concentrations above 14.5 g./100 ml. Thus Walker and Neligan (1955) found that ninety-five out of 181 affected infants had cord Hb concentrations above 14.8 g./100 ml. and the present author found that sixty-six out of 141 infants had cord haemoglobin concentrations about 14.5 g./100 ml.; further details of the distribution of cord haemoglobin values in the author's series are shown in Table LII.

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The distribution of cases is not expected to be exactly the same in any two series. For example, the higher the proportion of first-affected infants the greater will be the number of mildly affected cases.

TABLE LII

DISTRIBUTION OF CORD HAEMOBLOGIN VALUES IN A SERIES OF INFANTS WITH HAEMOLYTIC DISEASE OF THE NEWBORN (MOLLISON, 1956, p. 464)

Cord haemoglobin concentration g./100 ml.	*Number of cases*	*Per cent o total (approx.)*
>17.5	16	11
14.5–17.5	50	36
10.5–14.4	44	31
6.5–10.4	25	18
3.4– 6.4	6	4
	141	100

The Bilirubin Concentration of Cord Blood as a Criterion of Severity

The plasma bilirubin concentration of cord blood is quite closely related to severity as shown by Table LIII.

TABLE LIII

BILIRUBIN CONCENTRATION (CORD BLOOD) AND MORTALITY

No. of cases	*Plasma bilirubin mg./100 ml.*	*Deaths*	*Mortality* per cent*
12	0–2	1	8
29	2–4	4	14
17	4–6	8	47
4	6–8	3	75

* The figures are not representative for mature infants treated by exchange transfusion.

The data serve to show the relationship but should not be taken as generally representative since the series included many premature infants not all of whom were treated by exchange transfusion.

There is a negative correlation between the haemoglobin concentration and bilirubin concentration of cord blood in haemolytic disease of the newborn. That is to say, the more anaemic the infant, the more likely it is

to have a high bilirubin concentration. The correlation coefficient is of the order of −0.62 (Mollison and Cutbush, 1951).

The relationship between cord plasma bilirubin concentration and mortality is not as close as that between cord haemoglobin concentration and mortality. It might seem that the combination of cord haemoglobin concentration and bilirubin concentration would give a better index of severity than either taken separately. However statistical analysis (determination of the 'discriminant function') in a series studied by Mollison and Cutbush (1951) showed that consideration of the bilirubin concentration did not significantly improve the reliability of predictions based on the haemoglobin concentration alone (see Fig. 93). Nevertheless, this question deserves further study. The data suggested that the haemoglobin and bilirubin concentration considered together might give a better index even though the difference was too small to be 'significant'.

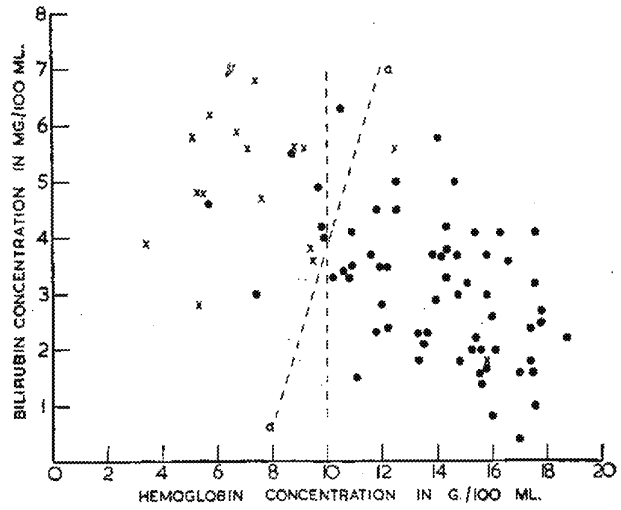

FIGURE 93. Haemoglobin concentration and plasma bilirubin concentration in the cord blood of seventy-nine infants with haemolytic disease of the newborn; there is a significant correlation between the two indices. The sloping line (a—a) represents the plane of best discrimination between a prediction of life and death, based on the estimates of haemoglobin and bilirubin considered together; this line does not differ significantly from the vertical, that is from a prediction based on haemoglobin concentration alone (from Mollison and Cutbush, 1951).

The cord plasma bilirubin concentration may be valuable index of

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severity when the infant's cord haemoglobin concentration is within normal limits (Walker and Murray, 1954). In such cases a cord level of 4 mg. per 100 ml. or more seems to be a clear indication for exchange transfusion (Walker, 1958).

Erythroblastaemia and Reticulocytosis

In infants whose cord haemoglobin concentration is well below the normal range the blood usually contains increased numbers of nucleated red cells and reticulocytes. In routine work there is little point in estimating their number since the haemoglobin concentration alone is so good an index of severity. When the cord haemoglobin concentration is within the normal range the count of nucleated red cells or of reticulocytes may be above the normal range, but in practice such counts have not proved to be a reliable guide in predicting whether a given infant will become jaundiced (personal observations).

The Presence of Haem Pigments in the Plasma

As explained in Chapter XIII a small amount of haem pigment is found in the plasma following the destruction of Rh-positive red cells by Rh antibody although the exact mechanism of the release of pigment is not yet known. It is therefore not surprising that in severe haemolytic disease of the newborn the amount of haem pigment in the plasma may exceed normal limits. Boggs and Abelson (1956) found a close correlation between severity and the amount of haem pigment in the plasma, as deduced simply from the optical density of plasma at 405-415 mμ, and they recommended that such estimates should be used as a guide to the need for exchange transfusion. Other workers have found a less satisfactory correlation.

In seven infants with severe haemolytic disease of the newborn (cord haemoglobin concentration less than 8 g./100 ml.) examined by Zetterström *et al.* (1956), five had amounts of haem pigment between 38 and 150 mg./100 ml. in the plasma. In these five, but not in the remaining two, methaemalbumin was detectable. (Since haptoglobin is absent from the plasma of most normal newborn infants methaemalbumin will be detectable whenever the level of haem pigment exceeds about 25 ml./100 ml.) In a series of infants with haemolytic disease of the newborn examined by Walker (1958) methaemalbumin was present in the plasma of seven out of eight infants with cord haemoglobin levels below 7.4 g./100 ml. It was present in sixteen out of forty-eight infants with a cord haemoglobin

concentration of between 7.4 and 14.8 g./100 ml. and in five out of thirty-two with a cord haemoglobin concentration of between 14.8 and 17.7 g./100 ml.; it was absent in all of four infants whose cord haemoglobin concentration exceeded 17.7 g./100 ml.

These findings make it clear that the presence or absence of haem pigment is not sufficiently well correlated with severity to be useful as a practical guide (although, as mentioned above, the opposite opinion was reached by Boggs and Abelson, 1956).

The Strength of the Direct Antiglobulin Test as a Guide to Severity

In most cases in which an Rh-positive infant is born to a woman whose serum contains Rh antibody the direct antiglobulin test on the infant's red cells is strongly positive. When the test is found to be weakly positive and it is known that the mother's serum contains Rh antibody there are two possible explanations: first that very little antibody has passed the placenta; in such cases the infant shows no signs whatever of haemolytic disease; second that the direct antiglobulin test has been improperly performed, and this should be suspected if the infant shows any signs whatever of haemolytic disease.

When a woman whose serum contains Rh antibody gives birth to an infant with only a weakly positive antiglobulin test there is one other possibility which should be considered; that is, that the infant is Rh-negative and the positive antiglobulin test is due to a second, immune blood group antibody in the mother's serum, for instance, anti-K.

When the infant is Rh-positive the strength of the direct antiglobulin reaction is of little value as a guide to severity, since mildly affected infants may have quite a strong reaction. This fits in with the result of experiments on injecting red cells sensitized *in vitro* (see Chapter XIII) in which it is found that red cells sensitized with an amount of Rh antibody insufficient to cause appreciable red cell destruction may nevertheless give a strongly positive antiglobulin test.

In a small series of cases referred to in the first edition of this book (Mollison, 1951, p. 382) small transfusions of Rh-positive blood was given to a few infants whose cord blood haemoglobin concentration was 15 g./100 ml. or more and whose red cells had a moderately strongly positive direct antiglobulin test. In most instances the survival of transfused red cells, estimated by the method of differential agglutination, was found to be normal although it was demonstrated by making 'extracts' of the transfused red cells from samples taken subsequently from the infant, that the cells had a positive direct antiglobulin test.

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Assessment of Severity when Cord Blood is not Available

The examination of cord blood has two particular advantages. First it is representative of the infant's blood before birth, before the confusing changes in haemoglobin concentration which follow birth have obscured the picture. Second the sample is available at the very moment of birth, so that diagnostic tests can be instituted at the earliest possible moment. In practice cord blood is not always available and the assessment must then be based partly on clinical findings and partly on the examination of blood samples taken after birth. As in normal infants, samples obtained more than a few minutes after birth have higher haemoglobin values than those of cord blood and samples of blood obtained by skin-prick have still higher values. An example of some comparisons is shown in Fig. 94. This particular infant had a cord blood haemoglobin concentration of 12.8 g./100 ml. A venous sample taken a few hours after birth had a haemoglobin concentration of 15.4 g./100 ml., and a skin-prick sample, taken simultaneously, had a haemoglobin concentration of 18 g./100 ml. Even greater changes are sometimes observed: for example, cord haemoglobin concentration, 12.2 g./100 ml.; venous sample 2 hours later, haemoglobin concentration, 18 g./100 ml.

The important point is that these changes are dependent on factors unconnected with the severity of the haemolytic process and therefore they confuse the picture.

When cord blood is not available a venous blood sample should be obtained. A haemoglobin concentration of less than 15 g./100 ml. (combined with a positive direct antiglobulin test) suggests that the infant has a haemolytic process and will probably require transfusion. But a haemoglobin concentration of 15 g./100 ml. or more does not by itself indicate that the infant is mildly affected, and the plasma bilirubin concentration should be determined.

The Plasma Bilirubin Concentration after Birth

In infants with haemolytic disease of the newborn the plasma bilirubin concentration usually increases progressively for the first 48 hours or so. There is general agreement that every effort should be made to keep the plasma bilirubin concentration below about 20 mg./100 ml. (Mollison and Cutbush, 1951), but in practice it is often difficult to predict what will happen in the next 24 hours.

Allen and Diamond (1958) have produced a chart which gives a tentative guide to the need for exchange transfusion at various ages after birth,

based solely on the level of 'indirect' bilirubin in the plasma. This is referred to below in considering the management of the individual case.

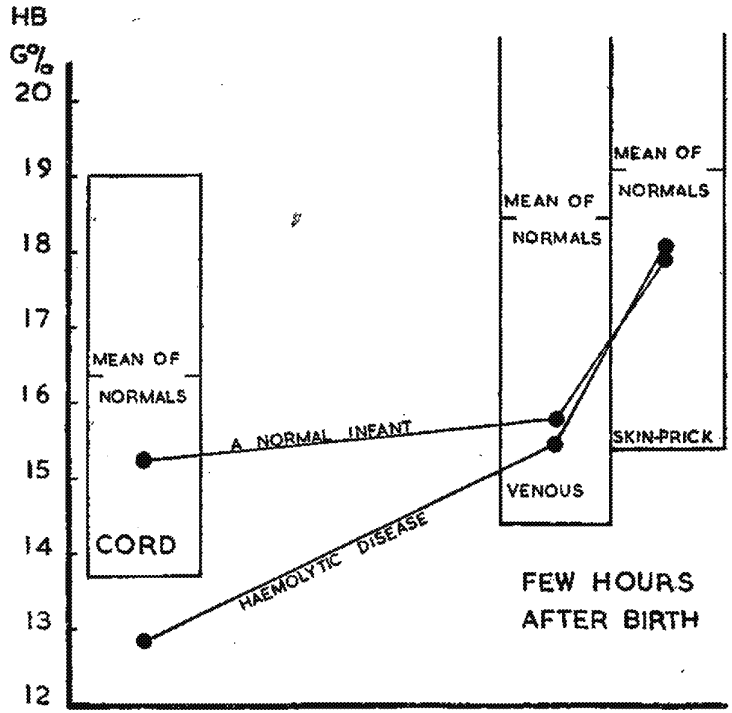

FIGURE 94. Changes in the haemoglobin concentration produced by the placental transfer of blood tend to mask mild degrees of anaemia in newborn infants. Often the haemoglobin concentration of skin-prick samples is much higher than that of venous samples and this is a second factor which may mask anaemia. In this case, an infant with haemolytic disease of the newborn has a cord haemoglobin concentration below the normal range, but after birth its haemoglobin concentration is as high as that of many normal infants (from Mollison and Cutbush, 1949a).

Changes in the Blood Picture in Untreated Infants after the First Twenty-four Hours of Life

For a period of 24 or 48 hours after birth, the output of red cells from the bone marrow, as judged by the count of nucleated red cells and reticulocytes, diminishes only to a small extent, and during the period there is no considerable change in the infant's haemoglobin concentration. At some time during the first week of life the output falls sharply as it does in

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normal infants. Since the rate of red cell destruction is greater than normal, the haemoglobin concentration starts to fall more rapidly than it does in a normal infant.

In infants who are more than a few days old, the number of immature red cells in the blood stream is usually small unless the haemoglobin concentration is less than about 11 g./100 ml. That is to say, the reticulo-

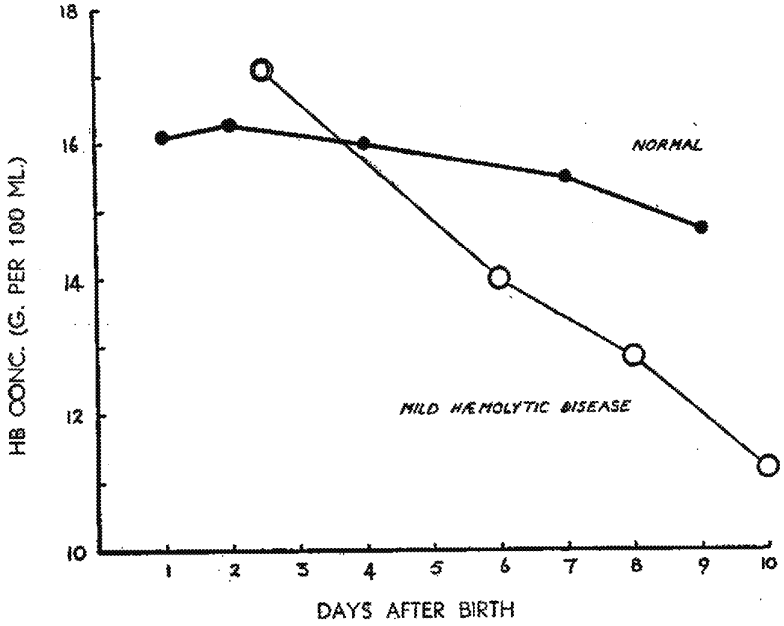

FIGURE 95. Rate of fall of haemoglobin concentration in an infant mildly affected with haemolytic disease of the newborn, contrasted with the changes in a normal infant.

cyte count depends primarily on the haemoglobin concentration and only indirectly on the rate of blood destruction.

It follows that in infants more than 3 or 4 days old, the only sign of a haemolytic process may be an unduly rapid fall in haemoglobin concentration. This is demonstrated in Fig. 95. This infant when first seen at the age of 3 days, had a haemoglobin concentration within the normal range and a reticulocyte count of less than 3 per cent. Nevertheless, serial estimates of haemoglobin concentration demonstrated beyond doubt that the rate of red cell destruction was increased.

Haemoglobin Changes in very Mild Cases

In very mild cases of haemolytic disease of the newborn, that is, infants with a positive direct Coombs reaction but a cord haemoglobin concentra-

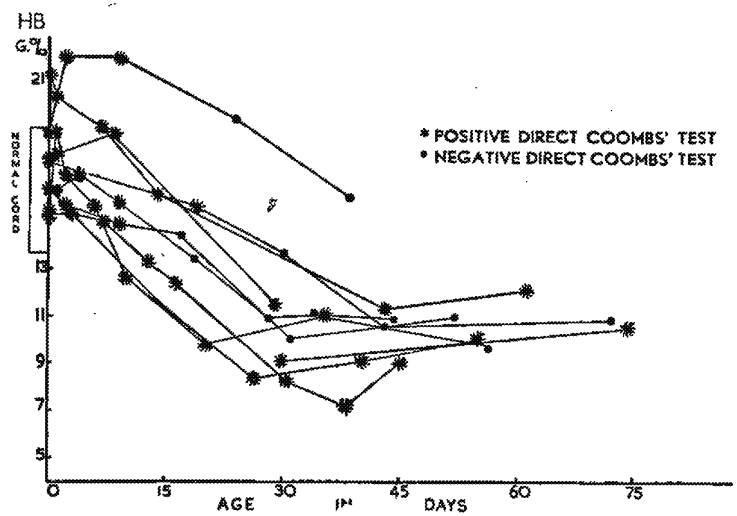

FIGURE 96. The changes in haemoglobin concentration, during the first 2 months of life, in ten infants very mildly affected with haemolytic disease of the newborn. The cord blood of seven of the infants was examined and the haemoglobin concentrations ranged from 15.1 to 18.7 g./100 ml. (from Mollison and Cutbush, 1949a).

tion exceeding 15 g./100 ml., the haemoglobin concentration may fall just a little more rapidly than normal and reach a slightly lower level than is reached by most normal infants. This is illustrated in Fig. 96.

Clinical Manifestations of Haemolytic Disease of the Newborn

Stillbirths

According to Walker and Murray (1956) the stillbirth rate in pregnant women whose serum contains Rh antibody is 12 per cent (based on a study of over 1300 Rh-immunized women). The relationship between the mother's previous history and the chance of stillbirths has been discussed above.

The stage of pregnancy at which stillbirths occur is discussed below in considering the question of premature induction of labour as a form of preventive treatment.

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Circulatory Changes accompanying Severe Anaemia

Infants whose cord haemoglobin concentration is about 8 g./100 ml. or less usually show circulatory changes similar to those seen in adults with severe chronic anaemia. It is an old observation that such infants may have large hearts (Schridde, 1910) and it has more recently been demonstrated that they also have a raised venous pressure (Mollison and Cutbush, 1949a).

The condition of such infants can usually be diagnosed at the moment of birth. The infant is often limp and pale. It may be slow to breathe and then develop irregular gasping respirations. Further examination is almost certain to reveal a grossly enlarged liver and spleen and some degree of oedema. The treatment of infants displaying these signs is considered later.

Kernicterus

As already mentioned, jaundice developing within the first 24 hours is very common in haemolytic disease of the newborn. In severely affected infants the jaundice rapidly becomes intense; the peak concentration may be reached as early as 36 hours in mature infants or may not be reached until the fifth or sixth day in a premature infant.

Deeply jaundiced infants often exhibit signs of damage to the central nervous system; these signs rarely develop before the age of 36 hours. At any time after this the infant, which has previously behaved normally, becomes increasingly lethargic and reluctant to feed and may exhibit opisthotonos. It may be noticed to be rolling its eyes and may develop a high-pitched cry. Its arms may be extended and the wrists fully pronated. At this stage in about 70 per cent of cases the respirations become irregular, bloody froth issues from the nose and the infant becomes cold and dies within 12 hours. In the remaining 30 per cent of cases these respiratory disturbances do not occur and the infant survives for months or years. Gerrard (1952) has pointed out that the later the onset of signs of cerebral damage the more likely is the child to live; thus of seven infants who developed signs on the second day of life, only one survived, whereas of ten infants who developed signs on the fifth day of life, seven survived.

Infants who develop only mild signs of cerebral damage in the first week of life may appear to recover completely, but sooner or later signs of permanent cerebral damage become apparent. Thus Armitage and Mollison (1953) reviewed the subsequent course of twenty infants who developed signs of kernicterus but survived for at least 6 months. At the age of 1 month eleven infants were obviously backward; of the remaining nine, seven were recognized to be backward at some time between the fifth and

eleventh month of life; the remaining two infants were not recognized as abnormal until some time between the first and third year of life. When high-frequency deafness is the only residual damage the infant is likely to appear completely normal until it is 2–3 years old.

TABLE LIV

RELATION BETWEEN MAXIMUM BILIRUBIN CONCENTRATION IN THE PLASMA AND KERNICTERUS (FROM MOLLISON AND CUTBUSH, 1954)

Maximum bilirubin concentration mg./100 ml.	*Total number of cases*	*Number with kernicterus*
30–40	11	8
25–29	12	4
19–24	13	1
10–18	24	0

The close relationship between deep jaundice and the onset of kernicterus is shown by the figures in Table LIV.

It has been shown that the brain contains lipid which takes up unconjugated bilirubin ('indirectly reacting' pigment) but does not take up conjugated bilirubin ('directly reacting' pigment). In kernicterus, the yellow staining material in the brain has been shown to be unconjugated bilirubin (Claireaux *et al.*, 1953).

TREATMENT OF HAEMOLYTIC DISEASE OF THE NEWBORN

Antenatal Measures

The Administration of Steroids

Because the administration of corticosteroids is so effective in controlling auto-immune haemolytic anaemia, it might seem that their administration to an Rh-immunized pregnant woman should mitigate the severity of the haemolytic disease in her foetus. In fact even when corticosteroids are injected for several months, the titre of the Rh antibody is usually unchanged and the infant is not protected against its effects. Thus stillbirths may occur in such cases even when cortisone has been administered for 3 months or more (Schmidt *et al.*, 1953; Hunter, 1944; Bowley, 1955). Walker (1958) gave large doses from the 14th week onwards to four women who had previous stillbirths from haemolytic disease but in all four, stillbirth occurred before the 28th week of pregnancy.

It appears that the administration of therapeutic doses of cortisone not

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only fails to reduce the titre of existing antibodies but may also fail to suppress secondary responses to antigens. The failure of cortisone to interfere with the effects of iso-antibodies on red cells has been discussed on p. 475.

Rh Haptene

The idea of Rh haptene, a theoretical substance capable of combining with or neutralizing Rh antibody, without stimulating the formation of new antibody is of course very attractive; and attempts have been made to isolate such a substance by making lipid extracts from human red cells (Calvin *et al.*, 1946; Carter, 1949). There is reason to believe that even if such a substance could be extracted from red cells very large quantities would be required for treatment. For example, to absorb a potent incomplete antibody from 1 volume of serum at least an equal volume of Rh-positive red cells is required. Thus an initial dose of haptene from about 2.5 litres of red cells would be needed to suppress a potent antibody and a similar dose would presumably be needed at intervals of 1 or 2 weeks thereafter through pregnancy. The red cells from at least 60 litres of blood would be needed during a pregnancy and this assumes that the extraction rate of haptene from red cells would be 100 per cent.

In fact it seems that the lipid substance extracted from red cells does not have Rh specificity (Murray *et al.*, 1950) and most workers have reluctantly concluded that it is of no value in treatment.

Premature Induction of Labour

Premature induction of labour has been advocated on two different grounds; firstly to lower the incidence of stillbirths and secondly to diminish the severity of haemolytic disease in liveborn infants.

The problem of preventing stillbirths will be considered first. The period of gestation at which stillbirths occur is shown in Table LV, which summarizes data published by Allen (1957). As the table shows, in Allen's series 56 per cent of all stillbirths from haemolytic disease of the newborn occurred in the first 35 weeks of pregnancy. This is rather higher than the figure of 40 per cent reported by Walker *et al.* (1957b). The figures for the proportion of stillbirths occurring up to the thirty-seventh week in the two series were 71 per cent (Allen, 1957) and 58 per cent (Walker *et al.*, 1957b).

The conclusion is that premature induction of labour carried out 1 month before term cannot possibly save more than half the infants who are at risk from stillbirth.

Only one trial has been carried out in which patients were selected at random for premature induction of labour (3 to 5 weeks before term) or for spontaneous delivery. In this trial among women delivered prematurely the incidence of stillbirths was about 8 per cent compared with 13 per cent in women left to go into labour spontaneously (Armitage and Mollison, 1953).

Before discussing the implications of these results, the effects of premature delivery on the severity of haemolytic disease of the newborn must be considered. It might seem that if premature induction of labour prevents some stillbirths it is probable that haemolytic disease must be less

TABLE LV

DATA RELATING TO STILLBIRTHS FROM HAEMOLYTIC DISEASE OF THE NEWBORN, INCLUDING THE PERIOD OF GESTATION AT WHICH STILLBIRTHS OCCUR; FROM ALLEN (1957)

	No previous stillbirths Anti-Rh titre:		*Previous stillbirths Anti-Rh titre:*		
	Less than 64	64 *or more*	*Less than* 64	64 *or more*	
Total number of women	174	186	25	59	
Total number having stillbirths	14 (8%)	54 (29%)	9 (36%)	46 (78%)	
Number with stillbirths					*Total*
Before thirty-third week	3	19	6	22	50 (41%)
Thirty-third to thirty-fifth week	4	10	0	5	19 (15%)
Thirty-fifth to thirty-seventh week	0	3	2	13	18 (15%)
After thirty-seventh week	7	22	1	6	36 (29%)
					123

severe at the thirty-fifth week of pregnancy than at the fortieth, but this is not necessarily so. An alternative explanation would be that haemolytic disease does not in fact increase in severity during the last few weeks of pregnancy, but that the conditions for intra-uterine survival become less favourable, at least for severely affected anaemic infants.

The evidence that haemolytic disease does not increase in severity during the last few weeks of pregnancy comes from the controlled trials of treatment referred to above. In these trials cases were chosen for premature induction of labour or spontaneous delivery by a system of random numbers, so that two strictly comparable groups of infants were available.

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The infants born at term did not have lower haemoglobin concentrations than those born at about 36 weeks. Thus, among the infants born at term seventy out of 100 (70 per cent) had cord haemoglobin concentrations of 11 gm./100 ml. or more, compared with fifty-one out of seventy-six (67 per cent) of the infants born at 36 weeks. (These figures are from unpublished data of Armitage and Mollison, 1953; stillborn infants were counted as having cord haemoglobin concentrations below 11 g./100 ml.).

It may be then that it is only when the disease is very severe that the infant's chance of survival diminishes with the period of gestation. There must of course be some exceptions because there are cases in which the mother's antibody titre increases during the last few weeks of pregnancy and in these the haemolytic process presumably becomes more severe.

One must next consider the effect of prematurity on the chance of survival of the infant born alive with haemolytic disease of the newborn. In the controlled trials already referred to, the diminished number of stillbirths in the 'prematurely delivered' group was offset by an increased number of neo-natal deaths, mainly from kernicterus (Armitage and Mollison, 1953). In these trials some infants were treated by simple transfusion and some by a single exchange, so that the results do not show what would happen if exchange transfusion were used in all cases and repeated when necessary. Although in the best hands kernicterus can be almost completely prevented by repeated exchange transfusion, there is no doubt that repeated exchange transfusions are difficult, and even dangerous, in premature infants; thus, taking into account the fact that not all infants can be treated at special centres, the infant with haemolytic disease must have a distinctly higher mortality when it is born prematurely.

Therefore, most clinicians have concluded that premature induction of labour should be carried out only when it is anticipated that if the woman is left till term her infant is likely to die *in utero*. As discussed previously, predictions have to be based firstly on the mother's previous history and secondly on her antibody titre. Table LV illustrates well the claim of Freisleben (1956) that given the titre the prognosis is related to the history; and that given the history the prognosis is related to the titre.

The risk of stillbirth according to the history alone is shown in Table LVI. As the table shows the risk of stillbirth is 55 per cent or more in a mother who has had a previous stillbirth or a very severely affected infant, and in such cases the history alone is a sufficient indication for premature induction of labour in a subsequent pregnancy.

Walker *et al.* (1957a) reported the results of a trial in which cases had been selected solely on history. The series consisted of 102 women who were seen early in pregnancy, with a history of previous stillbirths attributable to haemolytic disease of the newborn. Twenty-seven of the women had 'heterozygous' husbands and premature induction of labour was not carried out in these; of the twenty-seven infants fourteen were were Rh-negative and thirteen Rh-positive, of whom nine were stillborn. Fifteen more women were not induced because on further questioning the causes of the previous stillbirth seemed doubtful (only three of these had another stillbirth).

Of the remaining sixty women, twenty-three had a stillbirth before the thirty-fifth week of pregnancy; twelve more had stillbirths at about the thirty-fifth week of pregnancy; in the remaining twenty-five women premature induction of labour was carried at about the thirty-fifth week of pregnancy; two of the women had stillbirths, leaving twenty-three liveborn infants severely affected with haemolytic disease, of which eighteen recovered with treatment.

TABLE LVI

THE RISK THAT A PREGNANCY WITH AN RH-POSITIVE FOETUS IN AN RH-IMMUNIZED WOMAN WILL END IN A STILLBIRTH, RELATED TO THE PREVIOUS HISTORY
Walker *et al.*, 1957b)

	Risk of stillbirth in present pregnancy (per cent)
No previously affected infant	7
Previous mildly affected infant	2
Previous moderately or severely affected infant	20
Previous very severely affected infant (Cord Hb less than 9 g./100 ml.)	55
One previous stillbirth	70
More than one previous stillbirth	80

Thus, if all the women with a previous stillbirth were included the number of surviving infants was fifty out of 102, but if only those with a homozygous husband and a history of at least one stillbirth definitely due to haemolytic disease of the newborn were included the recovery rate was only eighteen out of sixty, or 30 per cent. Nevertheless, in view of the fact that many of the women had had two or more previous stillbirths due to haemolytic disease, the recovery rate of 30 per cent may have been twice that which would have been observed had all the mothers been left to go into labour spontaneously.

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Treatment of the Infant by Transfusion

Rationale

Deaths from haemolytic disease of the newborn tend to occur at three stages: first, within 12 or 24 hours of birth. The immediate cause of these deaths is cardiac failure, which in turn, is a consequence of prolonged anoxaemia. The administration of oxygen and venesection are valuable immediate measures. Although the infant urgently requires more red cells, simple transfusion is very dangerous because of the cardiac failure. Exchange transfusion is far safer because red cells can be introduced without increasing the infant's total blood volume. In fact, as described later, it is important to remove more blood than is injected, so as to lower the venous pressure.

The second stage at which deaths occur is at about 36 to 96 hours. The association of these deaths with deep jaundice has been discussed. It is firmly established that the development of deep jaundice and kernicterus is far less likely if exchange transfusion is carried out within a few hours of birth. In an exchange transfusion, most of the infant's red cells are removed and replaced by Rh-negative red cells which are not subject to rapid destruction. In a severe case of haemolytic disease of the newborn the life-span of the infant's red cells may be as short as 2 or 3 days (Mollison, 1943b). In an infant with a haemoglobin concentration of 6 g./100 ml. this would result in the production of approximately 500 mg. of bilirubin in 2 or 3 days. (This assumes that each gramme of haemoglobin is converted to approximately 40 mg. of bilirubin – see With, 1949.) By removing the majority of the infant's red cells the amount of bilirubin to be excreted is enormously reduced.

In an exchange transfusion a certain amount of bilirubin is also removed directly, in the plasma. In the first exchange transfusion carried out within a few hours of birth the plasma bilirubin concentration is not likely to be more than about 10 mg./100 ml., so that if 300 ml. of plasma are removed the total amount of bilirubin removed from the plasma will be less than 30 mg. which is very small compared with the amount withdrawn as potential bilirubin in red cells.

Different considerations apply to second and later exchange transfusions. Here the number of Rh-positive red cells removed is likely to be small – probably one-tenth or less of the number removed at the first exchange – whereas the amount of plasma bilirubin removed may be considerable. For example, suppose the number of Rh-positive cells at the time of a second exchange is equivalent to 0.5 g. Hb/100 ml. and the plasma bilirubin concentration is 18 mg./100 ml., and suppose that virtually all

the red cells are removed, together with 300 ml. of plasma, then the amount of 'potential bilirubin' will be of the order of 50 mg. and the amount of plasma bilirubin will be 54 mg. Inevitably, the effects of a second exchange are not likely to affect the course of haemolytic disease to the same extent as a first exchange.

When the plasma bilirubin concentration is appreciable (more than 10 mg./100 ml.) so that the amount of bilirubin removed in the plasma is an important consideration, one should take into account the 'rebound phenomenon'. By this is meant the rapid rise in plasma bilirubin concentration which occurs if an exchange transfusion is temporarily interrupted. For example, Brown, Zuelzer and Robinson (1957) measured bilirubin changes during and after an exchange transfusion and found that during pauses in the exchange the plasma bilirubin rose appreciably and at the end was sometimes as high as 40-60 per cent of the initial level. The 'rebound' was attributed to an extravascular 'reservoir' of bilirubin. They pointed out that whenever the removal of plasma bilirubin is the main object of an exchange it is necessary to exchange volumes of blood greater than those required for a satisfactory red cell exchange and that it is also advantageous to perform the exchange slowly.

Similarly, Forfar *et al.* (1958) calculated that during a slow exchange transfusion at least 45 per cent of the bilirubin removed came from the tissues; they suggested that the optimal rate of exchange was 0·8 ml. per lb. body weight per minute and that the optimal amount was about 90 ml. per lb. Valaes (1959) considered that the volume of blood exchanged was all-important and found that varying the time taken to exchange one 'blood volume' from 12 to 36 minutes made no difference to the amount of bilirubin removed (these rates correspond approximately to 3 ml. and 1 ml. per lb. per minute respectively). Valaes calculated that the total intravascular mass of bilirubin was one-third to one-quarter of the total bilirubin available for exchange and that of this total, one-third to one-half was removed by an exchange of 100 ml. per lb.

The conclusion is that when the infant's plasma bilirubin concentration is relatively high at the beginning of exchange transfusion the volume of blood exchanged should be about 90 ml. per lb. (200 ml. per kg.). Most clinicians would agree that an exchange transfusion of this volume should not be completed in less than about 90 minutes.

Evidence of the Value of Exchange Transfusion

Allen, Diamond and Vaughan (1950) reported that in a series of 109 affected infants treated by exchange transfusions there was only one case

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of kernicterus; they considered that kernicterus could be practically eliminated as a complication of haemolytic disease of the newborn.

In controlled trials of treatment carried out in Britain in 1950–52 the value of exchange transfusion was fully confirmed (Mollison and Walker, 1952). However, it was shown that the combination of exchange transfusion with premature induction of labour as a method of treatment was scarcely superior to simple transfusion in mature infants. This point, illustrated in Fig. 97, explains the confusion that at first existed about the merits of exchange transfusion. (It should perhaps be added that in this

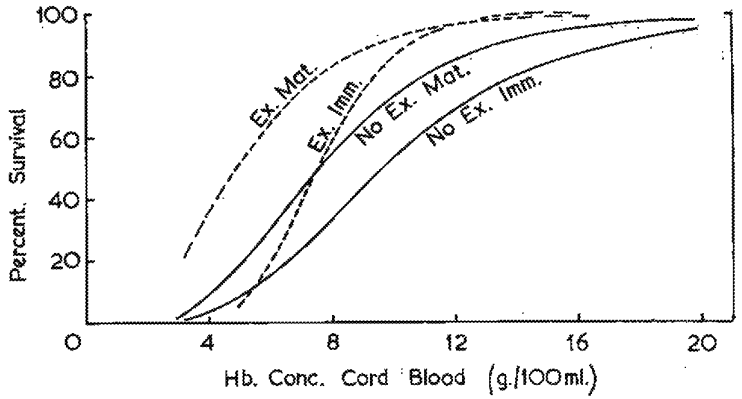

FIGURE 97. Curves relating the haemoglobin concentration of cord blood to the chance of survival, with different forms of treatment.
'Ex.' = exchange transfusion.
'No Ex.' = simple transfusion or no transfusion.
'Mat.' = mature, that is, born at term or within 10 days of term.
'Imm.' = immature, that is, born 35–18 days before term.
(From Armitage and Mollison, 1953.)

series only a single exchange transfusion, often of only 50 ml. per pound body weight or even less, was carried out.)

As Fig. 97 shows, at all levels of cord haemoglobin concentration the infants treated by exchange transfusion are more likely to survive than the infants treated by simple transfusion.

The very unfavourable effect of immaturity is illustrated in Fig. 97. Immaturity exerts an unfavourable effect in two ways. Firstly, the infant is less able to tolerate exchange transfusion. Secondly, it is far more liable to develop kernicterus. As described in Chapter XVI, the plasma bilirubin concentration increases to a much greater extent during the first few days of life in normal premature infants than in normal mature infants. This

may be taken as evidence that the premature infant has even less ability to excrete bilirubin than the mature infant and thus even a mild haemolytic process produces a deep jaundice.

Allen *et al.* (1950) mentioned that in the premature infant jaundice might be difficult to control. They described the case of an infant born at the thirtieth week of gestation who developed kernicterus despite two exchange transfusions. Similarly Walker and Neligan (1955) reported that kernicterus developed in three out of twenty-two premature infants with haemolytic disease of the newborn treated by early adequate exchange transfusion (at least 60 ml. per lb. body weight). By contrast kernicterus occurred in only two out of 196 mature infants treated similarly.

Evidence from the treatment of twins. There is evidence that when binovular Rh-positive twins are carried by a woman whose serum contains anti-Rh the severity of the haemolytic process may be very different in the two infants. Thus one may die *in utero* and the other be born alive and only mildly affected.

It might appear that uniovular twins would necessarily be affected to the same extent, but it must be remembered that at birth their weights may differ appreciably and that this may well affect the post-natal changes in plasma bilirubin concentration. Thus the outcome of the disease in uniovular twins could be different. Nevertheless if uniovular twins are treated differently and the smaller twin does better, it is reasonable to suppose that its treatment has been superior.

At the time when exchange transfusion was under trial, one of a pair of uniovular twins was treated by exchange and the other (heavier) twin by simple transfusion. Fig. 98 shows that in the twin treated by exchange transfusion the bilirubin concentration rose to a far smaller extent than in the twin treated by simple transfusion. These observations alone supply highly suggestive evidence of the value of exchange transfusion.

The use of Rh-positive blood for exchange transfusion. In a severe case of haemolytic disease of the newborn, if Rh-positive blood is transfused within a week of birth, all the transfused erythrocytes are removed from the circulation within a few days (Mollison, 1943b). The transfusion of Rh-positive blood produces only a transient relief of anaemia and may result in increased jaundice.

The use of Rh-positive blood for exchange transfusion was for a time advocated on the grounds that it might help to 'mop-up' free Rh antibody and thus bring the haemolytic process to an end more rapidly. There is

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little doubt that in practice a single exchange transfusion with Rh-positive blood is inferior to a single exchange transfusion with Rh-negative blood (see Mollison, 1956, pp. 481–2) and so far as the author knows the use of Rh-positive blood has been abandoned.

'Late' anaemia. Infants not treated by exchange transfusion may become profoundly anaemic between about 7 and 21 days after birth. In practice these cases are either ones which have gone undetected at birth, or are infants who have been judged at birth to have been too mildly affected to require exchange transfusion and have either not been treated at all or given only a small simple transfusion.

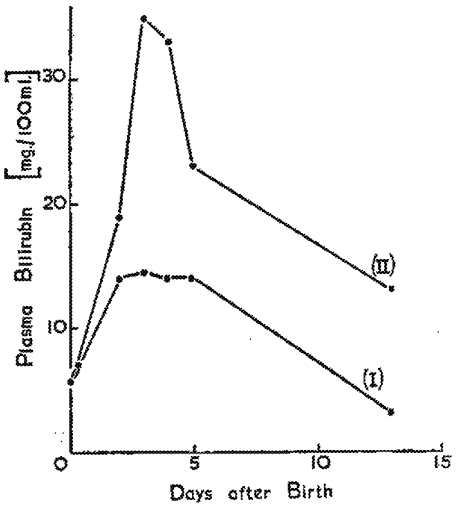

FIGURE 98. Changes in plasma bilirubin concentration during the first 2 weeks of life in uniovular twins with haemolytic disease of the newborn. Twin I was treated by exchange transfusion 5 hours after birth; twin II was treated by simple transfusion only (from Mollison, 1954b).

Of infants with a positive direct Coombs test and a cord haemoglobin concentration of 15 g. or more/100 ml. who are not treated by exchange transfusion about one in twenty become sufficiently anaemic 2 or 3 weeks after birth to require transfusion. In the treatment of late anaemia, the simple transfusion of Rh-negative blood is all that is required.

Infants treated by exchange transfusion also occasionally become sufficiently anaemic during the subsequent few weeks to require transfusion. It may therefore be helpful to describe briefly the changes in the blood picture which follow exchange transfusion.

Within a few days of exchange transfusion of Rh-negative blood, no Rh-positive cells remain in the circulation. Indeed if a large simple transfusion of Rh-negative blood is given to an infant with haemolytic disease, Rh-positive cells may completely disappear from the blood (Mollison, 1943b). This is due mainly to the fact that in newborn infants the production of new red cells remains very low as long as the haemoglobin concentration is above about 11 g./100 ml. However, in infants with haemolytic disease the persistence of Rh antibody undoubtedly plays some part in preventing the appearance of Rh-positive cells in the circulation during the first few weeks of life. Several observers have pointed out that during the first 2 months the haemoglobin concentration may fall to levels well below normal and that this is probably due to the persistence of Rh antibody. For example Ramaekers (1954) found that the Rh antibody could be detected for about 8 weeks in (Rh-positive) infants with haemolytic disease, following exchange transfusion with Rh-negative blood; he observed that anaemia did not develop in the absence of demonstrable persisting antibody. In a case described by Gasser (1955) an infant which had been treated by exchange transfusion was found at the age of 7 weeks to have a haemoglobin concentration of 30 per cent. There were no reticulocytes in the peripheral blood but the marrow contained numerous erythroblasts and reticulocytes. The serum contained a potent anti-Rh which was presumably destroying any Rh-positive cells which escaped into the circulation.

Giblett *et al.* (1956) described an infant with haemolytic disease of the newborn, treated by transfusion with Rh-negative blood, which had only 0.1 per cent reticulocytes in the peripheral blood despite a haemoglobin concentration of 6 g./100 ml. Injected Rh-positive cells, labelled with ^{51}Cr, were eliminated within $3\frac{1}{2}$ hours. Later, as the titre of Rh antibody fell, the number of reticulocytes rose. Fig. 102 (see p. 644) shows the changes in blood picture which occurred in three infants with haemolytic disease of the newborn during the first 2 or 3 months of life.

Because, following exchange transfusion, the destruction of Rh-positive cells in the infant's circulation may persist for many weeks it is advisable to supply the infant with sufficient Rh-negative cells to enable it to maintain a satisfactory haemoglobin level for a period of about 2 months. This is one reason for advocating the use of concentrated red cell suspensions for exchange transfusion, as described below.

One point to emphasize is that if an infant with haemolytic disease of

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the newborn requires transfusion at any time during the first 3 months of life Rh-negative blood should be used. Rapid destruction of Rh-positive cells has been described at the age of 6 weeks (Mollison, 1943b) and Rh antibody may persist in the circulation for 3 months.

In an infant who has been transfused with Rh-negative blood the fact that the direct antiglobulin test becomes negative must not be used as

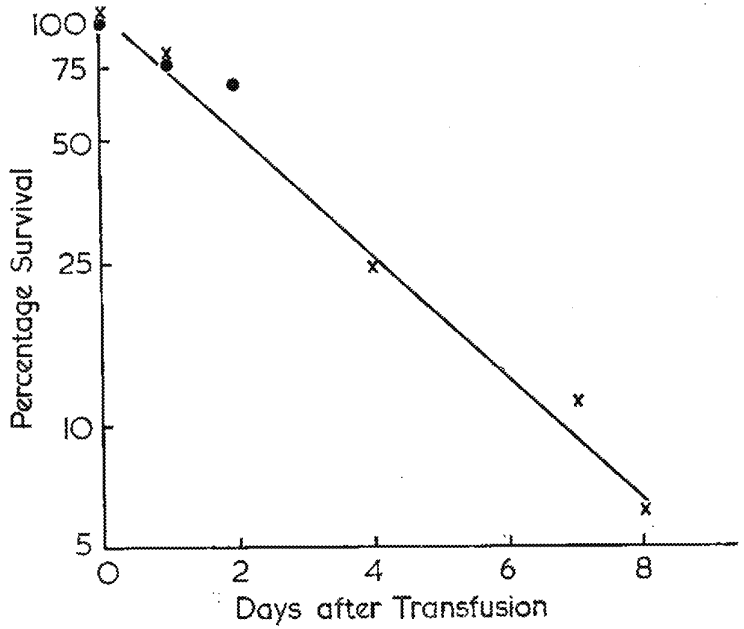

FIGURE 99. Survival of Rh-positive red cells after transfusion to two infants with haemolytic disease of the newborn. One infant, D (●), was 15 days old at the time of transfusion; as described in the text it had previously been transfused with Rh-negative cells and, before the Rh-positive transfusion, had a negative direct antiglobulin reaction, although the plasma contained 'free' Rh antibody. The second infant (×) was 9 days old at the time of transfusion (see Case XI, Mollison, 1943b). In both these infants the mean cell life was approximately three days.

evidence that the haemolytic process is at an end, since it may mean only that there are no Rh-positive red cells in the circulation. The following case illustrates the point:

Infant D. was a twin, which weighed 3 lb. at birth. The diagnosis of haemolytic disease of the newborn was not made until the infant was 48 hours old. On the third day of life a large transfusion of Rh-negative blood was given. On the fifteenth day of life the infant's haemoglobin concentration had fallen to 9 g./100

ml.; the direct Coombs reaction was negative. A transfusion of 25 ml. of Rh-positive blood was given; on the following day the direct Coombs reaction was noted to be strongly positive, and the transfused red cells were rapidly eliminated (see Fig. 99). Examination of a sample of plasma taken from the infant before transfusion revealed the presence of free, incomplete anti-Rh to a titre of 128.

The Survival of Rh-negative Red Cells

In general, Rh-negative erythrocytes survive as well in the circulation of infants with haemolytic disease as do normal adult erythrocytes trans-

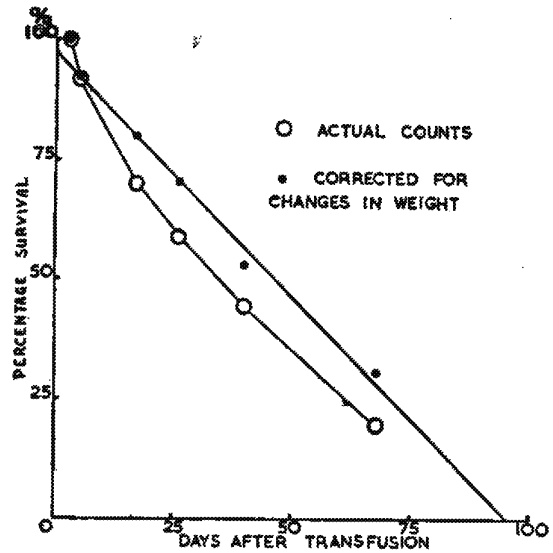

FIGURE 100. Survival of Rh-negative cells in an infant with haemolytic disease of the newborn. It may be assumed that blood volume increases in proportion to body weight and that this makes the slope curvilinear; when a correction for body weight is applied the slope becomes linear.

fused to 'normal' infants, i.e. infants not affected with haemolytic disease of the newborn (Mollison, 1943b). If the estimates of the number of cells surviving at different intervals are plotted against time, the slope of elimination usually shows some initial curvature. Pickles (1949) showed that this was due simply to the fact that the infant gained weight during the period of observation and presumably increased its blood volume. If it was assumed that blood volume bore a constant relation to weight, corrections could be applied, and when this was done the slope of elimination became linear. This is illustrated in Figs. 100 and 101.

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It will be observed that some of the slopes of elimination in the figures are initially convex; these were in infants whose haemoglobin concentration after transfusion exceeded 18 g./100 ml. The convexities are due to the shrinking of blood volume in relation to body weight, as the haemoglobin concentration falls.

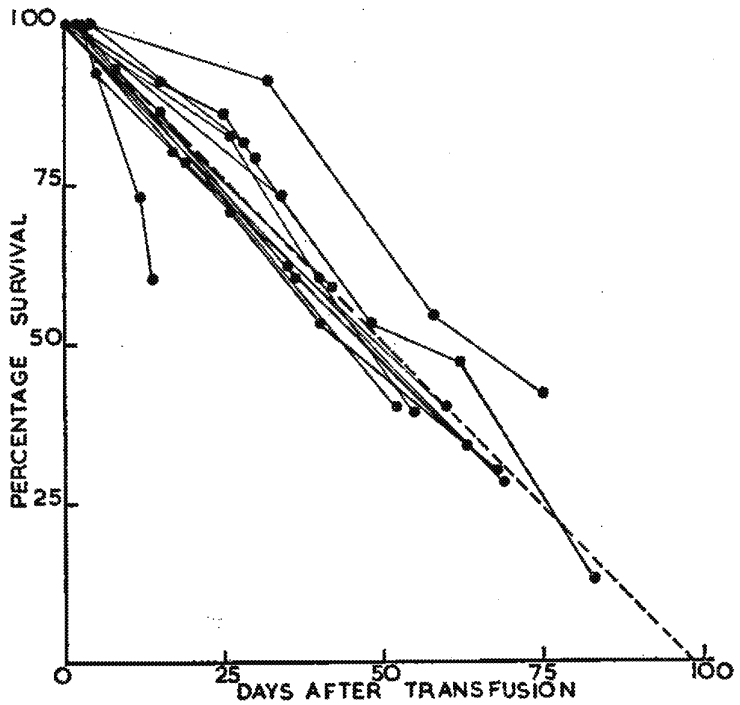

FIGURE 101. Survival of Rh-negative red cells after exchange transfusion in thirteen infants affected with haemolytic disease of the newborn. All the estimates have been corrected for changes in body weight. In all but one case the rate of elimination of transfused cells is about 1 per cent a day, or less; the anomalous case is discussed in the text. The initial convexity of some of the curves is due to transient plethora; as plethora diminishes, blood volume shrinks in relation to body weight. The effect is thus the converse of that illustrated in Fig. 100.

Active destruction of Rh-negative red cells. In one of the cases shown in Fig. 101, the rate of destruction significantly exceeded 1 per cent of the transfused cells per day. A similar case was described by the author (1943b) and these two anomalous cases will be briefly considered, together with one other published case.

In the case reported in 1943, Rh-negative cells transfused to one severely affected infant were eliminated very rapidly, the concentration falling from 1,000,000 cells per c.mm. to 300,000 per c.mm. during the week following transfusion. This rapid destruction could not be explained and it was concluded that perhaps in the more violent cases of haemolytic disease of the newborn there might be some non-specific destruction of cells.

In the anomalous case shown in Fig. 101 the destruction was not so rapid, the concentration of transfused Rh-negative cells falling from 4,000,000 per c.mm. 3 days after transfusion to 2,400,000 11 days later. Nevertheless it was significantly greater than 1 per cent a day. The possibility was considered that the donor might belong to the type 'D^{u}', but this was not the case. A sample of blood taken from the infant 11 days after transfusion consisted almost entirely of transfused cells; these could not be sensitized to an antiglobulin serum by incubation with a potent incomplete anti-Rh serum.

A possibility which ought to have been considered was that the destruction of the Rh-negative cells was due to a second blood group antibody acquired by the infant from its mother. However, even if such an antibody had been present it would probably not have been detected for at that time (1943) the antiglobulin had not been introduced.

Giblett *et al.* (1956) studied an infant which also appeared to destroy Rh-negative red cells, judged by the rapid fall in haemoglobin concentration after the transfusion of Rh-negative blood. They discussed the possibility that the destruction might have been due to the action of a second blood group antibody which they had failed to detect.

Changes in the Blood Picture following Exchange Transfusion

Red cells. The 'efficiency' of exchange transfusion is considered below, together with the technique of the operation. Following exchange transfusion of the order of 60 ml. per lb. body weight there will be not more than 500,000 Rh-positive cells per c.mm. in the infant's blood; the count of Rh-negative cells will depend mainly upon the red cell count of the donor blood. As already mentioned, in severely affected infants the output of new red cells continues for some days after transfusion and this is probably the main reason why some infants remain jaundiced despite exchange tranfusion. Nevertheless in all infants the production of new red cells greatly diminishes within a few days of birth provided that the total haemoglobin concentration remains above about 11 g./100 ml.

Platelets. Immediately after an exchange transfusion the platelet count is reduced, from a pre-transfusion value of approximately 300,000 per c.mm. to about 100,000 per c.mm. and returns to a value of 200,000 or more within 2 or 3 days (unpublished observations). Desforges and

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O'Connell (1955) found that platelets fell from 150,000 before exchange transfusion to 75,000 at the end of the exchange; normal values were regained in 1 week.

Jackson *et al.* (1956) observed a definite difference when using 1-day-old blood as opposed to 5-day-old blood. Thus when blood stored for 18-24 hours was used for exchange transfusion there was no immediate thrombocytopenia although the platelet count fell after exchange transfusion had reached a low level after 3-4 days. With 5-day-old blood, on the other hand, thrombocytopenia was noted immediately after exchange transfusion.

From experimental work in animals it seems likely that the fall in platelet count could be prevented by using whole fresh blood for exchange transfusion. Thus in rats, Matter *et al.* (1960) showed that if exchange transfusion equivalent to two blood volumes was carried out with virtually platelet-free blood, the platelet count at the end was about 15 per cent of the initial value. There was no rise in platelet count for about 3 days after exchange transfusion, indicating the absence of a reserve of platelets. If instead the exchange was carried out with fresh blood (taken into siliconized vessels) there was no fall in platelet count.

Bilirubin. During exchange transfusions of approximately 500 ml., the plasma bilirubin concentration falls to about 65 per cent of its initial value (Ramaekers, 1954). See also the discussion above of the 'rebound' phenomenon.

Management of the Individual Case

Diagnosis of Haemolytic Disease of the Newborn

If the mother's serum has been examined for anti-Rh at about the thirty-fifth week of pregnancy and found to contain none it is very unlikely that her infant will be affected with haemolytic disease of the newborn (due to anti-Rh).

Therefore, many clinicians are content to rely upon ante-natal serological tests and to omit the routine testing of cord blood samples. The author's experience is that very few cases indeed requiring treatment will be missed by relying on antenatal serological tests and that, provided the mother is going to have her infant in hospital where a constant watch can be kept for early signs of haemolytic disease, the routine testing of cord blood sample is unnecessary.

Dr W. Walker (personal communication) considers that for infants not born in hospital (that is, 50 per cent of all infants in Britain) a direct antiglobulin test on cord blood is desirable in all cases in which the mother is known to be Rh-negative or in which her Rh group is not known. Dr Walker adds that of all cases of haemolytic disease of the newborn detected in Durham and Northumberland in the past few years, 6 per cent were found by routine cord blood tests.

In all cases in which the mother's serum is known to contain Rh antibody and in all cases in which the mother's Rh group has not been determined antenatally, cord blood should be obtained from the infant at the time of birth.

As soon as the infant is born the cord should be clamped. A sample is then taken from the umbilical vein on the maternal side of the clamp, using a dry sterile syringe, and promptly mixed with a (dry) anticoagulant.

The direct antiglobulin test should first be performed. If the test is negative and control cells tested at the same time are found to be positive, and if the mother's serum contains anti-Rh, the infant is almost certainly Rh-negative. This should be verified by testing the cells with a potent anti-Rh serum. It is true that in Rh-positive infants whose cells are heavily coated with Rh antibody, the cells may fail to agglutinate with anti-Rh serum and thus appear to be Rh-negative but such cells will invariably give a strongly positive antiglobulin reaction. When the cells appear to be Rh-negative, they may in fact be Rh-negative and the positive antiglobulin reaction be due to an antibody other than anti-Rh. In all cases in which the infant's red cells give a positive antiglobulin reaction, it is essential to determine the specificity of the causal antibody in the mother's serum.

If the direct antiglobulin test is negative but jaundice or anaemia are present it is possible that the infant has haemolytic disease of the newborn due to anti-A (or anti-B) or some other disease. Methods of establishing the diagnosis of haemolytic disease of the newborn due to ABO incompatibility are discussed below.

Although a negative direct antiglobulin test excludes the diagnosis of haemolytic disease of the newborn due to anti-Rh or other antibodies outside the ABO system, a positive result does not imply that the infant will develop the clinical picture of haemolytic disease.

Indication for Exchange Transfusion

As already described, the best single estimate of severity is given by a determination of the haemoglobin concentration of the cord blood. Fig. 97 shows that the infant's chance of survival increases with the cord haemoglobin concentration and that when the haemoglobin concentration is above 15 g./100 ml. the infant's chance of recovery is about 95 per cent,

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if it is treated conservatively (i.e. not treated by exchange transfusion), whereas, with exchange, the chance of recovery is almost 100 per cent.

Although in experienced hands exchange transfusion carries a very low mortality, perhaps even as low as 1 per cent in reasonably fit infants, the mortality of the operation as it is generally practised may well be as high as 5 per cent. Therefore, when the infant's chance of dying without treatment is as low as 5 per cent, it is questionable whether exchange transfusion should be undertaken. In such a case the experience of the operator must be taken into account. It follows that there is a very strong argument for treating these infants at a few centres where operators can gain experience.

In any case, in these infants with cord haemoglobin concentrations in the region of 15 g./100 ml., waiting for 24 hours will clarify the picture. Many of the infants will not develop jaundice and can then safely be left untreated. A few infants will become jaundiced and in these the serum bilirubin concentration should be estimated and exchange transfusion carried out if necessary.

Walker and Murray (1954) suggested that in infants with a cord haemoglobin concentration between 14.9 and 17.7 g./100 ml., exchange transfusion should be carried out if the cord bilirubin concentration exceeded 2.7 mg./100 ml. This seems reasonable although evidence of the prognostic value of the cord bilirubin concentration in such infants is not very extensive. Moreover the estimation of bilirubin is not yet a carefully standardized procedure so that it might be more realistic to use values above about 3 mg./100 ml. as an indication for exchange transfusion in infants with a normal cord haemoglobin concentration.

Infants weighing less than about 6 lb. at birth who are found to have a positive direct antiglobulin test, should as a rule be treated by exchange transfusion since their risk of developing deep jaundice is greater than that of the mature infant. Nevertheless, when there are indications that the disease is very mild, e.g. cord haemoglobin concentration 17 g./100 ml. or more, or direct antiglobulin test only weakly positive, a decision can be deferred and based on estimates of serum bilirubin concentration.

Thus the decision whether to carry out exchange transfusion can be based on a few simple rules. Exchange transfusion should be carried out soon after birth (within a few hours) if the direct antiglobulin test is positive and (1) if the infant is mature and the cord haemoglobin concentration is less than about 15 g./100 ml., (2) if the infant weighs less than about 6 lb., unless there are indications that the disease is very mild (see above).

Dr W. Walker (personal communication) believes that the criteria for exchange transfusion based on the examination of cord blood can safely be applied to premature infants. The criteria he uses are: (1) cord haemoglobin 14.8 g. or less; (2) cord haemoglobin 14.8 g. to 17.7 g. per 100 ml. with cord plasma bilirubin concentration 2.8 mg. per 100 ml. or more; (3) irrespective of cord haemoglobin concentration cord plasma bilirubin concentration 4 mg. per 100 ml. or more. Of 111 infants with a positive direct antiglobulin test born at more than 25 days before term, only eighty-four were judged to need exchange transfusion. Of the twenty-seven not treated by exchange transfusion, seven were treated by simple transfusion and twenty were not treated at all and all these recovered uneventfully. Similarly out of 150 infants weighing 5½ lb. or less whose antiglobulin test was positive, forty-three were judged not to require treatment and all survived. Although Dr Walker's criteria are evidently the best for a large centre, it may be that for the hospital treating only a relatively small number of cases, it may be better to err on the safe side and treat most infants whose birth weight is less than 6 lb. as suggested previously.

After birth the decision whether or not to carry out exchange transfusion will depend mainly upon the infant's plasma bilirubin concentration.

As mentioned above, Allen and Diamond (1958) have produced a chart which gives a tentative guide to the need for exchange transfusion at various ages after birth, based solely on the level of indirect bilirubin in the plasma. Thus in mature infants aged 12 hours exchange transfusion is definitely indicated when the plasma bilirubin exceeds 11.5 mg./100 ml.; at 24 hours exchange transfusion is definitely indicated when the level exceeds 16 mg.; when the level is 12–16 mg. other factors should be taken into account.

Walker (1958) has suggested that exchange transfusion should be carried out if at any time during the first day of life the plasma bilirubin concentration exceeds the infant's age in hours (excluding the first few hours). A useful rule is to regard the appearance of jaundice within the first 12 of life as a definite indication for exchange transfusion.

Estimation of packed cell volume. The decision to carry out exchange transfusion often depends upon the estimate of the cord blood haemoglobin concentration. In borderline cases it is helpful to know the packed cell volume of the sample as this may suggest that the estimate of the haemoglobin concentration is in error. The MCHC of cord blood may be taken as 32 per cent and thus PCV×0.32 should correspond closely to the estimation of the haemoglobin concentration.

The MCHC of red cells from cord blood is a little lower than that of adult red cells (Veall and Mollison, 1950; Marks *et al.*, 1955), probably due to a higher water content of the cells; cf. the observations of Grant *et al.* (1958) in adults.

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The red cell count, as usually performed, is not a useful check on the haemoglobin value. In most laboratories only 500 cells are counted and the standard error of the estimate is then of the order of 10 per cent (Biggs and Macmillan, 1948).

Indications for Second Exchange Transfusion

Allen, Diamond and Vaughan (1950) suggested that if jaundice increased despite a first exchange transfusion the infant might benefit from a second exchange transfusion. Reasons for believing that second exchange transfusions must produce rather limited benefits have been discussed above. Nevertheless, if they produce any lowering of the bilirubin concentration at all, they must be considered in any infant whose plasma bilirubin concentration is approaching dangerous levels.

If an infant is more than slightly jaundiced 12 to 18 hours after a first exchange transfusion the plasma bilirubin concentration should be estimated. If the concentration is greater than 15 mg./100 ml. a second exchange transfusion should be carried out. 15 mg./100 ml. is not itself a dangerous level (see Table LIV) but if the infant is under 48 hours of age some further increase in bilirubin concentration must be expected. In infants weighing less than the average, the peak bilirubin concentration is reached well after the age of 48 hours (see previous chapter) and thus in such an infant a plasma bilirubin concentration of 15 mg./100 ml. at the age of about 36 hours would be an indication for a second exchange transfusion.

In considering whether exchange transfusions are needed after the first few days of life, it must be remembered that it is the amount of unconjugated bilirubin that is important rather than the total amount of bilirubin. Within the first 48 hours of life at least, most of the bilirubin is unconjugated and the total serum bilirubin concentration is a satisfactory guide to the need for exchange transfusion. Later on it should be remembered that occasionally infants with severe haemolytic disease of the newborn have very large amounts of conjugated bilirubin in their serum and that it is worth estimating conjugated and unconjugated pigment separately to avoid unnecessary repetition of exchange transfusion (Oppé and Valaes, 1959; see also Jirsova *et al.*, 1958).

In practice second exchange transfusions are seldom necessary in mature infants but are fairly often indicated in premature infants. Thus Walker and Neligan (1955) found that after a single exchange transfusion of 60 ml. per lb. body weight only two out of 196 mature infants developed kernicterus.

Treatment of Simple Anaemia

Occasionally an infant which has been judged to be too mildly affected to require exchange transfusion subsequently develops a serious degree of anaemia. This anaemia seldom develops very suddenly and provided that haemoglobin concentrations are estimated in any infants in this category who become obviously pale, the diagnosis is likely to be made before a dangerous degree of anaemia develops.

In infants treated by exchange transfusion, provided a concentrated suspension of red cells is used, the haemoglobin concentration is unlikely to fall to a level at which further treatment is required. Some typical findings will be found in Fig. 102. Although in Case A shown in that figure the haemoglobin concentration fell to 7 g./100 ml., recovery occurred without further treatment. This infant was severely affected (cord haemoglobin concentration 8.7 g./100 ml.) and was born 3 weeks before term, following premature induction of labour. As indicated on the figure, the amount of free Rh antibody in the infant's plasma had become very low by the age of 47 days and it was at about this point that the infant started to produce a substantial number of Rh-positive red cells again. From evidence discussed elsewhere in this chapter, it is unlikely that the infant will be able to maintain a large population of Rh-positive cells until the amount of free antibody in its serum has fallen to a low level. In most infants the haemolytic process has become fairly mild by the age of 6 weeks and, therefore, if the infant can be provided with a sufficient number of Rh-negative cells to maintain a good haemoglobin level for at least this period it is not likely to become seriously anaemic. Personal experience suggests that in most infants treated by exchange transfusion, using a concentrated suspension of Rh-negative cells, and thereby provided with a haemoglobin concentration of about 17 g./100 ml. at the end of exchange transfusion, the haemoglobin concentration does not at any subsequent time fall below about 8 or 9 g./100 ml.

Although in infants who are progressing well there seems to be no harm in allowing the haemoglobin concentration to fall as low as 7 g./100 ml., in those who are progressing poorly or who develop an infection, it may be justifiable to give simple transfusions as necessary to maintain the haemoglobin concentration at 10 or 11 g./100 ml.

Technique of Exchange Transfusion

Efficiency of Exchange Transfusion

The efficiency of the exchange is greater when the intermittent method is used, that is, alternate withdrawal and injection of blood, than when the

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continuous method is used, e.g. bleeding from an artery with simultaneous infusion of blood into a vein.

When the intermittent method is used, the efficiency of the exchange depends upon the number of 'operations' (withdrawal and reinjection of

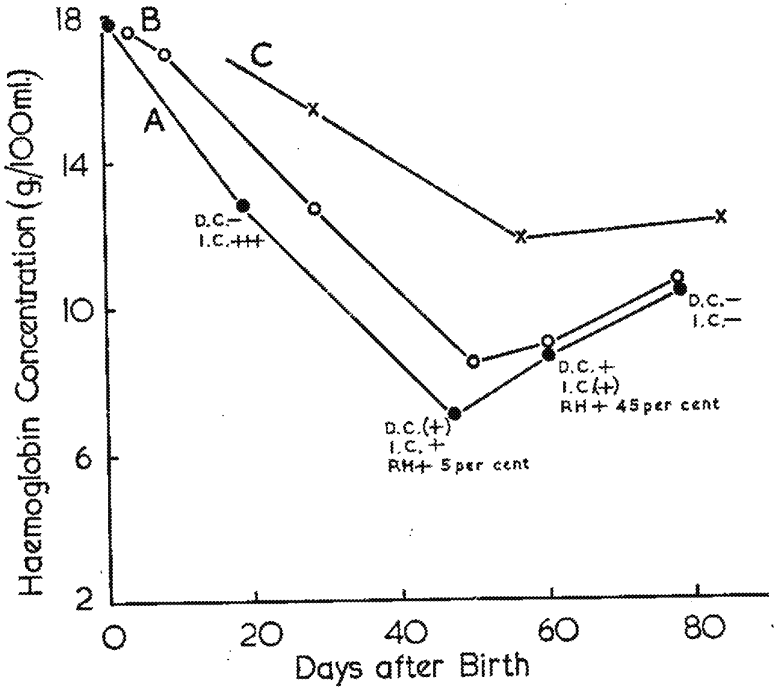

FIGURE 102. Changes in haemoglobin concentration during the first 3 months of life in one normal infant (C), and in two infants (A and B) with haemolytic disease of the newborn, treated by exchange transfusion. Infant B was only moderately severely affected (cord haemoglobin concentration, 12 g./100 ml.) but infant A was severely affected (cord haemoglobin concentration 8.7 g./100 ml.). As the figure shows, Rh-positive red cells disappeared from the circulation as a result of exchange transfusion and started to reappear about 50 days later. Recovery from severe anaemia coincided with the reappearance of large numbers of Rh-positive red cells in the circulation and the virtual disappearance of free Rh antibody. D.C. = direct Coombs test; I.C. = indirect Coombs test versus Rh-positive red cells, using infant's plasma; Rh+ = number of Rh-positive red cells present as a percentage of the total number of red cells.

blood) and upon the relationship between the volume of the syringe and the infant's blood volume (for example, if the volume of the syringe were equal to the infant's total blood volume, a single withdrawal and injection of blood would give 100 per cent exchange).

If the efficiency of exchange transfusion is considered in terms of red cells rather than whole blood then the infant's red cell volume must be taken into account since it is evident that it is far easier to reduce the absolute amount of red cells in the infant's circulation to a low level when it is anaemic to start with.

It is in fact possible to produce a formula which accurately predicts the results of exchange transfusion in terms of red cells by taking into account the above factors together with one refinement, namely the fact that the blood withdrawn has a haematocrit value which is higher than that in the body as a whole (Veall and Mollison, 1950). From their formula, Veall and Mollison produced a nomogram showing the amount to be exchanged to attain a particular objective, namely a final venous haematocrit of 50 per cent made up of Rh-positive 5 per cent and Rh-negative 45 per cent. A copy of the nomogram may be found in the previous edition of this book (Mollison, 1956, p. 495). The nomogram is helpful in understanding the effect of different factors on the efficiency of exchange transfusion, but it is of limited value as a guide to the amount to be used in practice, for the following reasons:

1. The consequences of leaving a certain small number of Rh-positive cells at the end of the exchange must vary with the severity of the disease. It is probably wise to compensate for this by exchanging just as much when the infant is anaemic as when it is mildly affected.
2. In practice blood is not dispensed in widely varying amounts, but in units of approximately 500 ml. so that the decision is usually between using red cells from one or from two units.

For these reasons it is advisable in practice to keep to some simple rule, e.g. exchanging a minimum of 60 ml. per lb. body weight (132 ml./kg.) when the infant is mildly affected and a minimum of 80 ml. per lb. (176 ml./kg.) when it is more severely affected.

As pointed out by Veall and Mollison (1950), it is desirable to use a concentrated suspension of red cells rather than whole citrated blood. The removal of approximately half the volume of citrate plasma not only reduces the risk of citrate toxicity but also leaves the infant at the end of the exchange with a haemoglobin concentration of at least 15 g./100 ml. and thus renders it unnecessary to inject a greater volume of blood than is withdrawn.

The standard bottle of citrated blood in use in Britain contains 420 ml. of blood and 120 ml. of citrate. This 420 ml. of blood is made up of approximately 180 ml. of cells and 240 ml. of plasma and thus there is

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360 ml. of citrate plasma. The removal of one-half of this (180 ml.) will leave approximately 360 ml. of a concentrated cell suspension with a haemoglobin concentration of approximately 10 per cent higher than that of the original blood.

Exchange Transfusion via the Umbilical Vein

The method of choice for exchange transfusion is that introduced by Diamond (1947). Blood is withdrawn and injected, intermittently, through a piece of plastic tubing passed up the umbilical vein.

Apparatus. The most convenient type of plastic tube is one originally prepared for feeding of premature infants (Fischer and Agerty, 1956; similar tubes are available from Edwards Surgical Supplies, Mortimer Street, London, W1.). The tip of this tube is sealed and rounded and there are openings in the side wall of the tube just proximal to the tip. At its other end the tube is fused into a Luer-fitting plastic socket for easy connections with a tap or syringe.

The remaining equipment required for exchange transfusion consists of a 20 ml. glass syringe with a metal tip and either a four-way tap or two three-way taps. A suitable apparatus with a four-way tap can be obtained from Edwards Surgical Supplies, Mortimer Street, London, W.1. In premature infants it is safer to use a 10 ml. syringe rather than a 20 ml. syringe.

The connections of the apparatus are shown in Fig. 103. Tap 1 is connected by a length of tubing to an empty sterile bottle and collects the discarded blood from the infant. Tap 2 is connected directly to the plastic catheter. Tap 3 is connected to a recipient set which in turn is connected to the bottle of donor blood. Tap 3 has a side arm, 3a, the nozzle of which is connected by a length of tubing to a reservoir containing heparin saline. (This may conveniently contain 500 ml. of saline to which 2000 i.u. of heparin are added.) This solution is used simply to flush out the apparatus initially and to rinse the syringe from time to time. It does not matter if a few millilitres of this solution are injected into the infant since the injection of at least 50 ml. would be required to produce a prolongation of the clotting time.

The technique is as follows. The umbilical cord is divided completely with a pair of scissors, about 1–2 cm. away from the point where it joins the skin. When this is done there may be a very little bleeding due to residual blood in the umbilical vein; usually only a negligible amount comes from the arteries though rarely there may be a small spurt when the

vessels are divided. Sometimes when the infant is in heart failure with cyanosis and a considerable rise in venous pressure, blood gushes from the umbilical vein when it is cut. The management of these cases is dealt with separately below. For the moment the procedure in the usual case in which virtually no blood flows from the open umbilical vein, will be described.

As a preliminary a pair of Spencer Wells forceps may be inserted into what seems to be the umbilical vein in order to confirm that it has been

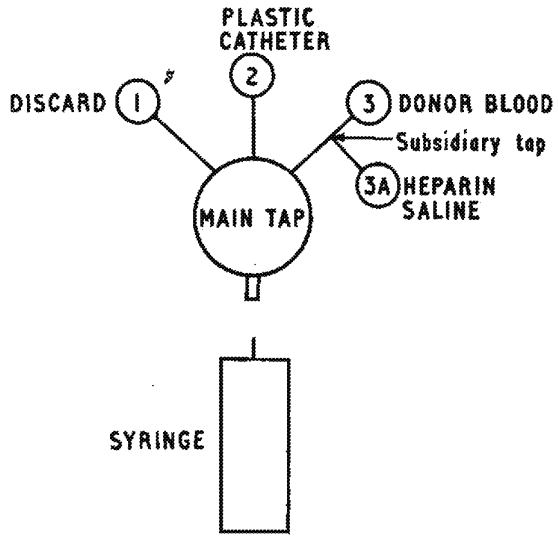

FIGURE 103. Diagram of a suitable tap for use in exchange transfusion in infants. The tap has one female Luer fitting into which the nozzle of the syringe is fitted and it has three male Luer fittings. The first leads to a tube connected with a discard bottle. The second is connected directly to the Luer fitting of the plastic catheter. The third is connected with the tubing from the transfusion bottle; this one has a side connection on to which can be fitted a tube leading to a reservoir of heparin-saline. This is very convenient as heparin-saline can be drawn into the apparatus and used to flush any of the connections.
(*Obtainable from Edward's Surgical Supplies, Mortimer Street, London, W.1.*)

properly identified. The blades of the forceps may be opened gently so as to dilate the vein and facilitate the passage of the catheter which in many cases may now be passed with difficulty. Occasionally the umbilical vein pursues a tortuous course in what is left of the umbilical cord and it may be necessary to shorten the cord even further. If necessary only a few millimetres need be left.

The catheter may stick at the point where the cord joins the skin. Slight

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pressure on the tip of the catheter from outside may help it to pass. Once the tip of the catheter is within the abdominal wall, the stump of the umbilical cord should be pulled downward towards the pubes. Now after gently trying to push the catheter in slightly different directions it will usually pass readily for a further 5 cm. Any further resistance is best overcome by gently rotating the catheter.

The catheter usually stops at a point 5 to 7.5 cm. from the surface and it can be shown by venous pressure measurements that the tip is still below the diaphragm. Usually the catheter is lying in one of the branches of the portal vein. In this position blood does not as a rule flow well if the catheter has been passed to its furthest extent, but a satisfactory flow can usually be obtained by withdrawing the catheter a few millimetres. Occasionally the catheter passes freely for 9 to 11 cm., indicating that the tip has passed through the ductus venosus into the vena cava. Venous pressure measurements may show that the head of pressure is lower than the front of the chest, and paradoxical movements of the column can be observed, the pressure falling on inspiration.

In practice the catheter should be passed in simply as far as is necessary to obtain a free flow of blood. It will often be found that strong suction collapses the vein so that no blood can be withdrawn. If instead very gentle suction is applied, a satisfactory flow will be obtained. The operation should be begun by withdrawing 20 ml. of blood. A part of this sample should be reserved for estimating the plasma bilirubin concentration, etc.; the remainder of the blood may be ejected into the discard bottle; now 20 ml. of donor blood is drawn into the syringe and injected into the baby, and so on.

At the beginning of each injection the catheter should be carefully watched for the passage of air bubbles. If any are seen to enter the catheter, they should be drawn back into the syringe. If the tip of the syringe is pointed downwards whilst this is done, any air bubbles will rise and come to lie against the end of the plunger. Throughout the exchange transfusion the syringe should be held in such a way that the plunger is higher than the tip of the syringe. Any air bubbles that accumulate can then be discarded at the end of the injection cycle by simply turning the tap leading to the discard bottle.

When the infant is more than 8 hours old and particularly if the infant is already 24 hours old, there is usually some blood clot in the vein. There is not likely to be any difficulty in passing the catheter, but when suction is applied, no blood can be withdrawn. If the catheter is now taken out it will be found that its tip has collected the clot on the way in.

Rinsing of the catheter in saline or passage of a new catheter will usually now be successful.

After the first 48 hours or so of life it becomes increasingly difficult to pass a catheter into the stump of the umbilical vein because of drying and shrivelling and the formation of clots within the lumen of the vein. It may then be best to cut down on the umbilical vein through the skin above the umbilicus. According to Pinkus (1948) the course of the vein is extraperitoneal for 2.5 to 4.0 cm. 'cephalad to the upper margin of the umbilicus'. Pinkus advocates a transverse incision 2 cm. long through the skin 1 cm. above the upper margin of the umbilicus. Upon separation of the subcutaneous tissue, the umbilical vein should be seen; a longitudinal incision, 0.5 cm. in length, is now made through the aponeurosis on each side of the vein which is then freed by blunt dissection. After passing a ligature round the vein a nick is made in its anterior wall and the catheter is inserted.

Hazards of Exchange Transfusion via the Umbilical Vein

Air embolism has been described (van Loghem *et al.*, 1949). As long as this possibility is considered, the danger should be averted. Even minute bubbles of air passing along the catheter can easily be seen and methods of ejecting them have been described above. There is a possibility of air being drawn up into the catheter whenever the latter is left in the umbilical vein unconnected to the multi-way tap and syringe. It is advisable when first passing the catheter to have a little saline in its lumen which will indicate the direction of flow in the catheter. During the exchange, the apparatus is disconnected from the catheter for rinsing, etc. The catheter should be closed or kept under constant supervision. Evidently the danger of air embolism arises only if the tip of the catheter is lying in a vessel in which the pressure falls below the atmosphere pressure; normally this happens only if the catheter passes into the ducta venosus so that the tip lies above the diaphragm but it may happen when the catheter is below the diaphragm and the infant is making powerful inspiratory efforts against some obstruction, for example, laryngeal spasm (see Mintz and Vallbona, 1960).

If bubbles of air are seen to pass along the catheter, the infant should immediately be picked up by the feet and held in that position for a few minutes to prevent cerebral embolism. Other first-aid measures in suspected air embolism are discussed in Chapter XV.

Perforation of the umbilical vein occurs occasionally (personal communications from three different sources). In these cases it occurred when using a

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catheter of hard plastic or when using a plastic catheter with a sharpened point. If a soft plastic catheter with a rounded end is used there can be little possibility of perforating the vein.

One case in which the umbilical vein was perforated and in which haemato-peritoneum was found at post-mortem has been described, by Sundal and Kåss (1954).

Septic thrombophlebitis is a serious complication. In a series including all deaths from haemolytic disease of the newborn reported in England and Wales in the years 1953 to 1955, five deaths attributable to it were found (Mollison and Walker, 1957). The clinical features are illustrated by the following case:

An infant who had received three exchange transfusions in the first few days of life suddenly became ill on the tenth day with dyspnoea and cyanosis; the abdomen was distended and the veins covering the abdomen were engorged; the liver was found to be enlarged. The infant was treated with penicillin and tetracycline but ascites developed; *staphylococcus pyogenes* was grown from the ascitic fluid. Signs of obstruction developed on the fifty-second day and laparotomy showed peritonitis with some pus formation (Andrews, 1957). The infant recovered but at the age of 4 has oesophageal varices and portal hypertension (personal communication from Professor P. R. Evans). Dr J. P. M. Tizard (personal communication) has seen an almost identical case.

Citrate toxicity has been discussed in Chapter XV. The use of concentrated suspensions of red cells rather than whole citrated blood greatly diminishes the amount of citrate injected into the infant. Nevertheless it is wise to inject 1 ml. of 10 per cent calcium gluconate after each 100 ml. of blood. The injection should be preceded and followed by the injection of a few millilitres of heparin-saline to avoid the production of clots through contact with citrated blood. (If the right amount of calcium is added to citrated stored blood, a clot will form within a few minutes.)

It is of course possible to avoid citrate toxicity completely by using fresh heparinized blood but this is often inconvenient.

Potassium toxicity has already been discussed in Chapter XV. As described there, it seems artificial to separate potassium toxicity from citrate toxicity. In any case a serious rise in plasma potassium is found only in blood stored for more than 2 or 3 weeks and, for exchange transfusion in infants, blood stored for not more than a few days should be used, to minimize the number of non-viable red cells introduced into the circulation.

The injection of ice-cold blood is probably not dangerous in view of results reported by Taylor *et al.* (1961). In experimental exchange transfusions in rabbits, blood at 1° C. was injected rapidly (10 ml. in about 30 seconds) into a catheter whose tip lay in the external jugular vein near to the right auricle. In a series of experiments designed to test the toxicity of citrate and potassium, blood transfused at 1° C. seemed to be no more dangerous than blood injected at 21° C. Indeed there was some evidence that the animal could tolerate larger amounts of citrated, high potassium blood when this was injected at 1° C.

Overloading of the circulation is mentioned below. In the author's experience, it occurs only in infants whose condition before transfusion is grave.

Other Causes of Sudden Death during Exchange Transfusion

Occasionally during exchange transfusion an infant rapidly becomes pale and silent and within a few minutes its heart stops beating and it dies. It is possible that two further factors, in addition to those already discussed, contribute to these deaths. One may be the use of stored red cells from ACD blood, which give up oxygen to the tissues less readily than fresh red cells (see Chapter I). Another may be the fact that the red cells of infants with haemolytic disease of the newborn carry less oxygen per unit amount of haemoglobin than the red cells of normal infants (Abrahamov and Smith, 1959); *in vitro* it can be shown that sensitization of Rh-positive red cells with Rh antibody interferes with the ability of the cells to take up oxygen (Abrahamov and Diamond, 1959).

Other Methods of Exchange Transfusion

Occasionally it proves impossible to obtain a satisfactory flow of blood from a catheter passed up the umbilical vein and the saphenous vein may then be used; the following description is taken from Arnold and Alford (1948): A line is drawn from the anterior superior spine of the ileum to the pubic tubercle. An incision is begun at a point 1½ cm. below the mid-point of this line and extended medially for 2 cm. When the great saphenous vein is identified it is not tied off as this would cause it to contract and make it much harder to catheterize. A nick is made in the vein just distal to the point where it passes deeply to join the femoral vein. The catheter is then passed upwards through the femoral vein, into the external iliac vein and on into the inferior vena cava.

Wiener and Wexler (1946) advocated bleeding the infant from the

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radial artery and introducing blood via the internal saphenous vein. A later description of this method (Wiener, Wexler and Brancato, 1954) is as follows:

A transverse skin incision is made over the internal saphenous vein at the ankle and a blunt gauge 20 (American) needle is inserted into the internal saphenous vein. This needle is tied into place and 200 units of heparin in 2 ml. of saline are injected. The radial artery is then exposed by making a transverse incision just proximal to the head of the radius; the artery is nicked and 50 ml. of blood is allowed to run out into a measuring vessel before beginning the injection of 10 ml. amounts of blood through a three-way stopcock connected to the canula in the internal saphenous vein. A concentrated suspension of Rh-negative red cells is used for injection. After every 100 ml., 1 ml. of 10 per cent calcium gluconate is injected. At the end of the operation, neither the artery nor the vein is ligated.

The radial artery-saphenous vein method should be considered when the umbilical vein route proves difficult but it is not to be preferred for routine use. The advantages of using the umbilical vein are: firstly, simplicity of technique and the avoidance of skin incisions; secondly the slightly greater efficiency, in terms of red cell exchange, of intermittent substitution compared with continuous substitution; and thirdly the ease of measuring precisely the amount of blood removed.

Special Procedure for Infants in Cardiac Failure

The most anaemic of the liveborn infants are often in heart failure and must in any case be regarded as having increased cardiac stress. It is known that in adults cardiac output rises as the haemoglobin concentration of the blood falls. Adults with severe chronic anaemia have a raised venous pressure and easily pass into cardiac failure if too large or too rapid a transfusion is given. Infants born with moderate or severe anaemia also have a raised venous pressure and also pass readily into cardiac failure (Mollison and Cutbush, 1949a). Moreover, many such infants are already in cardiac failure before transfusion:

Infant C. was a typical example. At birth it was very pale and slightly icteric; its cord blood haemoglobin concentration was 3.4 g./100 ml. One hour after birth the infant was making a continuous grunting noise. When the umbilical cord was cut across as a preliminary to exchange transfusion there was profuse bleeding. The venous pressure was measured and found to be raised (9 cm. above the front of the chest).

After 260 ml. of blood had been exchanged the infant appeared more cyanosed; then suddenly, after a further 20 ml. had been exchanged in 2 minutes, the infant became very cyanosed. The heart sounds could no longer be heard. The infant was now practically black and had become quite limp; respiration had ceased and the pupils were fixed and widely dilated. Artificial respiration was begun by intermittently compressing the chest. The heart then started to beat again very slowly and the infant gave a few slow gasps. About 10 ml. of blood were now slowly aspirated through the catheter and the heart rate increased strikingly. During the next 7 minutes some 35 ml. of blood were withdrawn with astonishing effect. The breathing and the heart rate returned to normal and the lips actually became pink. The venous pressure was now normal (+4 cm.). The exchange transfusion was continued very cautiously until a total of 400 ml. had been removed and 370 ml. injected. At the end the infant was a pale pink colour but still had a grunting cry. The infant was placed in an oxygen tent but died 10 hours later.

In infants with cord haemoglobin concentrations of less than 5 g./100 ml. recovery is unusual but in slightly less severely affected infants dramatic results are sometimes observed, particularly if 30–50 ml. of blood are withdrawn from the infant before beginning the exchange, as in the following case:

Infant W. was extremely pale at birth. Its respirations were shallow and there was oedema of the face, trunk and legs. The cord was jaundiced though the body was not. The liver was enlarged. Examination of the cord blood showed: direct antiglobulin test strongly positive; haemoglobin concentration 5.7 g./100 ml; bilirubin 4.6 mg./100 ml. The impression was that the infant was moribund.

Two hours after birth the cord was cut as a preliminary to exchange transfusion; blood spurted violently from the vein. Accordingly, after passing a catheter, 50 ml. of blood were withdrawn. Then 20 ml. amounts were cautiously replaced by equal amounts of a concentrated suspension of Rh-negative cells. In all, 575 ml. of blood were removed and 495 ml. injected. The infant was then placed in an oxygen tent. At the age of 12 hours the infant was better though it was still grunting on expiration. At the age of 24 hours the infant had improved remarkably and its colour and behaviour would have passed for normal.

The infant never became visibly jaundiced though its serum bilirubin concentration reached 7.7 mg./100 ml. 24 hours after birth. It left hospital within 2 weeks of birth. At the age of 18 months it appeared to be normal in all respects.

In all infants with cord haemoglobin concentrations below 10 g./100 ml. it is wise to measure the venous pressure before beginning an exchange transfusion. The most reliable method is to use the sort of device shown in Fig. 104. With such a device it is possible to be certain that there is a free flow of fluid in the system. An easier method is simply to hold up the umbilical catheter and note the level to which blood rises, using a small metal rule (Diamond *et al.*, 1951).

Whenever the venous pressure is found to be raised it is essential to take out more blood than is injected. The safest method is to do this in stages, for example, take out 40 ml., inject 20 ml., then exchange 20 ml.

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amounts until 120 ml. have been withdrawn and 100 ml. injected. Then take out an additional 20 ml. and continue till 240 ml. has been with-

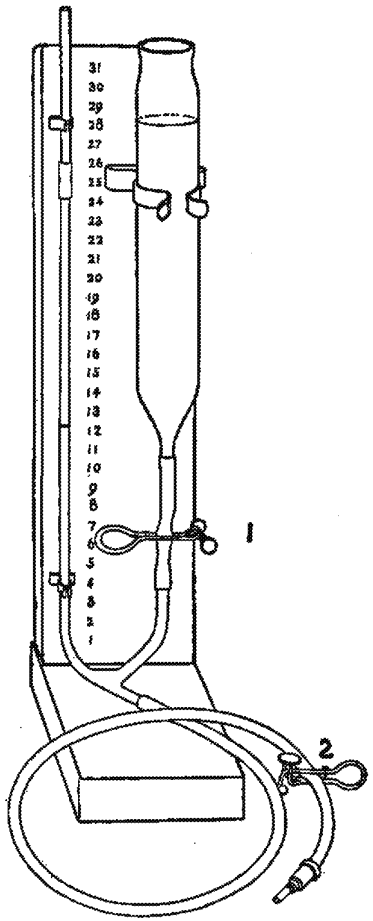

FIGURE 104. Saline manometer for measuring venous pressure. The column of the manometer can be raised by opening clip 1 and allowing fluid to run in from the reservoir. Readings are taken with clip 1 closed and clip 2 open. The position on the scale corresponding to the front of the infant's chest is taken as zero. This point is discovered by the use of a ruler and level.

drawn and 200 ml. injected. The infant's venous pressure and general condition must be a guide to the advisability of removing any further amounts of blood.

HAEMOLYTIC DISEASE OF THE NEWBORN DUE TO ABO INCOMPATIBILITY

In haemolytic disease of the newborn due to ABO incompatibility the serological findings are much less helpful in establishing the diagnosis than in haemolytic disease of the newborn due to anti-Rh. First, in the disease due to anti-Rh the direct antiglobulin test performed by standard methods is invariably positive whereas in the disease due to ABO incompatibility positive results are obtained only by using special modifications of the test. Secondly, in haemolytic disease of the newborn due to anti-Rh the disease can be predicted antenatally since the appearance of anti-Rh in the serum of a woman who has not been transfused with Rh-positive blood indicates that she has been sensitized by an Rh-positive infant; the finding of immune anti-A or anti-B in the mother's serum is of only limited prognostic significance since such antibodies may arise after a variety of stimuli apart from pregnancy or transfusion (see Chapter VIII). Because of this it is not as a rule considered worth while to test the serum of pregnant women for the presence of 'immune' anti-A and anti-B; and thus haemolytic disease of the newborn due to these antibodies is usually recognized only when the infant shows clinical signs of a haemolytic process.

Incidence

Halbrecht (1944) was the first to point out that jaundice developing within 24 hours of birth was frequently associated with ABO incompatibility; he gave the name 'icterus praecox' to this syndrome. He later reported that he had found ninety cases of the syndrome amongst approximately 16,000 newborn infants — an incidence of one in 180 births; thirty of the ninety infants were firstborn (Halbrecht, 1951).

Hsia and Gellis (1954) reported an essentially similar incidence of early jaundice (within 24 hours) associated with ABO incompatibility. Thus they found nineteen cases in approximately 2600 births — an incidence of approximately one in 140 births.

Valentine (1958) examined 1000 liveborn infants for the slightest sign of jaundice occurring in the first 24 hours of life and found twenty-one examples; seven of these had haemolytic disease of the newborn due to anti-Rh and fourteen haemolytic disease of the newborn due to anti-A or anti-B. The incidence of haemolytic disease of the newborn due to ABO incompatibility was therefore about one in seventy of all newborn infants.

As described below, Rosenfield and Ohno (1955) found that an even higher proportion of newborn infants (about one in thirty or three in 100)

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showed some laboratory evidence of a mild haemolytic process due to ABO incompatibility. This may be compared with the proportion at risk, that is the proportion of infants whose red cells are incompatible with their mother's serum and this is found to be approximately twenty per 100 (see Table LVII). In fact, as described below, haemolytic disease is found almost exclusively in group A (or B) infants born to group O mothers and these form about fifteen per 100 newborn infants. Thus it seems that a group A (or B) infant born to a group O mother has about a one in five (three in fifteen) chance of showing some evidence of a very mild haemolytic process. It should be added that very occasionally the haemolytic process is severe; see below.

If group A and B infants were equally liable to the disease, the ratio A/B in affected infants should be simply that of the gene frequencies of

TABLE LVII

CALCULATION OF PERCENTAGE OF 'HETEROSPECIFIC' PREGNANCIES IN ENGLAND

Maternal phenotype	*Phenotype frequency*	*Gene frequencies in random population*		*Heterospecific pregnancies*
		A	*B*	
O	0.47	0.26	0.06	$0.47 \times (0.26+0.06) = 0.150$
A	0.42		0.06	$0.42 \times (0.06) = 0.025$
B	0.086	0.26		$0.086 \times (0.26) = 0.022$
				Total = 0.197

A/B, that is approximately 0.26/0.06 or 4.3 to 1, see Table LVII. In the series of Rosenfield and Ohno (1955) the ratio was 31 group A to 7 group B, or 4.4 to 1. (The incidence in Newcastle has been almost identical, namely 18 A, 4 B, 1 AB; Dr W. Walker, personal communication.) Evidently then group B infants are as liable to be affected as are group A infants — a surprising conclusion, considering that 'immune' anti-A is so much commoner than 'immune' anti-B.

Cases of haemolytic disease of the newborn due to anti-A or anti-B which are severe enough to require treatment are rare. So rare indeed that for a long time many observers doubted that ABO incompatibility could cause haemolytic disease of the newborn. Perhaps the first really convincing cases were described by Grumbach and Gasser (1948), who recorded such features as reticulocytosis, erythroblastosis, microspherocytosis and in one case, the development of kernicterus. Rosenfield (1955), among

approximately 1500 newborn infants, found three which required simple transfusion but none which required exchange transfusion. Personal experience suggests that cases requiring treatment may be rarer than this. Thus during a 6-year period in which the blood of all obviously anaemic or deeply jaundiced infants was being tested in the author's laboratory, out of 14,000 consecutive births only seven infants had evidence of a moderate or severe haemolytic process due to ABO incompatibility. Three of these infants were considered to require transfusion and all recovered uneventfully.

Thus one may contrast the incidence of haemolytic disease of the newborn due to Rh incompatibility and ABO incompatibility as follows: haemolytic disease of the newborn due to anti-Rh has a *maximum* incidence of between one in 150 to one in 200 of all births; the word maximum is used since these infants never have signs of a haemolytic process unless the direct antiglobulin test is positive and then do not necessarily show signs. Approximately 60 per cent of the infants are sufficiently severely affected to require exchange transfusion. Thus, very approximately, one in 300 of all newborn infants have a sufficiently severe degree of haemolytic disease of the newborn due to anti-Rh to require treatment.

Haemolytic disease of the newborn due to ABO incompatibility has a *minimum* incidence of one in 150 births; the word minimum is used because the estimate is based on the number of infants who develop jaundice. It may be taken as certain that other infants have a mild haemolytic process due to ABO incompatibility but never display jaundice. Only about one in 3000 or less of all newborn infants have a sufficiently severe degree of haemolytic disease of the newborn due to ABO incompatibility to require treatment.

Familial Incidence

Although haemolytic disease due to ABO incompatibility may affect successive siblings in a family, the family incidence differs in two ways from that of haemolytic disease due to anti-Rh. First, haemolytic disease of the newborn due to ABO incompatibility often affects the firstborn infant and, second, it is common for unaffected (but ABO incompatible) infants to be born following the birth of an affected infant.

In reviewing a series of cases in the first edition of this book, the author concluded that in families in which haemolytic disease of the newborn due to ABO incompatibility was detected, the firstborn infant was affected in 40 per cent of families. In some of these families a group O infant was born before the birth of the first group A (or B) infant; if group O infants were

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omitted, the incidence in the first infant at risk became 50 per cent (in families in which haemolytic disease of the newborn due to ABO incompatibility was recognized).

Serological Findings

The Direct Antiglobulin Test

If the washed red cells of an infant with haemolytic disease due to ABO incompatibility are mixed with antiglobulin serum on a tile and observed for several minutes they may show no agglutination whatever.

In a series of eleven cases described by Crawford *et al.* (1953a) in all of which there were signs of a moderately severe haemolytic process, the direct Coombs test, performed in this way, was negative in four cases and only weakly positive in the remaining seven. The negative reactions were not due to the use of an inappropriate dilution of antiglobulin serum since the cells were in some instances tested against a range of dilutions. In twenty-one mild or moderate cases of haemolytic disease of the newborn due to ABO incompatibility described by Hsia and Gellis (1954), the direct Coombs test was invariably negative.

Rosenfield (1955) reported that if a modified antiglobulin test was used positive results were obtained much more often. The technique he used was as follows:

> 0.05 ml. of antiglobulin serum, diluted to the optimal extent, was added to 0.05 ml. of a 2 per cent suspension of four-times washed red cells in a small test tube. The mixture was immediately centrifuged at 2000 rev./min. for 10 seconds and then examined both macroscopically and microscopically. Aggregates of three or more red cells were considered to indicate a positive result.

Using this method on samples of cord blood, a positive result was obtained in eighty-nine out of 647 cases in which the infant's cells were incompatible with the mother's serum (Rosenfield and Ohno, 1955). The results of some haematological examinations made in these cases are shown in Table LVIII. The findings strongly suggest that the infants with the positive direct (modified) antiglobulin test have a mild haemolytic process.

Using Rosenfield's modification of the direct antiglobulin test, Hubinont *et al.* (1960) obtained a slightly lower incidence of positive results; among 549 infants whose red cells were incompatible with their mother's serum thirty-nine (7.1 per cent) had a positive test; in all the thirty-nine cases an antibody could be eluted from the infant's red cells by heating

stroma to 56° C.; this antibody would give a positive indirect antiglobulin test with red cells of appropriate ABO group.

Using a slightly modified 'spin-Coombs' test technique, Haberman *et al.* (1960) found 8 per cent of positives among almost 4000 infants whose red cells were incompatible with their mother's serum and succeeded in eluting antibody from the infant's red cells by the simple heat elution method of Landsteiner and Miller.

Using the capillary tube modification of the direct antiglobulin test, Dr Bruce Chown (personal communication) has found a positive direct antiglobulin test in a far higher proportion, namely 5 per cent of all new-

TABLE LVIII

EFFECT OF ABO GROUP INCOMPATIBILITY BETWEEN INFANT'S RED CELLS AND MOTHER'S SERUM ON CORD BLOOD FINDINGS
(From Rosenfield and Ohno, 1955)

	*No. of cord blood samples**	*Haemoglobin concentration g./100 ml.*	*Reticulocytes %*	*Bilirubin concentration mg./100 ml.*
A. Infant's red cells compatible with mother's serum	2256	16.05	4.34	2.11
B. Infant's red cells incompatible with mother's serum; infant's direct antiglobulin test negative	558	15.82	4.53	2.22
C. As B, but direct antiglobulin test positive	89	14.85	5.94	2.96

* Haemoglobin estimates were made on all samples, and reticulocyte counts and bilirubin estimates on almost all samples.

born infants (cord blood samples); that is to say, about one in four of all infants whose red cells are incompatible with their mother's serum. A positive test was usually obtained after 7 minutes in the capillary (see Chapter XII for further description). Dr Chown reports that not more than 1 per cent of infants with a positive capillary antiglobulin test develop sufficiently severe hyperbilirubinaemia to require exchange transfusion (i.e. less than one in 2000 of newborn infants).

Other Serological Findings in the Infant

Witebsky *et al.* (1947) found that when the whole oxalated blood of the infant with haemolytic disease of the newborn due to ABO incompatibility was mixed with 'inactivated' AB serum and spread on a glass slide, agglutination within 60-90 seconds was often observed. This test was

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found to be positive in ten out of ten cases examined by Shumway *et al.* (1955), but positive reactions may be very weak and false positives due to rouleaux are troublesome (Valentine, 1958).

Crawford *et al.* (1953a) noticed that 'blood freshly drawn from the infant (with AB disease) formed large clumps which were easily seen when the blood was allowed to flow down the side of a tube or was examined on an opal glass tile'. They pointed out that if blood is taken from a patient of group A_1 who has received a transfusion of plasma containing potent anti-A, it behaves in the same way.

Munk-Andersen (1958a) found that using his special test for clumping in a dextran-containing medium (see Chapter XII) positive results were obtained in thirty-five out of 1700 unselected samples of cord blood, that is, in approximately 1 in 10 of the infants at risk, a figure which agrees well with the results obtained with Rosenfield's modification of the direct antiglobulin test (see above). Nevertheless when both tests were used on the same series of infants, many discrepancies were observed (Dyggve and Munk-Andersen, 1960).

The phenomenon described by Lewi and Clarke (1960) presumably also demonstrates some change in the surface charge of the red cells of affected infants. It was found that red cells from infants suspected of having haemolytic disease of the newborn due to ABO incompatibility sedimented at an increased velocity. They described a test in which red cells from heparinized blood were washed three times in saline buffered with phosphate to neutrality. Then to 1 volume of packed washed cells, 3 volumes of PVP (25 per cent 'subtosan retard' in saline) was added, and the mixture was placed in a Westergren sedimentation tube. Whereas normal cells sedimented only 5 mm. in 10 minutes and 10 mm. in 15 minutes, samples of red cells from infants suspected of ABO disease sedimented 90 mm. or more in 10 minutes or showed a massive red cell deposit by the end of 15 minutes. By contrast in twenty cases of haemolytic disease of the newborn due to anti-Rh, the sedimentation rate was normal in seventeen and only slightly raised in three.

Free antibody is often demonstrable in the infant's serum; for example, Crawford *et al.* (1953a) found that it was present in eight out of eleven group A infants with haemolytic disease due to anti-A whose blood was examined in the first 3–4 days of life. The antibody was demonstrable only by the indirect antiglobulin test using A_1 red cells from adults; the infant's serum did not agglutinate or haemolyse adult A_1 cells.

Gunson (1957c) examined infants sooner after birth and used a different

test (Löw's enzyme method) and found free antibody in seventeen out of seventeen cord blood samples and in eighteen out of twenty samples taken between 24 and 48 hours after birth from infants with haemolytic disease of the newborn due to ABO incompatibility but in none of twenty-five normal cord blood samples. The same author suggested that the presence of free incompatible antibody (anti-A or anti-B) in an infant with haemolytic disease of the newborn due to anti-Rh indicated that the infant also had haemolytic disease of the newborn due to ABO incompatibility (Gunson, 1957b).

In a later more comprehensive investigation of 100 group A or B infants born to group O mothers, Gunson (1958) found twenty-two with free incompatible antibody in their serum. Ten of these developed jaundice within the first 24 hours of life. Of the remaining seventy-eight infants without free antibody, only two developed early jaundice. The findings of Stern (1958) were broadly similar; that is he found free antibody in 10 per cent of normal infants as well as in forty out of forty infants in whom the diagnosis of haemolytic disease of the newborn due to ABO incompatibility had been made. (For further references to the occurrence of free 'incompatible' antibody in the serum of normal infants, see pp. 597–598.)

Reactivity of Infant's Red Cells with Anti-A and Anti-B in vitro

As described in Chapter VIII, the red cells of newborn infants react relatively weakly with anti-A and anti-B *in vitro*. This relative weakness of the A and B receptors in newborn infants was first demonstrated by Kemp (1930); its possible role in protecting the newborn infant from the destructive effect of anti-A (and anti-B) has been discussed by Tovey (1945) and by Wiener, Wexler and Hurst (1949). As described in Chapter VIII, the relatively low reactivity is most obvious when the cells are tested with a partially neutralized incomplete anti-A serum. In this test the red cells of newborn infants characteristically behave like A_2 cells of adults, rather than A_1 cells.

Haemolytic disease of the newborn due to anti-A affects only A_1 infants (Zuelzer and Kaplan, 1954c), that is to say, only those who are genetically A_1. At the time of birth the red cells of such infants may fail to react with anti-A_1 although samples taken when the infant is a few months old do react (Crawford *et al.*, 1953a).

Serological Findings in Mother

When the infant has signs of a haemolytic process, the anti-A (or anti-B) in the mother's serum almost always displays 'immune characteristics'.

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Thus in a series of eleven cases in which the infant had definite signs of a haemolytic process, the mother's serum was invariably capable of haemolysing adult A_1 cells and of sensitizing them to an antiglobulin serum (Crawford *et al.*, 1953a). In Rosenfield's series of thirty-nine infants with a positive direct (modified) antiglobulin test, the mother's serum was capable of haemolysing 'normal cord cells' in thirty-seven instances. Similarly, in thirty-six cases of ABO disease investigated by Robinson *et al.* (1960), in which the mother was group O, her serum haemolysed A_1 red cells in all but one case. In seven of the cases the infant was group B and in these cases the mother's serum also haemolysed group B cells.

When the mother's serum contains 'immune' (i.e. haemolytic or incomplete) anti-A or anti-B and she carries an A or B infant there appears to be about one chance in three that the infant will show signs of haemolytic disease. Thus Reepmaker and van Loghem (1953), in the course of routine testing, found 240 mothers whose serum contained immune anti-A or anti-B and correlated this finding with signs observed in the infants: twenty-nine infants had typical haemolytic disease of the newborn, fifteen infants had doubtful haemolytic disease of the newborn and thirty-four had possible haemolytic disease of the newborn — that is, mild jaundice only. Thus in all, approximately one in three infants born to mothers whose serum contained immune anti-A or anti-B showed some signs of a haemolytic process.

The value of examining the mother's serum may be provisionally summarized as follows: if the mother's serum contains haemolytic anti-A or anti-B, incompatible with the infant's red cells, the infant may or may not show signs of a haemolytic process. If the mother's serum lacks haemolytic anti-A or anti-B, haemolytic disease in her infant due to ABO incompatibility is virtually excluded.

Rosenfield (1955) found that mothers of infants with haemolytic disease due to ABO incompatibility almost invariably belong to group O. He pointed out that if all incompatible blood group combinations were equally liable to lead to haemolytic disease of the newborn, then only 69 per cent of the mothers would be expected to be group O whereas in practice, among his thirty-nine infants with a positive direct antiglobulin test, thirty-eight had group O mothers. He pointed out that in two previous series of cases of haemolytic disease of the newborn due to ABO incompatibility all the mothers had been group O, though this had not been specially remarked by the authors (Wiener, Wexler and Hurst, 1949; Crawford *et al.*, 1953a).

This observation led Rosenfield to suggest that there was a peculiarity in the anti-A (or anti-B) activity of group O sera which was responsible for 'most of the A-B haemolytic disease problem'.

It appeared at first that this peculiarity of group O sera might be their content of 'cross-reacting antibody' but available evidence does not support this view. For example, Zuelzer *et al.* (1957) studied twins born to a group O mother; the first twin, whose group was B, was affected with haemolytic disease of the newborn; the other, whose group was A, was unaffected. Two pieces of evidence suggested that the antibody was specific anti-B and not 'cross-reacting antibody': first the fact that only the group B twin was affected and second the fact that the antibody eluted from red cells of this twin was specific anti-B.

Recent work has shown that the peculiarity of group O sera may simply be that, unlike group A and B sera, they frequently contain 7*S* anti-A and anti-B, although why this should be so is not yet known. Kochwa *et al.* (1961) found 7*S* anti-A or anti-B in fifty out of seventy-three group O mothers but in none of thirty-seven group A and B mothers.

Munk-Andersen (1958b) found that in a series of forty-five cases of ABO haemolytic disease the mother was group O in forty-three instances and belonged to subgroup A_2 in the remaining two. He found that A_2 mothers produced much stronger incomplete anti-B than A_1 mothers.

Haematological Findings

Haemoglobin Concentration

The syndrome of early jaundice associated with ABO incompatibility is evidently a very mild haemolytic process. From what is known of the evolution of mild cases of haemolytic disease of the newborn due to anti-Rh it is not to be expected that anaemia will be a prominent feature of such cases. Halbrecht (1944) in his first report found that only three out of fifty-seven infants were anaemic.

Occasionally, infants with moderately severe anaemia are encountered. Thus is a case described by Mollison and Cutbush (1949b) the infant's cord haemoglobin concentration was 10 g./100 ml.; cord blood was tested because the midwife noticed that the liquor amnii was very yellow. In this particular infant the cord haemoglobin concentration rose to 16 g./100 ml. during the few hours after birth — a reminder that minor degrees of anaemia demonstrable in cord blood are frequently masked after birth. Rosenfield's (1955) series of observations on cord blood shows that ABO

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incompatibility usually causes only a minor degree of anaemia; see also Table LVIII.

Even in moderately severe cases of haemolytic disease of the newborn due to anti-A it is unusual for the infant to develop severe anaemia after birth. Thus in the series of Crawford *et al.* one infant had a haemoglobin concentration of 9.9 g./100 ml. on the sixteenth day of life and one had a haemoglobin concentration of 9.7 g./100 ml. on the twenty-second day, but these were the lowest values encountered.

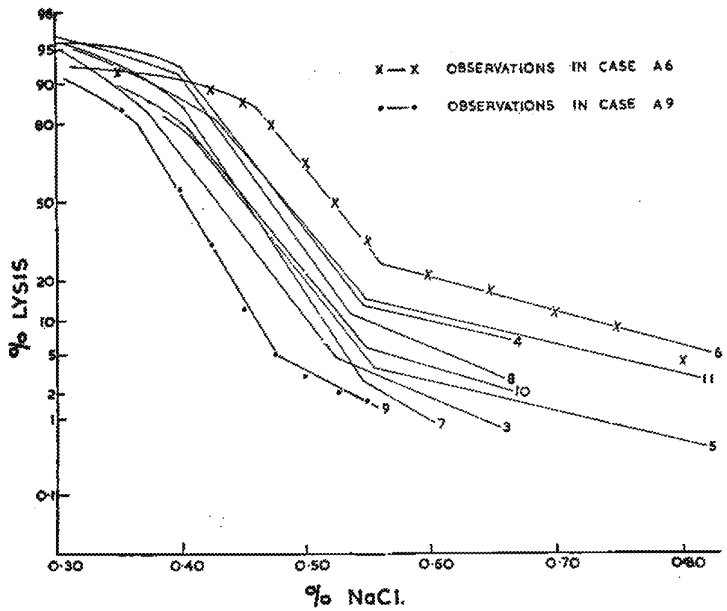

FIGURE 105. Osmotic fragility in nine infants with haemolytic disease of the newborn due to anti-A; percentage lysis is plotted on a scale of arithmetical probability (the figure is taken from Crawford *et al.*, 1953a, and the numbers marked against the curves refer to the case numbers in the original paper).

Reticulocytosis and Erythroblastaemia

A slight increase in reticulocytes is a common feature in haemolytic disease of the newborn due to ABO incompatibility (see Rosenfield, 1955). In the series of fairly severe cases collected by Crawford *et al.* (1953a) the reticulocyte count exceeded 15 per cent in six out of eleven cases. In five of these cases there were thirty or more nucleated red cells per 100 leucocytes.

Spherocytosis and Changes in Osmotic Fragility

Grumbach and Gasser (1948) drew attention to the presence of microspherocytes in the blood films of two cases of haemolytic disease of the newborn due to ABO incompatibility and found osmotic fragility to be increased in another case. Robinson *et al.* (1951) found osmotic fragility to be increased in four out of four cases. Crawford *et al.* (1953a) found that the median corpuscular fragility was above normal limits in ten out of

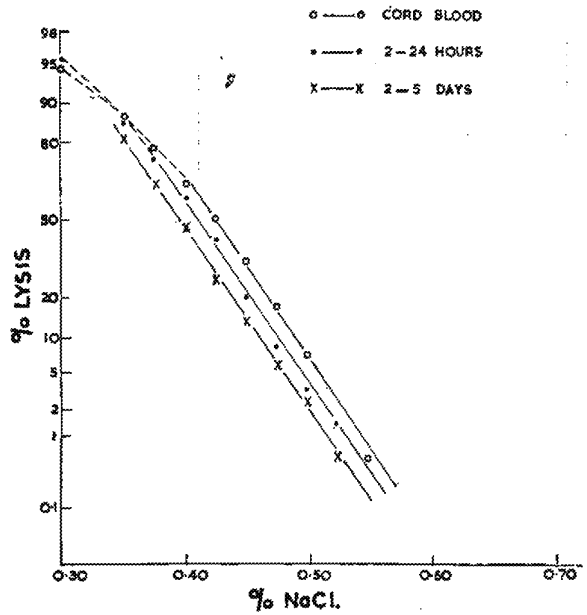

FIGURE 106. Osmotic fragility of the red cells of normal infants during the first 5 days of life; percentage lysis is plotted on a scale of arithmetical probability. The three groups are those referred to in Table LIX (from Crawford *et al.*, 1953a).

eleven cases and that in all eleven cases the fragility curves had an abnormal shape. These findings are illustrated in Fig. 105; for comparison Fig. 106 illustrates the findings of the same authors in normal newborn infants. Table LIX gives the mean and standard deviation for normal infants, using Parpart's method. Fig. 107 shows the findings in a series of cases of haemolytic disease of the newborn due to anti-Rh of greater than average severity; the changes in fragility are much less pronounced than in the cases due to ABO incompatibility.

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The saline concentrations corresponding to 50 per cent and 5 per cent lysis can be determined only by estimating lysis in a range of hypotonic

TABLE LIX

OSMOTIC FRAGILITY OF RED BLOOD CELLS IN NORMAL INFANTS (FROM CRAWFORD *et al.*, 1953a)

Age of infant	*Number of cases*	*50 per cent lysis (MCF) per cent NaCl*			*5 per cent lysis per cent NaCl*		
		Mean	*S.D.*	*Mean ± 2 S.D.*	*Mean*	*S.D.*	*Mean ± 2 S.D.*
Cord blood	16	0.422	0.021	0.380–0.464	0.502	0.022	0.458–0.546
2–24 hours	17	0.408	0.015	0.378–0.438	0.486	0.022	0.442–0.530
2–5 days	14	0.395	0.016	0.363–0.427	0.474	0.019	0.436–0.512
Normal adults	18	0.424	0.010	0.404–0.444	0.475	0.012	0.451–0.499

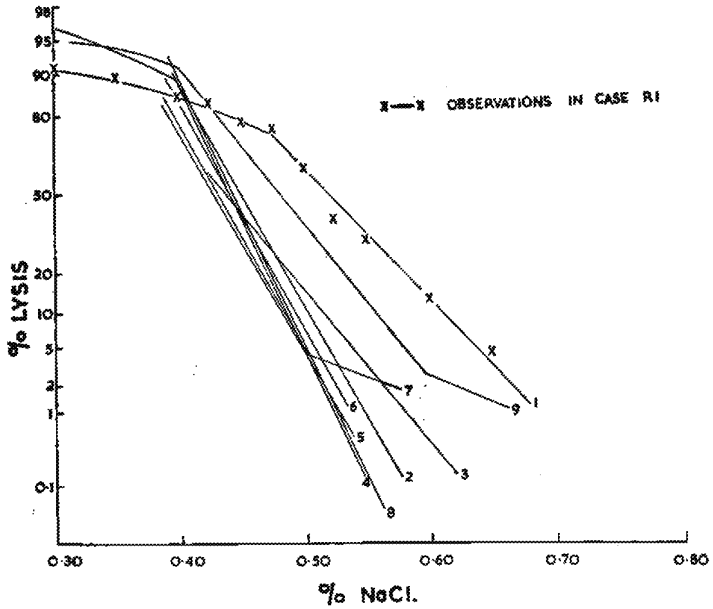

FIGURE 107. Osmotic fragility in nine infants with haemolytic disease of the newborn due to anti-Rh; percentage lysis is plotted on a scale of arithmetical probability (the figure is taken from Crawford *et al.*, 1953a, and the numbers marked against the curves refer to the case numbers in the original paper).

solutions. It is often sufficient to use only a few solutions and accordingly the range of lysis encountered in three solutions is given in Table LX.

Thus, for example, the findings that the red cells of an infant aged 12 hours showed 15 per cent lysis in 0.50 per cent saline and 3 per cent lysis in 0.55 per cent saline would be almost certainly abnormal.

TABLE LX

RANGE OF HAEMOLYSIS IN THREE DIFFERENT SALINE SOLUTIONS OF RED CELLS OF NORMAL NEWBORN INFANTS

(Based on data of Crawford *et al.*, 1953a)

Age of infant	*Percentage lysis in following three NaCl solutions*		
	0.55%	0.50%	0.45%
Cord blood	0–3	1–15	5–57
2–24 hours	0–2	1–9	4–42
2–5 days	0–1	1–7	4–22

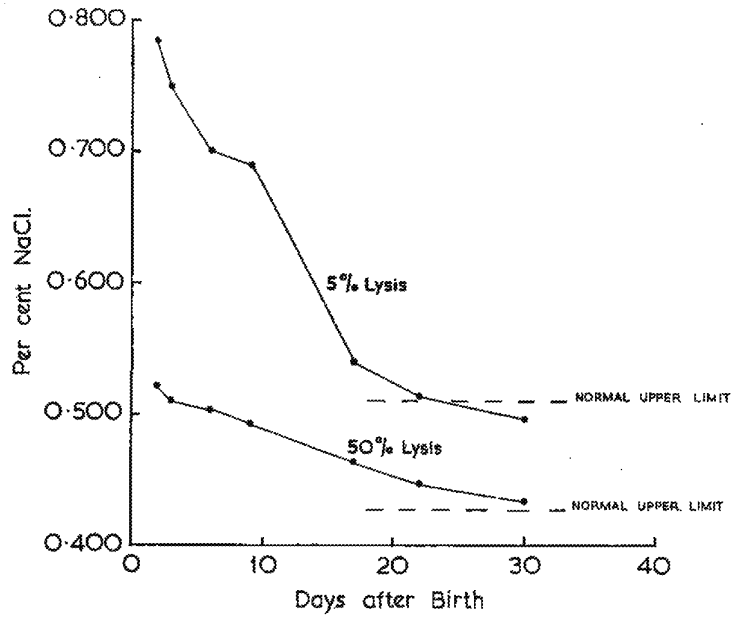

FIGURE 108. Changes in osmotic fragility during the first month of life in an infant with haemolytic disease of the newborn due to anti-A.

In haemolytic disease of the newborn due to ABO incompatibility, the changes in osmotic fragility may persist for as long as 2 or 3 weeks after birth, see Fig. 108. While osmotic fragility is distinctly increased, micro-

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spherocytosis can be observed in films of peripheral blood and the mean corpuscular haemoglobin concentration (MCHC) may be slightly increased (Crawford *et al.*, 1953a).

Changes in Bilirubin Concentration

Although jaundice appearing within 24 hours of birth in a mature infant should always suggest the diagnosis of haemolytic disease, absence of jaundice does not exclude haemolytic disease. Thus in the series reported by Crawford *et al.* (1953a) there were two infants (out of eleven) who never became jaundiced despite having evidence of a moderately severe haemolytic process (reticulocyte counts of approximately 15 per cent).

There are relatively few observations of the maximum bilirubin concentration attained in untreated haemolytic disease of the newborn due to ABO incompatibility. Values of 20-26 mg. were noted in two cases in the series of Crawford *et al.* (1953a). In a case described by Grumbach and Gasser (1948) kernicterus developed.

Survival of Transfused Red Cells

Red cells of the same ABO group as the infant undergo random destruction if transfused to an infant with haemolytic disease due to ABO incompatibility (Boorman, Dodd and Trinick, 1949; Mollison and Cutbush, 1949b; Crawford *et al.*, 1953a; Zuelzer and Kaplan, 1954c).

Fig. 109 shows observations of the survival of group A and group O red cells transfused to an affected infant. It should be emphasized that the donor was an adult of group A_1 and it may be taken as certain that the transfused red cells were destroyed more rapidly than the infant's own cells. In this connection a case reported by Carpentier and Meersseman (1956) is of interest. A group A infant with haemolytic disease due to anti-A was treated by exchange transfusion using A_1 blood from an adult. Following the exchange transfusion the direct antiglobulin test became positive and the infant developed haemoglobinuria; after a second exchange transfusion with A blood there was temporary improvement but haemoglobinuria then recurred and the infant died.

Diagnosis and Treatment of the Individual Case

Possible Screening Tests

Since almost all cases of haemolytic disease of the newborn due to ABO incompatibility occur in the offspring of group O mothers, ante-natal tests should presumably be confined to women of this group. The finding of

immune anti-A or anti-B in a pregnant group O woman implies only that her infant *may* be affected; according to Reepmaker and van Loghem (1953) two out of three infants born to such women appear to be completely normal. Nevertheless, a screening test to detect only the most potent examples of immune anti-A or anti-B, for example those with a haemolysin titre of 10 or more, might well prove valuable by predicting at least some cases of ABO haemolytic disease. In all probability estimation

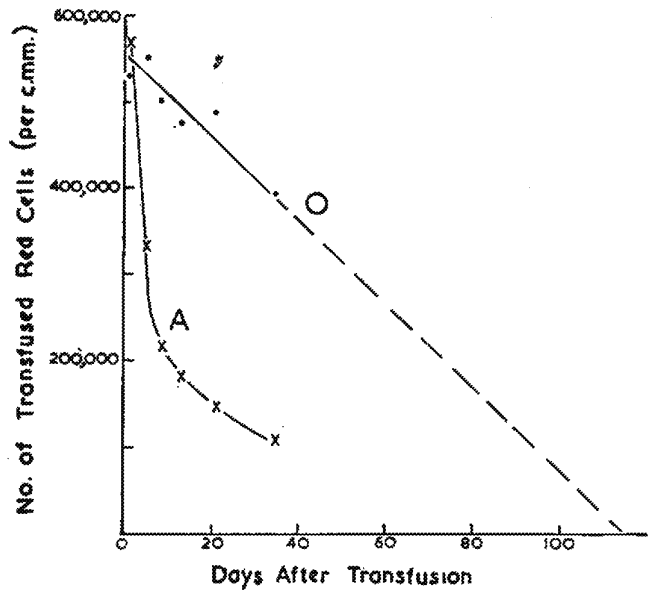

FIGURE 109. Survival of group A and group O cells, transfused 9 days after birth to an infant with haemolytic disease of the newborn due to anti-A; approximately equal numbers of the two types of cells were transfused. The estimates have been corrected for changes in body weight during the period of observation (from Crawford *et al.*, 1953a).

of the amount of 7*S* anti-A and anti-B, as suggested by Kochwa *et al.* (1961), would be the best method, although a simple technique is not yet available.

The routine testing of cord blood using Rosenfield's (1955) 'modified' direct Coombs test, might seem to be a better method of detecting cases. However, a very large number of tests would have to be made to detect a very few seriously affected infants. A positive test would not by itself be

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an indication for treatment but only for vigilance—that is to say, for keeping a special watch for the development of early jaundice.

In view of the rarity of severe cases of haemolytic disease of the newborn due to ABO incompatibility the most reasonable plan seems to be not to attempt to test all newborn infants, but instead to investigate only infants who develop early jaundice.

Tests in Suspected Cases

When a mature infant develops jaundice within the first 24 hours of life the diagnosis of haemolytic disease should be suspected. If the direct Coombs test, carried out by the standard method, is negative or only weakly positive, haemolytic disease due to ABO incompatibility becomes the most likely diagnosis. The ABO groups of the mother and infant should be determined. If the mother is group O and the infant A (or B) the diagnosis should be considered probable. The direct antiglobulin test, using the technique of Rosenfield, is likely to be positive and the indirect antiglobulin test on the infant's serum against adult A_1 (or B) cells should also be positive.

The mother's fresh serum should be incubated at 37° C. with the infant's red cells and with adult red cells of the same ABO group as the infant. Failure of the mother's serum to haemolyse the infant's red cells makes the diagnosis of haemolytic disease of the newborn due to ABO incompatibility unlikely; failure of the mother's serum to haemolyse adult red cells of the same ABO group as the infant virtually excludes the diagnosis.

The infant's haemoglobin concentration should be determined but is likely to be within normal limits; the reticulocytes should be counted; if the infant is less than 48 hours old they may be above normal limits, but only if the infant is rather severely affected. Red cell osmotic fragility should be determined; a raised osmotic fragility is often the only definitely abnormal sign.

In any case, the infant's plasma bilirubin concentration should be determined since if the value seems likely to reach 20 mg./100 ml. or more, exchange transfusion must be considered.

Syndromes which may have to be distinguished from Haemolytic Disease of the Newborn

Deep Jaundice in Premature Infants

As described in the previous chapter, post-natal hyperbilirubinaemia is more intense in premature infants than in mature infants, presumably due

to the slower development in premature infants of the enzyme which conjugates bilirubin. In premature infants with no evidence of a haemolytic process the serum bilirubin may rise to levels at which kernicterus develops (Aidin *et al.*, 1950; Zuelzer and Mudgett, 1950). Although when deep jaundice develops in a premature infant without serological evidence of haemolytic disease of the newborn it may be impossible to find any particular cause for the jaundice, the following conditions which may also affect mature infants should be considered.

Overdosage with Synthetic Vitamin K Analogues

The first definite warning of the danger of injecting large doses of vitamin K into newborn infants came from Allison (1955). He reported that in a series of cases of haemolytic disease of the newborn in premature infants, treated with 30 mg. of 'synkavit' a day, signs of haemolytic anaemia and kernicterus had developed; he noted that up to 67 per cent of the red cells contained Heinz bodies. Gasser and Karrer (1948) and Gasser (1953) had previously described a haemolytic anaemia with inclusion bodies in premature infants which may well have been due to vitamin K. Indeed Gasser (1953) carefully considered this possibility, but felt that the failure of vitamin K to induce Heinz body formation in rats suggested that a different agent was responsible.

Allison *et al.* (1956) have shown that in vitamin E deficient rats 'synkavit' will cause red cell destruction with haemoglobinaemia and that in the rat only occasional Heinz bodies are seen.

It had previously been shown by György *et al.* (1952) that the red cells of newborn infants were far more susceptible to damage by hydrogen peroxide than the red cells of adults and that this susceptibility could be prevented by the administration of vitamin E. Gordon and de Metz (1952) showed that this susceptibility to haemolysis by hydrogen peroxide was greater in premature infants, suggesting that they were more deficient in vitamin E.

The hazard of giving large doses of vitamin K to premature infants was confirmed by Laurance (1955). He found that on changing from a dosage of 10 mg. of 'synkavit' once on admission to 10 mg. t.d.s. for 3 days, within a short period six infants developed clinical signs of kernicterus and died.

It appears that the administration of 10 mg. of 'synkavit' a day for 3 days is probably harmful (Bound and Telfer, 1956; Meyer and Angus, 1956) although the evidence suggests that the effect is not very striking. In any case it is certainly unnecessary to give as much as 10 mg. of 'syn-

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kavit' a day for there is evidence that a single dose of 0.5 mg. is as effective as a dose of 5 mg. in influencing the prothrombin time of newborn infants (Lehmann, 1944). Moreover it is possible that 'synkavit' is more toxic than some other synthetic vitamin K analogues (Allison *et al.*, 1956). For example, Corner *et al.* (1960) have used instead vikastab (dipotassium menaphthone bisulphate). In a series of premature infants treated with as much as 20-30 mg. of this drug given as 5 mg. doses at 12-hour intervals serum bilirubin levels were no higher than in infants receiving none of the drug at all.

Hereditary Spherocytosis in Newborn Infants

In reviewing a series of some forty infants with hereditary spherocytosis, collected from the literature, Burman (1958) noted that 50 per cent were jaundiced in the first few days of life.

Even in infants with little or no jaundice the characteristic signs of the disease are likely to be present at birth as in the following case:

> The cord blood of infant M. was tested because its mother was known to have hereditary spherocytosis; the results were as follows: haemoglobin concentration, 15 g./100 ml.; packed cell volume, 44 per cent; reticulocytes, 8 per cent. Blood group O, Rh-positive; direct Coombs test, negative. Blood film: microspherocytosis ++. Osmotic fragility, increased (50 per cent lysis in 0.48 per cent NaCl; 5 per cent lysis in 0.61 per cent NaCl). At the age of 48 hours the serum bilirubin concentration was 3.7 mg./100 ml. and the infant was faintly jaundiced. At the age of 3 weeks the haemoglobin concentration was 7.5 g./100 ml. but it did not subsequently fall any further.

Deep jaundice has been described in newborn infants with hereditary spherocytosis; Smith and Franklin (1957) reported a case in which the bilirubin concentration reached 26 mg./100 ml. and exchange transfusion was successfully carried out.

Congenital Non-Spherocytic Haemolytic Anaemia

Infants with this condition may suffer from neo-natal jaundice (for references see Erlandson and Hilgartner, 1959).

Deficiency of Glucose-6-Phosphate Dehydrogenase (G-6-P D)

A deficiency of this enzyme is encountered as a sex-linked inherited trait. The red cells of affected subjects are suspectible to damage by a wide variety of substances (e.g. primaquine (an anti-malarial drug), sulphonamides, fava beans). The frequency of the gene is relatively high in parts of the world where malaria is, or has recently been, prevalent

(apparently because the enzyme deficiency confers some protection against malaria). In parts of Sardinia the incidence of the gene reaches 30 per cent (Siniscalco, 1959) and it was in Sardinia that some of the first cases of neonatal jaundice associated with the enzyme-deficiency were reported (see Panizon, 1960).

G-6-P D deficiency appears to be a fairly common cause of neonatal jaundice not only in Sardinia but also in Malaya (Smith and Vella, 1960; Wetherall, 1960) and in Greece (Doxiadis *et al.*, 1961). In most cases no external sensitizing agents (e.g. drugs) can be identified.

The incidence of G-6-P D deficiency is very low in people of northern European origin.

Galactosaemia

In this rare condition the liver is enlarged and there is hyperbilirubinaemia. A case requiring exchange transfusion was described by Norman (1957).

Bruising

Rausen and Diamond (1961) described four infants with severe ecchymoses with bilirubin levels reaching 20-25 mg./100 ml. They supposed that the high bilirubin levels were due to pigment derived from the extravasated blood.

Jaundice in Infants of Pre-Diabetic Mothers

Miller *et al.* (1944) showed that erythroblastaemia was a very common feature in infants born to diabetic mothers and to pre-diabetic mothers and Zetterström *et al.* (1958) showed that hyperbilirubinaemia was common in these infants.

Congenital Syphilis

Congenital syphilis when severe may cause jaundice, anaemia and erythroblastaemia, but it is then likely to produce skin lesions and other characteristic stigmata; the diagnosis can be established by demonstrating the X-ray appearance of osteochondritis and by the finding of a positive Wassermann reaction.

Sepsis

In many published accounts of sepsis as a cause for jaundice and anaemia, other causes, particularly serological causes or congenital red cell defects, have not been adequately excluded. Nevertheless, it is generally believed

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that sepsis will by itself cause jaundice and anaemia (see Erlandson and Hilgartner, 1959).

Hepatitis in Newborn Infants

Occasionally, an infant is born alive with hepatitis and may appear at first sight to be affected with haemolytic disease of the newborn.

Infant MacM., a second infant, became jaundiced 5 hours after birth. When admitted to hospital at the age of 24 hours, it was deeply jaundiced and the liver and spleen were enlarged. Venous blood from the infant gave a negative direct antiglobulin test; the blood group was O. Other findings: haemoglobin concentration, 19.7 g./100 ml.; reticulocytes, 6 per cent; bilirubin concentration, 21.6 mg./100 ml.

A negative direct antiglobulin test in a group O infant excludes all forms of haemolytic disease due to blood group antibodies; in the present case the normal haemoglobin concentration and reticulocyte count suggested that the infant did not have any form of haemolytic anaemia.

The infant died at the age of 36 hours and was found at autopsy to have severe fibrosis of the liver (further details are given as 'Case 2' by Dible *et al.*, 1954).

Exchange Transfusion for Hyperbilirubinaemia unassociated with Haemolytic Disease of the Newborn

Killander *et al.* (1960a) reported that in their experience 2-3 per cent of full-term and 9-10 per cent of premature infants developed bilirubin values of 20 mg./100 ml. or more. They compared bilirubin levels in forty-two untreated mature infants with those of thirty-one mature infants treated by exchange transfusion (in four cases treated by more than one exchange transfusion). In no case, either in the treated or untreated series, did kernicterus occur subsequently.

They also treated by exchange transfusion twenty-three premature infants with serum bilirubin concentrations reaching 20 mg./100 ml. All these recovered without kernicterus and the authors compared this with their experience in the previous 2-year period during which exchange transfusion had not been used, when there had been six deaths (presumably from kernicterus).

When exchange transfusion is used solely to remove bilirubin (rather than to remove damaged red cells as well), it seems important to use fresh blood. For example, suppose in an exchange transfusion 300 ml. of plasma with a mean bilirubin concentration of 15 mg. were removed, that is, a total of 45 mg. of bilirubin; and suppose that following the transfusion,

because the blood has previously been stored for a few days, 3 per cent of the red cells remaining in the circulation at the end of the exchange were removed during the next few hours by the R.E. system. If the blood volume were 250 ml., the amount of haemoglobin broken down would be very approximately $\frac{3}{100}$ (2.5×15) g. or about 1.2 g. This would yield approximately 50 mg. of bilirubin (see Chapter XIV), that is to say, as much as was removed in the plasma. Nevertheless Killander *et al.* (1960b), using blood which had been stored for up to 72 hours, found that infants (without haemolytic disease) treated by exchange transfusion had lower bilirubin levels than controls. No difference was observed between cases in which whole blood was used for the exchange and those in which a concentrated red cell suspension was used.

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RCPE0002068_0388

INDEX

A and B antigens
  added to group O plasma, 485–7
  in animal products, 279–81
  in body fluids, 257
  in cervical secretion, 230
  in epidermis, 260
  in meconium, 140, 278
  immunizing effect, 140, 224–5, 273–82
  in leucocytes and platelets, 259–60
  in red cells, 380–1
  in saliva, 257–9, 383–4
  in serum, 254, 379
  in sperm, 230, 260
  in various secretions, 257
  'partial neutralization', 268
A_1 and A_2 (also A_1B and A_2B) antigens
  distinction *in vitro*, 262, 381–2
  distinction *in vivo*, 262–3, 442, 453–4
  in haemolytic disease of the newborn, 668
  in newborn infants, 255, 661
A_3, A_4, A_5, A_m, A_0 and A_x antigens, 251–3, 256, 259, 275–6, 454
A^P antigen (and anti-A^P), 272, 400
ABO system
  and disease, 215–16, 255–6
  antibodies, *see* Anti-A_1, -A, -B, -H(O)
  antigens, *see* A and B, etc.
  determination of ABO group, 380–1
  discovery, 211
  gene frequencies, 214
  sub-groups, 250–3, 381–2
ABO incompatibility
  destroying spermatozoa, 230–1
  effect on response to Rh antigen, 312–13, 323
  in blood transfusion, 441–3, 445–7, 453–4, 456–60, 484–95, 497–500, 508
  in preganancy, 281–2, 655–8
Absorption
  of antiglobulin serum, 405–6
  of blood group antibodies, 428–9
ACD, *see* Acid-citrate-dextrose solution
Acid-citrate-dextrose solution
  modifications, 17–20
  original solution, 17
  swelling of red cells in, 17
Acidification of blood for preservation, 16–17
Acidification of serum
  in detecting cold haemolysins, 355
  in detecting incomplete warm antibodies, 357
Acquired haemolytic anaemia
  antiglobulin test, 356–7
  cold antibody type, 200, 354–6
  compatibility tests, 426–7
  estimation of red cell life-span, 189–202
  survival of transfused red cells, 194–200
  warm antibody type, 198–202, 357–8
Adenine
  as red cell preservative, 22–3
Adenosine
  addition to stored blood, 20–4
  presence in shed blood, 570
  toxicity 23, 570
Adenosine triphosphate (ATP) in stored red cells, 13, 20, 22, 31
Adjuvants
  in 'raising' antiglobulin sera, 404
Ag group, 248
Agammaglobulinaemia, *see* Hypogammaglobulinaemia
Age of red cells
  effect on storage properties, 11
Agglutinated red cells
  harmful effects *in vivo*, 488
  survival after transfusion, 459, 487–8
Agglutinogens, *see* A_1, Rh, etc.
Agglutinins, *see* Anti-A, etc.; *see also* Auto-antibodies; *see also* 'Non-specific' anti-bodies
'Agla' syringe, 53
AHF, *see* Haemophilia
Air embolism
  during exchange transfusion, 649
  fatal amounts in animals, 574
  in donors, 5
  in recipients, 574–6
  signs, 574–5
Albumin
  effect of injections on plasma volume, 98
  fate after injection, 36, 55
  for detecting 'immune' anti-A, 271
  for detecting incomplete antibodies, 236
  in treatment of cirrhosis, nephrosis, 36–8
  'turnover', 55

RCPE0002068_0389

Albumin-agglutinating antibodies, *see* Incomplete antibodies
Allergic subjects
  as donors, 3, 547–8
  as recipients, 546–8
Allergic reactions, 546–9
  due to nickel sensitivity, 547
  effect of antihistamines, 548
  effect of steroids, 548–9
Alpha globulin, 307
Alsever's solution, 18
Amidopyrin, *see* Febrile reactions
Ammonia in transfused blood, 570
Amniotic fluid
  anti-Rh in, 599
  bleeding into, from foetus, 589–90
  blood-group substances, 257, 304
  colour in haemolytic disease of the newborn, 610–11
  gamma globulin in, 599–600
Amorphic genes, 213
Amount of blood to transfuse
  in exchange transfusion in infants, 629, 645–6
  in treating anaemia in adults, 114–15
  in treating anaemia in infants, 595
  in treatment of acute haemorrhage, 101
Anaemia
  effect on circulation, 97–8
Analbuminaemia, congenital, 38
Anaphylactic shock, *see also* Allergic reactions
  prevention by steroids, 548
Animal blood groups
  cattle, 283
  rabbits, 139
  sheep, 283
Animal transfusion experiments
  effect of incompatible plasma transfused to dogs, 493
  incompatible transfusions in dogs, effect on kidneys, 501
  inter-species transfusions, 441, 496–7, 501
  normal red cell survival in dogs, 147
  normal red cell survival in rabbits, 170–1
  survival of incompatible red cells in dogs, 440, 449, 457
  to assess viability of stored red cells, 15
Antenatal tests
  to predict ABO haemolytic disease, 662
  to predict Rh haemolytic disease, 607–10
Anti-A and anti-B
  absence in hypogammaglobulinaemia, 260–1
  absorption following transfusion, 226, 274–5
  applied to differential agglutination, 135–41
  as cause of haemolytic disease of the newborn, 661–3
  destruction of donor's red cells by, 441–3, 446–7, 453–4, 457–9
  destruction of recipient's red cells by, 485–95
  development of, 266
  effect of age on iso-agglutinin titre, 266
  haemolysin, 219, 267–8, 270, 272–3, 597, 661–3
  haemolysis of 'PNH' cells, 268
  immune anti-A stimulated by A_4 cells, 275–6
  in cervical secretions, 230–1
  in leukaemia, 261
  in myelomatosis, 261
  in newborn, 265–6
  incomplete form, 267, 387, 415
  'linked' anti-AB, 261
  'missing' isoagglutinins, 260–1
  persistence of 'immune' anti-A, 224, 281, 283
  placental transfer, 597–8, 661–3
  production in rabbits, 139
  relation of isoagglutinin titre to immune characteristics, 273
  seasonal fluctuations, 223
  serological characteristics, 267–72
  7*S* and 19*S*, 219, 267, 597, 661–3
  stimulated by meconium, 140, 278
  stimulated by saliva, 278
  stimulated by urine, 278
  stimulation by animal substances, 224, 279–81
  stimulation by incompatible blood, 225, 273–8
  stimulation by vaccines, 280–1
  stimulation in heterospecific pregnancy, 281–2
  stimulation of anti-B by A substance, 279
Anti-A_1
  clinical importance, 262–3
  in seeds of *Dolichos biflorus*, 262, 382
  influence of inheritance, 262
  isolation by absorption of group B serum, 382
  occurrence in serum of A_2, etc., 262–3
  reactions with red cells of newborn, 255, 661
  stimulated by A_1 blood, 271–2

Anti-A^P, 272, 400
Antibodies, *see also* Anti-A, Anti-Rh, etc.; *see also* Incomplete antibodies; *see also* Antibody production
  absorbed by incompatible transfusion, 444–9
  absorption *in vitro*, 428–9
  against adsorbed gamma globulin, 246–8
  distinction between naturally-occurring and immune, 216–17
  effect of storage, 376–7
  elution, 430–2
  isolation, 427–32
  identification, 432–5
  labelled with ^{131}I, 308–9
  naturally-occurring, 216–17, 220–3
  relative clinical importance, 220–1, 227–8
  7*S* and 19*S*, 217–20, 235, 596–9
Anti-B, *see* Anti-A and anti-B
Anti-Be^a, *see* Be^a antigen
Antibody production; *see also* Antigenicity
  competition of antigens, 313
  effect of heredity, 262–6
  effect of dose of antigen, 223
  in newborn, 220, 265–6, 425
  individual variation, 226
  inhibited by excess antigen, 228
  persistence of anti-Rh, 224
  persistence of 'immune' anti-A, 224
  primary and secondary response, 223–4
  role of spleen, 226–7
  seasonal fluctuations, 223
Anti-By^a, *see* By^a antigen
Anti-C
  as a single antibody, 297, 305, 316
  occurring with anti-D, 315–16
  term applied by Moss to antibody in group O serum, 261
Anti-C^w, 310, 316
Anti-C^x, 310
Anti-c, 297, 305, 316–17, 324–5, 418, 435, 457, 496
Anti-cellano, *see* Kell blood group system
Anticoagulants
  anti-complementary effect, 234–5, 412–13
  citrate, 8
  ethylene diamine tetra-acetic acid (EDTA), 9–10, 20, 234–5, 412–13
  heparin, 8, 234
  oxalate, 60
  sequestrene, *see* EDTA
  versene, *see* EDTA
Anti-complement
  anti-β_1 globulin, 238–41, 402–3, 407–8
  immuno-conglutinin, 244–6
Anti-D (*see also* anti-Rh)
  formed by D-positive persons, 314–15
Anti-d, 295, 317
Anti-Di^a, *see* Diego blood group system
Anti-E
  immune, 315–17, 324–5
  naturally-occurring, 309
Anti-e
  in haemolytic anaemia, 198–9, 316
Anti-f (ce), 302, 316
Anti-Fy^a (and -Fy^b), *see* Duffy blood group system
Anti-G, 302–3, 315–16
Antigen sites
  number on a single Rh-positive red cell, 309
Antigenicity
  of C, 315–16
  of c, 316–17, 324–5, 418
  of D, 310–11, 321–5
  of D^u, 313, 315
  of E, 316–17, 325
  of e, 316
  of various blood group antigens, 227–8
Antigens, blood group, *see* A, B, Rh, etc.
Antiglobulin test, 237–43, 402–17
  anti-beta$_1$ (complement), 239–42, 244–5, 402–3, 407–8, 412–14
  as compatibility test, 421–3
  consumption of antiglobulin, 232, 304, 436
  detecting 7*S* and 19*S* antibodies, 241–2, 402–3, 407–8, 416–17
  direct test, 409–11
  'false' positive, 372, 406
  gamma-globulin neutralization test, 238, 416–17, 435
  in acquired haemolytic anaemia, 416
  indirect test, 411–16
  in haemolytic disease of the newborn (ABO) 658–60
  in haemolytic disease of the newborn (Rh), 617, 621, 634–5, 644
  'mixed' antiglobulin test, 143, 416
  positive after cell-storage in frozen state, 376
  positive after transfusing incompatible plasma, 491, 494–5
  positive after transfusing incompatible red cells, 447–8, 529
  positive in normal subjects, 358–9

RCPE0002068_0390

Antiglobulin test (*contd*)
preliminary testing of reagent, 407–9
production of antiserum, 403–5
prozones in, 414
removal of heteroagglutinins, 405–7
role of complement in, 238–41, 407–8, 412–14
with enzyme-treated cells, 285, 344, 415–16
Anti-H(O)
accompanying anti-Le^b, 290
cold agglutinin, 263–5
identification, 434
in 'Bombay bloods', 258, 265
in eel serum, 382–3
Lotus tetragonolobus, 265, 382
normal incomplete cold antibody, 265, 352–4
Ulex europaeus, 265, 382
Anti-haemophilic globulin, *see* Haemophilia
Anti-histamine drugs
effect on red cell preservation, 13
in prophylaxis and treatment of allergic reactions, 548
in febrile reactions, 545
Anti-I, anti-i; *see* Ii antigens
Anti-Jk^a (and -Jk^b), *see* Kidd blood group system
Anti-K (and anti-k), *see* Kell blood group system
Anti-Le^a (and -Le^b and anti-Le^c), *see* Lewis blood group system
Anti-Lu^a (and anti-Lu^b), *see* Lutheran blood group system
Anti-M (and anti-N), *see* MNSs blood group system
Anti-O, *see also* Anti-H(O)
destroying red cells *in vivo*, 464
Anti-P, anti-P$_1$, etc., *see* P blood group system
Anti-Rh (anti-D or anti-Rh$_0$)
albumin-agglutinating, *see* incomplete
as cause of haemolytic disease of the newborn, 602–3
blocking, *see* incomplete
destruction of red cells *in vivo*, 447–51, 469–75, 509
detected by antiglobulin test, 307
detected with enzyme-treated cells, 307
effect of poliomyelitis vaccine, 313
formed by D^u and other D-positive persons, 314–15
incidence in random Rh-negative blood donors, 419
incomplete, 224, 236, 305–9
in differential agglutination, 136–8
in transfused plasma, 495–6
labelled with ^{131}I, 300, 308–9, 472
maternal titre and severity of haemolytic disease of the newborn, 607–10
naturally-occurring, 309–10
persistence in immunized persons, 325–6
placental transfer, 598, 607
'prozones' in titrating, 308
saline agglutinin, 224, 236
similar antibody in animals, 326–7
stimulation affected by ABO group, 312–13, 323
stimulation affected by dose of antigen, 224
stimulation by D^u blood, 313–15
stimulation by intramuscular blood, 311–12
stimulation by pregnancy, 317–25
stimulation by transfusion, 310–11, 314–15
stimulation in volunteers, 310–13
Anti-S (and anti-s), *see* MNS blood group system
Anti-7*S* and anti-19*S* gamma globulin; *see* Antiglobulin test
Anti-T, *see* T receptor
Antiseptics
for skin, 3, 4
Anti-tetanus serum, 269, 281, 491
Anti-Tj^a, *see* P blood group system
Anti-U, *see* MNSs blood group system
Anti-V, 316–17
Anti-Vw, *see* MNSs blood group system
Anti-Vel, *see* Vel antigen
Anti-Wr^a, *see* Wr^a antigen
Anti-X, *see* Lewis blood group system
Anti-Yt^a, *see* Yt^a antigen
Anuria
incompatible transfusion as cause, 500–1
treatment, 502–4
Arterial transfusion, 100–1
Ascorbid acid
to stop uptake of ^{51}Cr, 154–5
Ashby method, *see* Differential agglutination
Aspirin, *see* Febrile reactions
Atebrin, as a leucocyte label, 32
Atopens in transfused plasma, 546
Auto-antibodies
active only in albumin, 359
cold agglutinins, normal, 350–1
cold antibodies, pathological, 354–7
cold antibodies in paroxysmal haemoglobinuria, 356–7

cold, incomplete, normal, 265, 352–4
warm, incomplete, pathological, 357–8
with I, i specificity, 348–9, 351, 354
with Rh-specificity, 357–8
Automatic pipette, 137
Auto-transfusion
of blood shed into peritoneal cavity, 122
Azide, *see* Sodium azide

B antigen, *see* A and B antigens
B$_3$ and Bw antigens, 253
B-like antigen, 256–7
Bacillus cereus
leading to positive antiglobulin reaction, 369
Bacteria causing haemagglutination, 364–9
Bacteria in stored blood
effect of antibiotics, 550
effect of storage period, 555
fatal effects, 552–3, 555
minimum number detectable on microscopy, 551–2
positive blood culture following transfusion, 553
psychrophilic organisms, 553
Bauhinea purpurea, anti-N in seeds, 332
Be^a antigen and anti-Be^a, 346
Benzidine method for estimating haemoglobin, 520–1
Beta-$_1$ globulin, 239–40
Beta-$_{1A}$ globulin, 239–40
Beta-$_{1C}$ globulin, 239–40
Beta-$_{2A}$ globulin, 43, 218
Beta-$_{2M}$ globulin, *see* Gamma globulin, 19*S*
Beta-propiolactone, 559
Bilirubin, amount derived from haemoglobin, 628
Bilirubin excretion in normal newborn infants, 586–7
Bilirubin in plasma
conjugated and unconjugated, 623, 642
estimation, 585–6
increased, *see* Hyperbilirubinaemia
normal values in newborn, 586–7
Biological tests for red cell compatibility
differential agglutination, 524–9
injection of isotope-labelled red cells, 439 *et seq.*
Oehlecker's test, 438
pigment changes (Wiener *et al.*), 521
Bivalent antibodies, *see* Saline agglutinins
'Blocking' antibodies, *see* Incomplete antibodies
Blood group substances, *see* Group specific substances
Blood grouping techniques
ABO grouping, 380–1
antiglobulin test, *see* Antiglobulin test
capillary-tube method, *see* Capillary tubes for blood group tests
Diamond's rapid slide test, 387
enzymes in, *see* Enzymes
grouping cells already agglutinated, 383
on cards, 388–9
slide tests, 380, 387–8
Stratton's sandwich, 388
titration of sera, 389–91
tube method, 379–80
use of colloid media, 390–2
Blood groups, *see also* ABO, Rh, etc.
discovery, 211–13
inheritance, 213–15
related to disease, 215–16, 255–7
terminology, 211
Blood pressure, *see* Systolic blood pressure
Blood volume (*see also* Plasma volume, *and* Red cell volume)
and body specific gravity, 80
and lean body mass, 80
after haemorrhage, 87, 90–5
changes following transfusion, 128–30
in anaemia, 80–1, 84
in cardiac failure, 84–5
in infants, 81–2
in normal adults, 79–80
in obese subjects, 79–80
in pernicious anaemia, 84
in polycythaemia, 83–4
in pregnancy, 82–3
in starvation, 85–6
predicted from height and weight, 79–80
related to haematocrit, 80–1
'Body' haematocrit, *see* Haematocrit, 'Body'
'Bombay' blood, 258–9, 353
Bone marrow
collection for transfusion, 126–7
effect of transfusion on, 116
maximum red cell output from, 210
preservation of, 126–7
transfusion of, 123–7
Bromelin, 396–8
Brucellosis, 564
Burns
anaemia following, 93
area of as guide to transfusion, 102
effect on red cells, 93, 103

RCPE0002068_0391

Burns (*contd*)
plasma transfusion in, 102-3
reduction of plasma volume in, 92-3
By (Batty) antigen, 346-7

C, c antigens (of Rh system), 295-6; *see also* C antigen of Moss, 261
C^w, C^x antigens, 299-300, 316, 347
Ce, ce antigens, etc., 301-3
^{14}C as red cell label, 174
Cadaver blood, 6
Calcium
in citrate toxicity, 564-6
Capillary tubes for blood group tests
agglutination of enzyme-treated cells, 395, 397
agglutination of untreated cells, 384-6
direct antiglobulin, 411
Carbowax, 432
Carcinomatosis
red cell survival in, 203
Cardiac by-pass
anticoagulants, 9-10
compatibility tests, 424
haemoglobinaemia following, 513
Cardiac failure
blood-volume in, 57
precipitated by transfusion, 97-8, 101-2
Cards, blood grouping on, *see* Blood grouping techniques
Chimaeras, 124-5, 229
Chlorhexidine, 4
Chown's test, *see* Capillary tubes for blood group tests
Christmas factor (Factor IX)
transfusion to correct deficiency, 41-2
Chromic chloride
causing red cell agglutination, 361
radioactive, 56
Chromate, *see* Radioactive chromium
Circulatory overloading following transfusion
in haemolytic disease of newborn, 652-4
in hypotension due to infection, 101-2
in severe anaemia, 97-8
signs and symptoms, 132
Citrate
amount to prevent clotting, 8
preservative solutions for blood, *see* Acid-citrate-dextrose
promoting 'cold' agglutination, 265, 351
reducing effect on chromate, 63
toxic effects, 564-6, 568-9, 650
Citrate-phosphate-dextrose solutions
for blood preservation (CPD), 20
for washing red cells prior to 'labelling', 67
Cleaning solution
to remove traces of radioactivity, 70
Clotting factors in stored blood, 38-42
'Cold' antibodies
effect on survival of red cells, 262-3, 265, 459-63, 466
in compatibility tests, 421-2
non-specific, *see* Auto-antibodies
specific cold agglutinins, *see* Anti-A, -A_1, -B, -H, -Le^a, -Le^b, -M, -N, -P_1, etc.
Cold blood, transfusion of
effect on incidence of reactions, 556
in exchange transfusion, 651
producing venespasm, 594
Colloid media, for detecting incomplete antibodies, 236
Colloidal silica,
effect on red cells, 361-2
'Coming-back' phenomenon, *see* Reappearance of absorbed antibodies
Compatibility tests
between donors, 424
effect of pooling cells, or serum, 424
in acquired haemolytic anaemia, 199, 426-7
indirect antiglobulin test, 422-3
in newborn infants, 425-6
in patients having repeated transfusions, 424-5
in urgent cases, 422-4
'minor cross match', 419-20
recommended procedures, 420-7
request form, 420
screening of donors, 419
using mother's serum, 426
Complement
absorption by kaolin, 235, 412
activation inhibited by EDTA, 234-5, 412-13
anti-complementary sera, 234-5
components, 233-4
destruction by heating, 233
fixation by immune anti-A, 267
limiting haemolysis *in vivo*, 449
rate of deterioration during storage, 377-8
role in antiglobulin test, 239-40, 244-5, 402-3, 407-8, 412-14
role in binding antibodies, 240-1, 412, 432
role in conglutination reaction, 239, 244-5
role in haemolysis *in vitro*, 233, 245,
tests compared, 245
use of guinea-pig serum, 140, 243, 268
use of horse serum, 268, 352, 401
use of rabbit serum, 243, 245, 268, 352, 401, 402
Complementoid, 234-5
Complete antibodies, *see* Saline agglutinins
Concentrated red cell suspensions
preparation, 133-4, 646
use for exchange transfusion, 645-6
use in severe anaemia, 133
Congenital haemolytic anaemia, *see* Hereditary spherocytosis, Thalassaemia, etc.
Conglutination
as used by Wiener, 236-7
original sense, 237, 244-6
Cooling
effect of rate on red cells, 26
Coombs test, *see* Antiglobulin test
Corticosteroids
in allergic reactions, 548-9
in incompatible transfusions, 475, 545
in haemolytic anaemias, 113
in Rh-immunized women, 623-4
^{51}Cr, *see* Radioactive chromium
Cross-matching, *see* Compatibility tests

D antigen, 294-5 (*see also* Rh antigens, Anti-Rh(D), etc.)
D^u antigen, 298-301
Dangerous 'universal' (group O) donors
antibodies concerned, 487-92
screening tests, 273-4
Defibrination of blood
in vitro, 377
in vivo, 38-9
Density differences between old and young red cells, 147, 530
Destruction of recipient's red cells by transfused plasma
animal experiments, 493
by anti-A and anti-B, 485-95
by anti-D(Rh) 495-6
by anti-T, 367-8
by other blood-group antibodies, 495-6
summary of clinical effects, 493-6
tests to establish, 532-3
Destruction of incompatible red cells
abnormal bleeding, 499-500
by anti-A and anti-B, 441-3, 446-7, 453-4, 475-9
by anti-A_1, 459-60, 484-5
by anti-C, 484
by anti-c, 451-2, 457, 476
by anti-E, 475-6, 484
by anti-Fy^a, 448, 466-8, 484, 509
by anti-Jk^a, 456, 476, 478-9
by anti-K, 484, 509
by anti-K_p^a, 478
by anti-Le^a and anti-Le^b, 455, 463-5, 509
by anti-Lu^b, 444
by anti-M, 456-7, 460-2
by anti-O(H), 464
by anti-P_1, 457, 466
by anti-Rh(-D), 447-51, 469-75, 509
by anti-S, 478, 509
by anti-s, 478
by anti-Tj^a (-P+-P_1), 458
effect on white cells, 499
in hypogammaglobulinaemia, 459, 498
pigment changes, *see* Hyperbilirubinaemia, etc.
renal failure, 500-2
role of spleen, *see* Spleen
symptoms produced by, 496-8
without demonstrable antibodies, 168, 476-7
Dextran
abnormal bleeding following infusions, 110-11
as an antigen, 110
as cause of rouleaux formation, 108
as medium for detecting incomplete antibodies, 236, 387
as plasma substitute, 107-11
Dextraven, 109
in estimating plasma volume, 56-7
Intradex, 108-9, 111
Macrodex, 108-9
molecular weight, 'weight average' and 'number average', 107-9
reactions following injection, 109-11
'sludging' following transfusion, 108
Dextrose
as cause of post-transfusion haemolysis, 510
consumption by red cells, 11
in red cell preservation, 15-16
in treatment of anuria, 502-3
Dialysis, to remove glycerol, 375-6
Di^a antigen, *see* Diego blood group system
Diego (Di) blood group system, 344-5
$DF^{32}P$, *see* Radioactive Di-isopropyl-fluorophosphate

RCPE0002068_0392

Differential agglutination
antisera for, 136, 138–40
as test of compatibility, 524–9
detecting foetal cells in mother, 588
direct method, 142–4
error, 140–2
for estimating red cell life-span, 134–52
indirect method, 134–42
Differential haemolysis, 135, 140
Dimethyl sulphoxide
storage of marrow, 127
storage of red cells, 30
Diphosphoglycerate (DPG) in red cells, 16
Direct Coombs test, *see* Antiglobulin test
Disinfection of skin, 3
Disseminated lupus erythematosus
red cell survival in, 205
tendency to form antibodies, 226
Dolichos biflorus
anti-A_1 in seeds, 226, 382
Donath-Landsteiner reaction, 241, 356
Donors
air embolism in, 5
dangerous 'universal', *see* Dangerous universal donors
embolism by stilette of needle, 5
exclusion of D^u, 300–1
fainting in, 2
false positive serological reactions in, 6
genotyping of Rh-negative, 300–1
hepatitis carrier rate, 556–7
history of jaundice in, 3
hypertensive, 2
iron requirements of, 95–7
jaundice in, 3
liver function tests in, 557–8
minimum Hb concentration, 2
protection of, 1
selection of, 1
screening for unusual antibodies, 419
Duffy blood group system, 341–3
behaviour of anti-Fy^a *in vitro*, 342–3
effect of anti-Fy^a *in vivo*, 342–3

E, e antigens, 295
Echinococcus cyst fluid, 336, 434
'Edglugate-Mg' solution, 10
EDTA (*see also* Anticoagulants)
as a blood preservative, 20
as anticoagulant, 9–10
excess causing cell shrinking, 60
to block the activation of complement, 234–5, 412–13
toxicity, 567
Eel serum, 382–3
Elliptocytes, transfusions of, 176
Elliptocytosis
inheritance linked to *Rh*, 215
Elution of antibodies
in compatibility tests, 199, 427
in detecting weak antigens, 253
role of complement, 240, 412
'Elution' of chromium, *see* Radioactive chromium
Embolism
by air, *see* Air embolism
by stilette of needle, 5
Enhancement of agglutination by serum, 270–1
Enzymes,
bromelin, 396–8
enhancing haemolysis, 243, 398
ficin, 395–6
in detecting auto-antibodies, 357
Löw's method, 396–7
papain, 394
treated cells for absorption, 406, 429
trypsin, 393–4
Epidermis
blood group antigens, 260
embolic particles, 577
Erythroblastaemia
following haemorrhage *in utero*, 589
in haemolytic disease of the newborn, 616
in infants born to pre-diabetic mothers, 673
in normal newborn infants, 581
Erythroblastosis foetalis, *see* Haemolytic disease of the newborn
Erythroctye, *see* Red cell
Ethylene diamine tetra-acetic acid (EDTA), *see* Anticoagulants
Evans blue, *see* T-1824
Exchange transfusion in adults, 113–14, 132
Exchange transfusion in infants
apparatus, 646–7
blood picture following, 637–8
efficiency of, 643–5
evidence of value, 629–31, 674–5
hazards of, 649–52
indications for, 639–42, 674–5
nomogram for predicting red cell exchange, 645
radial artery, 651–2
rationale, 628–9
saphenous vein, 645, 651

umbilical vein, 646–50
use of Rh-positive blood, 631–2
Extravascular destruction
definition, 505
haemoglobinaemia following, 472–4, 508–9
of stored red cells, 514–16
pigment katabolism following, 507

F, f antigens, 302
Fainting after blood loss, 87–9
Febrile reactions
anti-histamines, 545
amidopyrin, 535
aspirin, 544–5
caused by incompatible red cells, 498–9, 538
caused by incompatible white cells, 538–40
caused by incompatible platelets, 540–1
caused by incompatible antigens in plasma, 541
caused by non-viable white cells, 542
characteristics, 535–7
herpes simplex, 537–8
induced experimentally, 535–7
in infants, 537
latency of pyrogen fever, 537
prophylaxis, 542–5
pyrogens, 535–7
Fibrinogen
effect on rouleaux formation, 359–60
injections in hypofibrinogenaemia, 38–9
Fibrinolysis
post-mortem, 6
Ficin, *see* Enzymes
Filters, for transfusion sets, 576
Fluorescent antibodies
to detect foreign red cells, 318
Foetal haemoglobin
in haemolytic disease of the newborn, 604
in relation to Cr binding, 168
Foetal red cells
detected in maternal circulation, 317–19, 587–9
life-span, 150–2, 166–8
Formol-treated red cells, 429
Forssman antigen, 279
Fragility, *see* Osmotic fragility
'Free' (incompatible) antibody
in haemolytic anaemia ('warm'), 357–8
in haemolytic disease of the newborn, 633–5, 644, 660–1
in normal newborn infants, 597–8
Freezing, *see* Storage of blood
Fresh blood versus stored, 42
Freshly-washed red cells
antibody for, 364
Freund's adjuvant
in preparing antiglobulin serum, 404
'Frühgift', 570
Fy^a, Fy^b, *see* Duffy blood-group system

G antigen, 302–3, 315–16
g (force due to gravity)
effect on cell packing, 59
Galactosaemia
as a cause of neonatal jaundice, 673
Gamma globulin; *see also* Antiglobulin test
denatured by heating, 247
estimation in serum, 416–17
Gm groups, 246–7
immunizing dose in rabbits, 404
in newborn, 43, 220
in treating hypogammaglobulinaemia, 43–4
in prophylaxis of viral infections, 44–5
labelled with ^{131}I, 43, 596
7*S* and 19*S* gamma globulins, 42–3, 217–20, 246, 416–17, 596–9
turnover rate, 43
uptake after intramuscular injection 7*S*, 44
Gamma-1 globulin, 217–19
Gamma-2 globulin, see Gamma globulin, 7*S*, etc.
Gastro-intestinal haemorrhage
estimated with ^{51}Cr, 207–8
Ge antigen, 347–8
Gelatin
as plasma substitute, 110
Gene frequencies, 214; *see also* ABO, Rh
Genotypes, *see* ABO blood group system, etc.
Glass particles
injected intravenously, 576–7
Globulin, *see* Gamma globulin, etc.
Glucose, *see* Dextrose
Glucose-6-phosphate dehydrogenase deficiency as cause of neonatal jaundice, 672–3
effect on red cell life-span, 189
Glycine
^{14}C-labelled, 174
^{15}N-labelled, 172–4
inhibiting rouleaux formation, 360
Glycerol
for storing frozen marrow, 126–7
for storing frozen red cells, 27–30, 373–6
Glycolysis in red cells, 11

RCPE0002068_0393

Gm serum groups, 246-7
Goats
  production of anti-human globulin in, 405
  slow mixing of injected red cells, 65
Guanosine, 21-2, 24
Guinea-pig serum, *see* Complement
Gum arabic
  as plasma substitute, 107

H substance
  in red cells of adults, 253
  in red cells of infants, 255
  in saliva, 257-8
  relation to Le^b substance, 284-6
  secretion related to Lewis group, 284-7
Haptoglobin
  binding plasma haemoglobin, 505-6, 509
  genetics, 245
Haematocrit, 'Body'
  relation to venous haematocrit, 46-8, 76-9
Haematocrit, venous
  cell swelling in ACD, 17
  changes following burns, 102-3
  changes following haemorrhage, 94, 108-9
  effect of oxygenation, 59
  effect of posture, 60-1, 85
  effect of stasis, 61-2
  estimation, 57-62
  normal values in adults, 102
  normal values in children, 102
  normal values in cord blood, 581-2
  normal values in foetuses, 582-3
  relation to 'body' haematocrit, 46-8, 76-9
  relation to plasma volume, 72-3
  relation to red cell volume, 74-5
  relation to total blood-volume, 80-1
  trapped plasma, 57-8
Haemochromatosis, *see also* Haemosiderosis
  effect of repeated bleeding, 97
Haemoglobin
  changes following acute haemorrhage, 93-4
  changes following blood donation, 95-7
  cord blood of normal infants, 581-2
  different in skin-prick and vein, 579-80, 583
  in immature infants, 582, 584
  in normal newborn, 578-84
  in plasma, *see* Haemoglobinaemia
  in pregnancy, 115
  level demanding transfusion, 98, 114-15
  minimum for blood donors, 2
  minimum for injured patients, 98
  minimum for woman near term, 115
  normal variation, 95
  readjustment following transfusion, 127-30
  regeneration after haemorrhage, 95-7
  standardization, 580-1
Haemoglobinaemia and Haemoglobinuria (*see also* Intravascular destruction *and* Extravascular destruction)
  clearance of haemoglobin from the plasma, 505-7
  estimation of plasma Hb, 519-21
  following intravenous water, 510-11
  following red cells through fine needle, 512-13
  following transfusion of lysed red cells, 511-17
  in haemolytic disease of the newborn, 616-17, 668
  pigments in urine, 522-3
  role of haptoglobin, 505-6, 509, 616
Haemolysins
  anti-A and anti-B, 219, 245, 267-8, 270, 398-401
  anti-A neutralized by A substance, 267
  anti-Fy^a, 245
  anti-Jk^a, 398
  anti-K, 245
  anti-Le^a and Le^b, 219, 245, 270, 398
  anti-P+−P_1 (−Tj^a), 219, 336
  anti-P_1, 245, 335
  anti-Vel, 347
  delicate test, 400
  detected by ^{51}Cr release, 400
  Donath-Landsteiner antibody, 241, 356
  effect of heating *in vitro*, 270
  non-specific, *see* Auto-antibodies
  role of complement, 267-8, 270
Haemolysis
  avoidance in taking blood samples, 519
  by bacteria in stored blood, 551, 553
  by saliva, 415
  in stored blood, *see* Stored blood
  in vivo, *see* Haemoglobinaemia
Haemolytic anaemias, *see also* Life-span of red cells *and* Survival of transfused red cells,
  auto-antibodies in, *see* Auto-antibodies (pathological)
  compatibility tests in, 199, 426-7
  role of transfusion, 112-13

Haemolytic disease of the newborn
  amniotic fluid, 610-11
  bilirubin changes after birth, 618-19
  bilirubin changes during exchange transfusion, 638
  bilirubin concentration in cord blood, 614-16
  bilirubin concentration in mother's blood, 611
  circulatory changes, 622, 628, 652-4
  cortisone to mother, 623-4
  deafness as sequel, 623
  definition, 602
  diagnosis and assessment, 638-42
  direct antiglobulin test in, 617
  disease in successive siblings, 604-6
  due to ABO incompatibility, 387, 655-70
  due to anti-Rh, 693-54
  due to other antibodies, *see under various blood group systems*
  effect of immaturity, 612
  effect of Rh phenotype, 606
  efficiency of exchange transfusion, 643-5
  erythroblastaemia in, 616
  exchange transfusion, 628-32, 637-8, 642-54
  father's genotype, 297-9
  foetal haemoglobin, 604
  following transfusion to mother, 606
  haemoglobin changes after birth, 618-21, 632
  haemoglobin changes after exchange transfusion, 632-3, 643
  haemoglobin concentration in cord blood, 603, 612-15, 663
  haem pigments in plasma, 616-17, 668
  hydrops foetalis, 603
  icterus gravis neonatorum, 604
  icterus praecox, 655
  in a D^u infant, 301
  in first pregnancies, 319-21
  in twins, 631-2
  incidence, 602-3
  kernicterus, 613, 622-3, 628
  methods of grading cases, 612-18
  mortality, 612, 614-15
  osmotic fragility of red cells, 665-8
  packed cell volume in, 641
  platelet deficiency following exchange, 637-8
  premature induction of labour, 624-7
  red cell clumping in, 387
  replacement transfusion, *see* Exchange transfusion
  DDD
  reticulocyte count, 604, 616
  Rh-negative transfusions, 632-7, 643
  Rh-positive transfusions, 634-5
  severity in successive siblings, 604-6, 610, 657-8
  severity related to maternal titre, 607-10
  sex difference, 612
  stillbirths, 605, 621, 624-7
  treatment, 628
  treatment of individual case, 638-43
Haemolytic transfusion reactions, *see also* Extravascular destruction *and* Intravascular destruction
  difficulties in investigating, 517-18
  due to incompatibility, *see* Destruction of recipient's red cells, *also* Destruction of transfused red cells
  methods of investigating, 519-29
  routine investigation, 529-33
Haemophilia
  concentrate of animal AHF, 41
  concentrate of human AHF, 41
  plasma transfusions in, 39-40
Haemorrhage
  affecting red cell survival measurements, 207-8
  after massive transfusion, 106
  assessed by wound size, 90, 99
  associated with operations, 92
  blood-pressure as a guide, 91, 99-100
  during and after operation, 92
  effect on body haematocrit : venous haematocrit ratio, 79
  effect on circulation, 87-9, 91
  fatal amount, 87
  following dextran, 110-11
  following incompatible transfusions, 499-500
  from cord, 591-2
  from foetus during parturition, 590-1
  from foetus into maternal circulation, 317-19, 587-9
  from placenta during Caesarean section, 590-1
  gastro-intestinal, estimated with ^{51}Cr, 207-8
  haemoglobin values after acute, 93-5
  in newborn infants, 42, 591-2
  in wound shock, 90-1
  spontaneous restoration of blood volume, 93-5
  treatment by plasma substitutes, 106-11
  treatment by transfusion, 100-2

RCPE0002068_0394

Haemorrhagic disease of the newborn, 42
Haemosiderin in urine, 523
Haemosiderosis
  caused by multiple transfusions, 570–2
  effect of bleeding, 571–2
Haptoglobin
  complex with haemoglobin, 505–6
  different types, 248
  in newborn infants, 248, 616
  quantitative estimation, 522
Heart-lung machines, *see* Cardiac by-pass
Heated red cells
  effect on Rh antigen, 326
  haemoglobinaemia following, 511
  survival *in vivo*, 511–12
Heating serum
  anti-complementary properties, 270
  effect on anti-A, 268–70
  effect on anti-Rh, 306
  effect on complement, 233, 412
  effect on haemolysins, 270
  to destroy heterologous agglutinins, 406
Heparin, *see also* Anticoagulants
  affecting optical density of plasma samples, 59–60
  dose to 'heparinize' a patient, 113
  in thrombophlebitis, 574
  to prevent clots in canulae, 594–5
  use in samples for plasma volume estimation, 53
Hepatitis, *see* Serum hepatitis
Hereditary non-spherocytic haemolytic disease
  in newborn, 672
Hereditary spherocytosis
  in adults, 185–6
  in newborn, 672
Herpes simplex, 537–8
Heteroagglutinins
  absence in germ-free chicks, 222
  destruction by heating, 406
  relation to cold auto-agglutinins, 350
  removal by absorption, 405–6
'Heterospecific' pregnancies, 656
Hexokinase
  in stored red cells, 20
High incidence antigens, 347–9
  use of relatives as donors, 427
Hog pepsin stimulating anti-A, 279–81
Homologous serum jaundice, *see* Serum Hepatitis
Horse serum, *see also* Complement
  stimulating anti-A, 281
Hübener–Thomsen-Friedenreich phenomenon, 365
Hyaluronidase, 362
Hydatid cyst, *see* Echinococcus cyst
Hydrops foetalis, *see* Haemolytic disease of newborn
Hyperbilirubinaemia
  after transfusion of 'incompatible' plasma, 488, 491–2, 494, 496, 517
  after transfusion of incompatible red cells, 448, 464, 516, 524
  after transfusion of stored red cells, 513, 515–17
  following intravascular haemolysis, 507
  in haemolytic disease of the newborn, 614–16, 618, 622–3, 628–31, 640–2
  in normal newborn infants, 586–7, 674
  in normal premature infants, 670–1, 674
  in pregnant Rh-immunized women, 611
  relation to kernicterus, 604, 622–3
  some causes in newborn, 671–4
Hypersplenism, 202
Hypertensive response, following haemorrhage, 89
Hyper- and Hypothyroidism
  red cell survival in, 207
  red cell volume in, 84
Hypoalbuminaemia, *see* Albumin
Hypochromic anaemia,
  effect on red cell life-span, 188
Hypofibrinogenaemia, 38–9
Hypogammaglobulinaemia, 42–4
  absence of isoagglutinins in, 260–1
  estimation of gamma-globulin concention, 416–17
  failure to respond to B substance, 227
  survival of incompatible red cells, 459, 498
'Hypoprothrombinaemia', 42

Ii, 200, 348–9, 351, 354–6
^{131}I, ^{132}I *see* Radioactive iodine
Iberis amara
  anti-M in seeds, 331
Icterus gravis neonatorum, *see* Haemolytic disease of the newborn
Icterus praecox, *see* Haemolytic disease of the newborn
Identification of blood-group antibodies, 432–5
  testing patient's red cells, 530–1
  testing patient's saliva, 530
Immune antibodies
  definition of, 216–17

Immune responses, *see* Antibody production
Immuno-conglutinin, 244–6, 352, 401–2
'Immunoglobulins'
  absent in hypogammaglobulinaemia, 43, 260–1
  definition, 218
Immunological paralysis, 228–9
Immunological tolerance, 229–30, 350
  to A, 281
  to Rh, 323–4
'Inagglutinable counts', *see* Differential agglutination
'Inapparent haemolysis', 525
Incompatible transfusions, *see also* Destruction of transfused red cells, *also* Destruction of recipient's red cells
  abnormal bleeding, 499–500
  between different species, 441, 496–7
  effect on leucocytes, 499
  intramuscular, 277
  pyrexia, 497–8
  renal failure, 500–4
  steroids, 475
  symptoms, 496–8
Incomplete antibodies, 236–43 (*see also* Anti-A, etc.)
  Anti-A and anti-Rh compared, 597
  composed of 7*S* or 19*S* γ, 218–19, 242
  definition, 217
  discovery, 236
  discrepancies between different tests, 243
  terms used, 306
  tests for, *see* Antiglobulin test, *see also* Enzymes and *see also* Colloid media
Indirect antiglobulin test, *see* Antiglobulin test
Infected blood
  blood-grouping hazard, 364–9
  transfusion hazard, 549–56 (*see also* Bacteria in stored blood)
Inheritance
  ability to form antibody, 262–6
  blood group antigens, 213–15
Injection of particulate matter
  'debris' in stored blood, 576
  glass, 576–7
  skin, 577
Inosine, 22–4
Inseparable antibodies, 261, 305, 329, 344
Intra-arterial transfusion, 100–1
Intramuscular injections
  of blood or red cells, 122–3, 276–7, 312
Intraperitoneal transfusion, 119–22
Intrasternal transfusions, 122
'Intravascular agglutination'
  following O to A transfusions, 486 494
  in ABO haemolytic disease, 659–60
Intravascular destruction (haemolysis) associated with symptoms, 497–8
  definition, 505
  following ABO incompatible red cells, 441
  following intravenous water, 510–11
  following red cells mixed with glucose, 510
  following transfusion between species, 441, 497–8, 508
InV, 246
Iron
  deficiency in pregnancy, 115
  gained from transfusion, 118–19
  haemoglobin regeneration, 95–7
  overload following transfusions, *see* Haemosiderosis
  radioactive, *see* Radioactive iron
  serum levels in donors, 95–6
Irradiation
  effect on red cells, 203
Iso-agglutinins, *see* Anti-A, anti-B, etc.
Isolation of antibodies, 427–32

J blood group system in cattle, 283
Jk^a, Jk^b, *see* Kidd blood group system
Js^a, *see* Sutter blood group system

K, k, *see* Kell blood group system
Kaolin
  to absorb complement and complementoid, 235, 412
Kell (K) blood group system, 339–41
  antigenicity of K, 339–40
  anti-K in transfused plasma, 496
  behaviour of anti-K *in vitro*, 341
  destruction of red cells *in vivo*, 341
  haemolytic disease of newborn due to anti-K, 341, 603
Kernicterus, 604, 613, 622–3
Kidd (Jk) blood group system, 343–44
  behaviour of antibodies *in vitro*, 344
  effect of anti-Jk^a *in vivo*, 343–4, 455
Koeppe's criterion
  for complete red cell packing, 59

Le^a and Le^b, *see* Lewis blood group system
Lectins, *see Dolichos biflorus*, etc.

RCPE0002068_0395

Leuco-agglutinins
stimulated by pregnancy, 231-2
stimulated by transfusion, 231-2
Leucocytes
blood group antigens in, 231, 259
detection of leuco-agglutinins, 435-6
normal life-span of, 31-2
post-transfusion survival, 32-3
Leucopenia
following incompatible red cells, 499
Leukaemia
change of ABO group in, 255-6
survival of red cells in, 203, 209
transfusion in, 105-6, 113-14, 209
weak iso-agglutinins in, 261
Levay antigen, 345
Lewis blood group system
adsorption of Lewis substance by red cells, 283, 288
antibodies (anti-Le^a, anti-Le^b, anti-Le^c, anti-X, *Magard*), 285-93
changes in pregnancy, 287
'changing' of Lewis groups, 287-8
detection of antibodies, 240-2, 245, 292, 412-13, 415
destruction of red cells *in vivo*, 293, 454-5, 465
genetics, 284
haemolysis *in vitro*, 289, 292
in relation to haemolytic disease of the newborn, 293
phenotypes of adults, 284-7
phenotypes of infants, 287-8
substances in saliva, 284-8, 291, 384, 530
substances in serum, 284-8
Life-span of red cells, *see also* Survival of transfused red cells
correction for blood-volume changes, 150-1, 160-1, 194
correction for haemorrhage, 207-8
deduced from survival of transfused normal cells, 194-8
effect of transfused red cells, 182-3
in acquired haemolytic anaemia, 189-207
in children, 166
in elliptocytosis, 176, 186
in G-6-PD deficiency, 189
in Hb C disease, 181
in hepatic cirrhosis, 206
in hereditary spherocytosis, 185, 186
in hyperthyroidism, 207
in intrinsic red cell defects, 176-81, 183-5
in iron-deficiency anaemia, 188
in lymphadenoma, 203
in newborn, 150-2, 166-8
in normal adults, 144-50
in P.N.H., 181, 185-7
in pernicious anaemia, 187
in phenacetin overdosage, 207
in polycythaemia vera, 189
in scurvy, 206
in sickle-cell anaemia, 181, 185-6
in sickle-cell trait, 188-9
in thalassaemia, 181, 185, 189
measured by differential agglutination, 145-6
measured with ^{14}C-glycine, 174
measured with ^{15}N-glycine, 172-4
measured with ^{51}Cr, 156-64
measured with ^{59}Fe, 169-70
measured with sulphhaemoglobin, 174-5
red cell production deduced from, 117-18, 205, 210
Linkage
of blood group genes, 215
Lipids, red cell
loss on storage, 13
loss on washing, 364
Lotus tetragonolobus, anti-H in seeds, 265, 382
Low incidence antigens, 345-7
Löw's enzyme method, 396-7
Lu^a, Lu^b, *see* Lutheran blood group system
Lumbar pain, 496-8
Lupus erythematosus, *see Disseminated Lupus Erythematosus*
Lutheran blood group system, 337-9
antibodies *in vitro*, 337
antibodies *in vivo*, 338-9
incidence of anti-Lu^a, anti-Lu^b, 337-8
RNA derivatives and specificity, 304
Lymphadenoma, 203

M and N, *see* MNSs blood group system
Magard antibody, see Lewis blood group system
Malaria
in blood donors, 563
parasites in stored blood, 562-3
red cell compatibility in transmission, 563-4
red cell destruction in, 206
transmitted by intramuscular blood, 312
transmitted by transfusion, 563
Mercapto-ethanol
dissociating macroglobulins, 218-19
Methaemalbumin, 521-2, 616-17

Methaemoglobin
affecting red cell survival, 166
recognition in urine, 522-3
Milk, antibodies in, 600
'Minor cell population'
techniques for demonstrating, 143-4
Mixed agglutination, specific, 259, 260, 304, 392
Mixed antiglobulin reaction
detecting leucocyte antibodies, 416
detecting leucocyte antigens, 304
detecting 'minor population', 143-4
detecting platelet antibodies, 416, 602
detecting platelet antigens, 232
'Mixing' time
of labelled plasma, 50-1
of labelled red cells, 50-1, 64-6
in cardiac failure, 51, 64
in oligaemic shock, 65
in splenomegaly, 51, 64-5, 209
MNSs blood group system, 328-33
anti-M, 329-31
anti-M in plants, 331
anti-M in transfused plasma, 496
anti-M_1, 331
anti-M^6, 333
anti-Mi^a, 329-33
anti-N, 331-2
anti-N in plants, 332
anti-S and anti-s, 332-3
anti-Vw, 329, 333
destruction of red cells *in vivo*, 457
differences between *MM* and *MN*, 455
haemolytic disease of newborn, 330-3, 599
production of antibodies in rabbits, 331
Multiple antibodies
identification of, 435
production of, 226
Mutation, somatic, possible
demonstrated in normal blood, 254
in leukaemia, 255-6
Myxoedema
red cell volume in, 84
N, *see* MNSs blood group system
^{15}N-glycine, 172-4

Naturally-occurring antibodies
definition, 216-17
effect of antigenic stimulus, 224-6
in ABO system, 220, 223, 260-6
in other systems, 221-3
origin, 222
seasonal fluctuations, 223
Needles
bore-sizes, British and American, 104
limiting rate of transfusion, 103-4
injecting procaine, 4
scalp-vein transfusion, 593
taking blood, 4
Neonatal neutropenia, 601-2
Neonatal thrombocytopenia, 602
Nephritis
blood volume in, 85
red cell destruction in, 205
red cell production in, 205
Neuraminidase, 237
'Non-gamma' globulin
shown to be complement, 239
Non-specific antibodies, *see also* Auto-antibodies
versus freshly-washed cells, 364
versus stored cells, 363-4
Non-spherocytic congenital haemolytic anaemia
in newborn, 672

Osmotic fragility of red cells
following incompatible plasma, 458, 488-9, 493, 495-6
following transfusion of stored cells, 31
in ABO haemolytic disease, 665-8
in Rh haemolytic disease, 666
in normal infants, 665-7
in stored blood, 13
old and young cells, 147
Oxalate, *see also* Anticoagulants
Heller and Paul's mixture, 60

P blood group system, 333-7
anti-P_1, 334-5
anti-Tj^a (—P+—P_1), 334, 336-7
no immune response, 225, 335
P-like substance in hydatid cysts, 336
Packed cell volume, *see* Haematocrit, venous
Packed cells, *see* Red cell suspensions
Panagglutination, 369
'Panel', of red cells, 433
Papain, *see* Enzymes
Parabiosis, 492-3
Paroxysmal nocturnal haemoglobinuria ('PNH')
effect of large transfusions, 117
life-span of red cells, 185-7
survival of normal red cells, 186
transfusion of washed red cells, 133
use of PNH cells in testing, 268, 352, 357

RCPE0002068_0396

'Partial neutralization' of anti-A and anti-B, 268–9, 391–2, 414–15
Pelger-Hüet abnormality
as leucocyte label, 32
Penicillin
antibody against penicillin-treated red cells, 362–3
Peritoneal cavity, absorption of red cells from, 119–22
Persistence of immune antibodies
anti-A, 224, 283
anti-Rh, 325–6
Persistence of passively acquired antibodies, 44, 600–1
Phenacetin
overdosage affecting red cell survival, 207
Phosphate buffers, recipes, *see also* Citrate-phosphate-dextrose solution
pH 7.7, 393
pH 7.4 (Hendry), 396
pH 7.3, 395
pH 5.4, 396
Phosphorus, *see* Radioactive phosphorus
Placental permeability to red cells, 317–19, 587–9
Placental transfer of antibodies, 596–9 (*see also* Haemolytic disease of newborn)
anti-A and anti-B, 597–8
anti-M, 330–1, 599
anti-P+—P1, 336
anti-Rh, 598
bacterial antibodies, 596–7
normal incomplete cold, 599
relation to molecular size, 596
Plasma substitutes, *see* Dextran, Gelatin, Gum arabic, Polyvinylpyrrolidone
Plasma (or serum) transfusions
atopens, 546
causing hepatitis, *see* Serum hepatitis
effect on plasma volume, 98, 546
in burns, 102
in defibrination syndrome, 39–40
in Factor IX deficiency, 41–2
in haemophilia, 40–2
in haemorrhage, 98
incompatible, *see* Destruction of recipient's red cells by transfused plasma
plasma antigens, 541
Plasma volume, *see also* Blood-volume
after haemorrhage, 93–5, 98
changes following birth, 578–9
effect of bed rest on, 82
effect of posture on, 60, 81–2, 85
effect of salt loss, 84
estimation of, 49–57
following burns, 92–3
in cardiac failure, 57, 84–5
in children, 72
in newborn, 72–3
in pregnancy, 82
in severe anaemia, 73–4
in starvation, 85–6
normal, 72–3
readjustment following transfusion, 127–30
relation to circulating protein, 85, 98
relation to venous haematocrit, 72–3
Plastic containers
advantages, 7
effect on red cell preservation, 7
Plastic tubing
for umbilical vein, 646, 649–50
instead of metal canulae, 594
scalp vein transfusion, 593
toxic substances in, 7
Platelets
antibodies against, 232
blood group antigens in, 232, 259–60
deficiency following transfusion, 106, 637–8
deficiency in newborn, 602
deficiency, transfusion for, 105–6
effect of storage, 34–6
in transfusion reactions, 540–1
labelled with ^{51}Cr, 33–4
labelled with $DF^{32}P$, 33
labelled with ^{32}P (PO_4), 34
lyophilized, 105
methods of transfusing, 34–5
normal life-span of, 33
sequestration after transfusion, 34
survival after transfusion, 33
Plethora, *see also* Polycythaemia
transient, affecting red cell survival curves, 152
Polyagglutinability, 365–8
Polybrene, 9
Polycythaemia
blood volume in, 74–5, 81, 83–4, 636
effect on red cell production, 116–17
red cell survival in, 152, 189
Polyvinylpyrrolidone (PVP)
as plasma substitute, 111
Pooled plasma
causing red cell destruction, 489, 492
virus of hepatitis, 558

Potassium
content of plasma in stored blood, 30
in stored red cells, 30–1
toxicity, 567–70
Potency, relative, of blood group antigens, 227–8
Pregnancy
as disqualification for blood donation, 1
blood-volume in, 82–3
immunization by foetus, 317–19
transfusion in, 115
Premature induction of labour, *see* Haemolytic disease of the newborn
Preservation, *see* Storage
'Private' antigens, *see* Low incidence antigens
Procaine
skin necrosis after intradermal injection, 4
Properdin, 235
Protamine, 9
Prothrombin
deficiency in newborn, 42
in stored blood, 42
Prozone
in titrating anti-A, 138
in titrating antibody-containing sera and using antiglobulin test, 414
in titrating antiglobulin sera, 356, 407–8, 410
in titrating anti-Rh, 307–8
Pseudoagglutination, *see* Rouleaux formation
'Public' antigens, *see* High incidence antigens
Purine nucleosides, *see* Adenosine, etc.
Pyrexia, *see* Febrile reactions
Pyrogens, *see* Febrile reactions

R blood group system in sheep, 283
Rabbits
for production of anti-A, 139
for production of anti-human globulin, 404–5
for production of anti-M and anti-N, 140
venesection of, 405
Radiation dosage
^{51}Cr, 153, 209
^{32}P, 71
Radioactive chromium (^{51}Cr)
absence of re-utilization, 166
as label for haemoglobin, 443
as label for incompatible red cells, 439–40
as label for platelets, 33–4
ascorbic acid to stop uptake, 154
blood loss in stools, 207–8
bound by 'beta' chains of Hb, 168
dosage for red cell survival, 152–3
dosage for red cell volume, 63
elution from red cells, 156–9, 161, 163–4, 172
estimation of plasma volume, 56
estimation of red cell survival, 152–68
estimation of red cell volume, 62–6
half-life of isotope, 152
interpretation of ^{51}Cr curves, 190–4
labelling red cells *in vitro*, 161–2
labelling red cels *in vivo*, 156, 164
radiation dosage, 153, 209
'surface counting', 208–9
'$T\frac{1}{2}Cr$', 156, 159–64
toxic effects on red cells, 164–6
Radioactive Di-isopropylfluorophosphonate ($DF^{32}P$)
as label for platelets, 33
as label for red cells, 170–2
as label for white cells, 32
Radioactive iodine (^{131}I and ^{132}I)
as label for albumin to estimate plasma volume, 55–6
as label for antibodies, 300, 308–9, 472
as label for gamma globulin, 43, 596
Radioactive iodophenylhydroxylamine, 175–6
Radioactive iron (^{59}Fe and ^{55}Fe)
as label for human red cells, 169–70
as label for rabbit red cells, 171
as label for rat red cells, 150
Radioactive phosphate (^{32}P)
as label for leucocytes, 32
as label for platelets, 34
as label for red cells, 66–71
estimation of red cell volume, 66–71
half-life, 67
radiation dosage from, 71
Rate of transfusion
affecting febrile reactions, 545
citrate toxicity, 564–6
in comparatively-well patients, 130
in haemorrhage, 100
in infants, 592–3
in severe anaemia, 97, 129–32
limited by bore of needle, 103–4
measured by drip-rate, 130
Reactions
allergic, *see* Allergic reactions
febrile, *see* Febrile reactions
haemolytic, *see* Haemolytic reactions
Reappearance of absorbed antibodies, 429

RCPE0002068_0397

Red cell age
related to density, 147, 530
related to osmotic fragility, 147
Red cell life-span, *see* Life-span of red cells
Red cell preservation, *see* Storage of blood
Red cell production,
deduced from rate of destruction, 210
effect of oxygen, 116
Red cell survival, *see* Life-span of red cells *and* Survival of transfused red cells
Red cell suspensions
concentrated, for transfusion, 133
for testing, *see* Suspensions of red cells
washed, for transfusion, 133–4
Red cell volume, *see also* Blood volume
calculation of, 66
deduced from plasma volume and haematocrit, 71–2
in myxoedema, 84
in newborn, 75
in normal subjects, 73–5
in polycythaemia, 74–5, 81, 83–4, 636
in thyrotoxicosis, 84
relation to venous haematocrit, 75
use of ^{51}Cr, 62–6
use of ^{32}P, 62, 66–71
use of stored red cells, 66–7
Renal failure, *see* Anuria, *also* Nephritis
Replacement transfusion, *see* Exchange transfusion
Reticulo-endothelial system
'saturation' of, 449–50
Reticulocytes
following blood donation, 95
in foetuses, 583
in haemolytic disease of the newborn, 616
in normal infants, 581
in plethora, 116
positive antiglobulin reaction, 406
Rh antigens (*see* D, Ce, etc.)
absence from white cells, platelets and saliva, 304
Rh blood-group system
antibodies, *see* Anti-Rh(D), —C, etc.
antigens, *see* D, C, etc.
chemical nature of specificity, 304
detection of antibodies, 307
genes, 295–7
genotypes, 297–8
nomenclature, 296–7, 304–5
phenotypes, 297–8
Rh 'haptene', 624
Rh-negative blood donors
emergency use of, 418
exclusion of D^u, 300–1
genotyping of, 300–1
Rh antibody in, 419
Rheumatoid factor, 246–7
accelerating removal of Rh-sensitized cells, 472
Rouleaux formation
effect of dextran, 360
effect of fibrinogen, 359–60
effect of globulin, 359–60
effect of glycine, 360
effect of sodium salicylate, 360
Rotary pumps to hasten transfusion, 105

S, s, S^u, *see* MNSs blood group system
Saline infusions
effect on plasma volume, 95, 108–9
Saliva
ABH in, 257–9
absence of Rh, 304
causing haemolysis in testing, 415
in demonstrating immune anti-A, 414–15
injections to stimulate anti-A, 278
Lewis substances in, 283–8, 385
testing, 383–4
Salk vaccine, 313
Saphenous vein
for drip transfusion, 593–5
for exchange transfusion, 651–2
Scalp vein transfusion, 592–3
Schumm's test, 522
Screening tests
for antibodies in donors, 419
for 'dangerous' O donors, 273–4
Scurvy
red cell survival in, 206–7
'Secretors', *see* Saliva (ABH)
'Sensitized' (antibody-coated) red cells; survival of *in vivo*, 200–2, 440, 467–75
Serum groups, *see* Gm, Tf, Ag
Serum hepatitis
attempted prophylaxis with gamma globulin, 560–1
carriers, 556–7
following plasma fractions, 560–1
following pooled plasma, 558–9
in blood-bank workers, 561
in transfusion recipients, 558–9
incubation period, 556
methods of destroying virus, 559–60
minimum infective dose of plasma, 559

persistence of carrier state, 557
screening of donors, 557–8
'Syringe jaundice', 561
transmitted to foetus, 561, 674
Serum transfusions, *see* Plasma (and Serum) transfusions
Sequestrene, *see* Anticoagulants
'Shock', 91, *see also* Haemorrhage
Short-lived red cells
difficulty of detecting, 148–9
Sialic acid, 237
Sialidase, 237
Sickle-cell anaemia, 186
Sickle-cell trait red cells
life-span, 188
survival after storage, 188
Silica, effect on red cells, 361
Skin-fragments, in lumen of needles, 577
Skin-grafting, 277
Skin-prick samples, 579–80, 618–19
Skin disinfection, 3–4
Slide tests, *see* Blood-grouping techniques
Sodium, in stored red cells, 30–1
Sodium azide, 377
Specific gravity of blood in donors, 2
Specific mixed agglutination, 259–60, 277
Spirochaetes, survival in stored blood, 562
Spleen
affecting normal red cell life-span, 150
in acquired haemolytic anaemia, 208–9
in antibody formation, 227
in removal of incompatible cells, 469–73
mixing of red cells in, 51, 64–5, 209
Splenectomy
effect on haematocrit ratio, 78
effect on survival of normal red cells, 150, 209
effect on survival of Rh-sensitized red cells, 472–3
Splenomegaly,
effect on body haematocrit : venous haematocrit ratio, 78
effect on red cell mixing, 51, 65–6
Sterilization of apparatus, 553–4
Steroids, *see* Corticosteroids
Storage of blood (red cells)
acid-citrate-dextrose, 16–19
adenine, 22–3
adenosine, 20–3
dextrose (glucose), 15
EDTA, 20
Edglugate-Mg solution, 10
effect of initial incubation, 25
effect of rate of cooling, 26
effect of temperature, 24
electrolyte changes, 13, 30–1
freezing, 26–8, 30
gas-transport of stored red cells, 30, 99
guanosine, 21, 24
in plastic containers, 7
inosine, 20–4
reactions, 542
sucrose, 12
trisodium citrate, 15–16
Storage of blood grouping sera, 376–8
Stored red cells
antibody specific for, 363–4
for blood group tests, 371–6
potassium and sodium in, 30–1
'Stratton's Sandwich', *see* Blood grouping techniques
Stroma
effects *in vivo*, 498
preparation, 429, 432
Subcutaneous injections of red cells, 122–3
Subgroups of A, *see* ABO blood group system
Sucrose, *see* Storage of blood
Sulphhaemoglobin, as red cell label, 174–5
Sulphydryl compounds
in dissociating 19*S* gamma globulin, 218–19, 607
Suppression of D in certain Rh genotypes, 301
Suppressor genes, 258–9
Survival of transfused red cells, *see also* Life-span of red cells
after storage, 13–28
compared with that of patient's own cells, 194–9
correction for loss of blood, 207–8
deduced from PCV changes after transfusion, 117–18
effect of blood-volume changes, 150, 194, 635–6
effect of haemorrhage, 207–8
effect of repeated 'washing', 164
effect of splenomegaly, 186
from donors with acquired haemolytic anaemia, 200–2
in acquired haemolytic anaemia, 189–207
in carcinomatosis, 203
in disseminated lupus erythematosus, 205
in eclampsia, 206

RCPE0002068_0398

Survival of transfused red cells (*contd*)
in haemolytic disease of the newborn, 617, 631–7, 668–9
in hepatic cirrhosis, 206
in hereditary spherocytosis, 186
in hypersplenism, 202
in infections, 206
in leukaemia, 203
in malaria, 206
in nephritis, 205
in normal adults, 144–50
in normal newborn infants, 150–2
in paroxysmal nocturnal haemoglobinuria, 186
in pernicious anaemia, 187–8
in plethora, 152
in rheumatic fever, 203
in rheumatoid arthritis, 203–5
in sickle-cell anaemia, 186
in thalassaemia, 186
'sensitized' *in vitro*, 200–2
slight random destruction in normal subjects, 149–50
Suspensions of red cells
agglutination tests, 378–9, 386
antiglobulin test, 409–10
capillary tube test, 385
differential agglutination, 136
for transfusion, 133–4
haemolysin tests, 398
Sutter (Js) blood group system, 345
Sw^a (Swann) antigen, 346–7
Syncope, *see* Fainting
Syphilis
false 'positives' in donors, 6
transmitted by transfusion, 561–2
Systolic blood-pressure
guide to haemorrhage, 89, 91

T receptor (and anti-T), 365–8, 599
T$\frac{1}{2}$Cr, *see* Radioactive chromium
Tn receptor, 366
T-1824
affecting skin-colour, 50
binding with albumin, 49
comparison with ^{131}I, 49, 51, 55
estimates in cardiac failure, 57, 84
estimation in plasma, 53–4
extraction from plasma, 55
injection of dye, 49–50
misleading results in rabbits, 51, 78
rate of disappearance from circulation, 51–2
rate of mixing after injection, 50–51
TAB, *see* Vaccines
Tannic acid
effect on red cells, 361–2
Terminology
blood group systems, 211
in tissue transplantation, 123
Rh blood groups, 296–7, 304–5
Tetanus anti-serum, stimulating anti-A, 281, 491
Tetanus toxoid, *see* Vaccines
Tetany in blood donors, 6
Tf groups, 248
Thomsen phenomenon, *see* Hübener-Thomsen-Friedenreich phenomenon
Thrombocytes, *see* Platelets
Thrombophlebitis
effect of duration of infusion, 572–4
effect of glucose, 573–4
effect of heparin, 574
effect of transfusion tubing, 572–4
following use of umbilical vein, 650
Thyrotoxicosis
red cell survival in, 207
red cell volume in, 84
Titration of sera, 389–92
Tj^a, *see* P blood-group system
Tolerance, *see* Immunological tolerance
Transfer of antibody from one red cell to another, 471–2
Transferrin groups, 248
Trypsin, *see* Enzymes
Typhoid vaccine, *see* Vaccines

Ulex europaeus
anti-H in seeds, 265, 382
Umbilical vein for transfusion, 592, 646–50
'Universal' donors, dangerous, *see* Dangerous 'universal' donors, *see also* Destruction of recipient's red cells (by transfused plasma), *see also* Screening tests
Urine, haem pigments in, 522–3
Urticaria, *see* Allergic reactions

V antigen, 302
Vaccines, stimulating anti-A, 280–1
Vaso-active substances, 104–5, 570
Vaso-vagal attack, 87–9
Vel antigen (and anti-Ve^a), 347
Venepuncture, *see also* Needles
precautions against haemolysis, 519
technique of, 4–5

Venous pressure
apparatus, 654
following haemorrhage, 89
following rapid infusions, 101–2
in chronic severe anaemia, 97
in haemolytic disease of the newborn, 622, 652–4
Venous spasm, 104–5, 570
associated with oligaemia, 104
caused by vaso-constrictor substances in blood, 104–5, 570
Versene, *see* Anticoagulants
Vi antigen
red cells sensitized with, 206
Vicia graminea, anti-N in seeds, 332
Vitamin K
haemolytic effect, 671–2
in haemorrhagic disease of the newborn, 42

Warming blood for transfusion, 556, 594, 651
Warming the patient
to diminish risk of overloading, 131–2
to modify febrile reactions, 545
Washed red cells
antibody against, 364
for transfusion, 133–4
Water injected intravenously, 510–11
Wharton's jelly, 362
White cells, *see* Leucocytes
Wound size as guide to blood loss, 90
Wr^a antigen (and anti-Wr^a), 346–7

X, *x* genes, 258–9

Y, *y* genes, 259
Yt^a antigen (and anti-Yt^a), 347

'Zoning', in titrations, *see* Prozone
Zymosan, 233

RCPE0002068_0399