REFERENCE 7

of haematological counting
90, 342.
d by Soper (1914).
Blood Cell Diameters, pp. 47,
University Press, London.
J. Z. (1952). 'The flying-spot
instr elect. Engrs, 99, Part IIIA, 747.
'Blood-counting machine.' Lancet,
Tables of Poisson's exponential bino-
ka, 10, 25.
n the error of counting with a hae-
trika, 5, 351.
'Neue Methode der quantitativen
nalyse des Blutes.' Arch. Physiol.
Zählungen der Blutkörperchen des
hysiol Heilk., 11, 327.
'Untersuchungen über die Fehler-
ltung der Blutkörperchen.' Arch.
54
Ueber Blutuntersuchungen.'
See p. 14.)
prakt. Heilk., Prague, 44, 11.
72).
Grösse, Zahl, Volum, Oberfläche
Körperchen bei Menschen und bei
J. 3rd ser., 20, 257.
950). Clinical Hematology, 4th ed.,
Kimpton, London.
, apparatus for counting small
distribution, with special reference to
Nature, Lond 165, 967.
and BEHRENS, D. J. (1950).
No. 595/50

Brit. J. Haemat., 1959, 5, 17.

Assay of Plasma Antihaemophilic Globulin (AHG)*

JUDITH G. POOL† AND JEAN ROBINSON

Department of Medicine, Stanford University School of Medicine, San Francisco, California

THE introduction of the thromboplastin-generation test by Biggs and Douglas (1953) provided an entirely new and sorely needed basis for the quantitative assay of antihaemophilic globulin (AHG). The procedure described by Biggs, Eveling and Richards (1955), modified by Pitney (1956) and by the original authors (Biggs and Macfarlane, 1957; Biggs, 1957), presents only two major impediments to adoption by the research laboratory engaged in long-term studies on AHG: the day-to-day reproducibility is insufficiently good, and there is poor correlation between *in-vitro* and *in-vivo* measurements of AHG. The latter difficulty is, unfortunately, a serious one, because the validity of an AHG assay can be demonstrated only in an AHG-deficient patient. Unless an anticoagulant is present, an assay should be able to measure the low level in the haemophiliac, measure the level in normal plasma to be used for transfusion and confirm the predicted rise in the patient's level following the transfusion.

This report describes further modifications of the reagents and procedure of Biggs, Eveling and Richards (1955), as well as statistical studies of the modified method which demonstrate its high level of precision and transfusion studies which demonstrate its validity.

MATERIALS AND METHOD OF ASSAY

Standard and Test Plasma (AHG Source)

A supply of standard plasma is obtained every 2 weeks from the same donor and frozen at −15–20° C. in 0.2-ml. volumes. Nine parts of blood are mixed with one part of 3.2 per cent (w/v) trisodium citrate and the cells separated by 10 minutes' centrifugation at 3000 r.p.m. Each new batch of plasma is checked against several previous ones; uniformity and stability for at least 2 weeks have repeatedly been confirmed.

The plasma is prepared for use as follows: 0.8 ml. of saline is added to 0.2 ml. of the test or standard plasma to make 1 ml. of 20 per cent plasma. A quantity of standard aluminium hydroxide ($Al(OH)_3$) gel (Biggs and Macfarlane, 1957) is also diluted 1 in 5 with four parts of distilled water, for use as adsorbing reagent for the diluted plasma. One ml. of dilute plasma is placed in the 37° C. water-bath for 1 minute after which 0.1 ml. of the diluted gel is added to it. The mixture is shaken, then incubated at 37° C. for 3 minutes. The tube is then centrifuged at 3000 r.p.m. for 10 minutes and the supernatant plasma promptly decanted into a cold test-tube. From this 20 per cent adsorbed preparation dilutions with saline are made for determining curves such as that shown in Fig. 1. The test plasma is prepared in the same way; the small volume required (0.2 ml.) permits studies on small animals.

Serum

Normal human serum is used as the source of Factor VII and plasma thromboplastin component (PTC). 10-ml. volumes of blood from ten donors are incubated at 37° C. for 4 hours

* This project was supported by a grant from the Bank of America-Giannini Foundation.
† Postdoctoral Fellow of the Bank of America-Giannini Foundation.

and then stored at 4° C. overnight. The next morning the sera are separated from their clots, pooled and 50 ml. of the pooled serum are lyophilized and stored at −15–20° C. The activity of this material remains stable for about 3 months. For use, 50 mg. of the lyophilized material are dissolved in 10 ml. of imidazole buffer, pH 7.3 (Mertz and Owen, 1940), and stored at 4° C. for 60 hours to permit complete 'dilution-activation'. Once activated, such a preparation remains stable and potent for 3 days if kept at temperatures less than 10° C.

Cephalin

A preparation from human brain, as described by Bell and Alton (1954), diluted 1 in 100 with saline before use, is used as a platelet substitute.

Adsorbed, Precipitated Beef Serum (Ac-Globulin or Factor V)

The serum is first adsorbed with barium carbonate, as described by Ware and Seegers (1949), in order to remove prothrombin, PTC and Factor VII. Since our assay indicated that there is some residual AHG in beef serum, this is precipitated by 33 per cent saturation with ammonium sulphate and discarded. The ammonium sulphate saturation of the supernate is then increased to 50 per cent in order to precipitate the Ac-globulin activity. This precipitate is then dissolved in saline (in volume one-fourth of that of the original serum) and dialysed overnight at 4° C. against saline. After dialysis, the preparation may be frozen and stored at −15–20° C. for about a year. The volumes frozen need not be particularly small since the activity of the material is not affected by repeated freezing and thawing. The final volume of the material is approximately half that of the original serum and its Ac-globulin activity as determined by the one-stage method of Lewis and Ware (Tocantins, 1955) is approximately 150 per cent of that of normal human plasma. As a source of Ac-globulin in the assay this preparation is used diluted to 5 per cent in the imidazole buffer.

Calcium Chloride

0.025 M-$CaCl_2$ in distilled water is used.

Substrate

A large quantity of fresh platelet-free citrated beef plasma (1 part of 3.2 per cent trisodium citrate to 9 parts of beef blood), stored frozen in convenient volumes, is used as-substrate. It remains stable for about 3 months and is diluted with an equal volume of saline just before each experiment.

METHOD OF ASSAY

From a stock kept in melting ice, 0.1 ml. of each of the following reagents is pipetted into a warm test-tube in the 37° C. water-bath: 5 per cent adsorbed, precipitated beef serum; diluted human serum; 1 in 100 cephalin, and 0.025 M-$CaCl_2$. A stop-watch is started with the addition of the $CaCl_2$. When the reagents have been incubated for 6 minutes, 0.2 ml. of the cold standard or test plasma is added, the tube is agitated slightly, and a pipette placed in it. Another tube already containing 0.1 ml. of $CaCl_2$ is then placed in the water-bath; this is used in the final measurement of the amount of 'thromboplastin' generated. At 10 minutes 40 seconds after starting the stop-watch, 0.1 ml. of the generating mixture is added to the tube containing the $CaCl_2$ and at 11 minutes 0.1 ml. of the cold diluted substrate is added. The clotting time of the mixture is determined with an extra stop-watch. By starting

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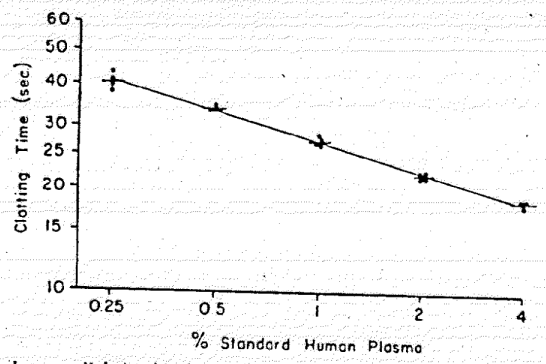

FIG. 1. Relationship between the concentration of the source of AHG and the clotting time of the substrate.

RESULTS

Standard Curve

Fig. 1 shows the relationship between the concentration of the standard plasma and the clotting time of the substrate, when plotted on double logarithmic paper. For our standard plasma the plot produces a straight line between 4 per cent and 0.25 per cent, with a plateau at each extreme; the saline-control time for the system is 55–60 seconds. The points shown in the figure were obtained from a Youden square (latin square with one column omitted) and permit a study of the precision of the method (Davies, 1954)*. Analysis of variance with co-variance of these data confirm that the relation of log clotting time to log concentration is linear; the slope of the line of best fit is 0.2844; there is no significant variability from run to run or from order to order within a run, and the standard deviation of duplicate determinations of log. clotting time (the actual measurements made) is 0.00938, making the coefficient of variation of estimate of AHG concentration 7.6 per cent. That there is no tendency for the coefficient of variation to depend on the level of AHG measured was demonstrated by a plot of the standard deviation for 20 duplicate, 2 triplicate and 7 quadruplicate determinations against the means for each set. The index of precision, λ (the ratio of the standard deviation to slope) was determined from a total of 146 measurements to be $\frac{0.0106}{0.29} = 0.0366$.

The above statistics all relate to determinations carried out within a given day. It would be convenient if we could assay AHG by measuring the clotting time of a specimen on any day and read the AHG value from a permanent 'standard line', the average of many previous determinations. The precision of estimation done in this way was gauged for our assay as follows: over a period of 6 weeks there were accumulated thirteen determinations at

* The five dilutions (4, 2, 1, 0.5 and 0.25 per cent) were tested in five separate runs. The limitations of incubation time prevented more than four determinations being made in any one run. To balance the order of testing within the run, a Youden square design was used, this having the property that each of the dilutions was omitted from one of the five runs and that the four trials of any dilution occupied the first, second, third and fourth positions in four different runs.

each of four known concentrations. The coefficients of variation were: at 4 per cent dilution, 16.1 per cent; at 2 per cent dilution, 4.4 per cent; at 1 per cent dilution, 11.4 per cent; and at 0.5 per cent dilution, 14.6 per cent.

In practice, since it takes only 16 minutes to prepare a 'standard curve of the day', this is done; however, no more than a 2 seconds deviation for 2 per cent plasma from the Youden square value is acceptable. If there is a larger discrepancy than this, it is assumed that one of the reagents is defective and should be replaced.

Studies on Transfusion of Haemophilic Patients

To evaluate the assay, haemophilic patients were transfused. The material to be used for transfusion and the patient's AHG level before and after transfusion were assayed. The patient's plasma volume was estimated so that the level of circulating AHG which should have resulted from transfusion of a given volume of plasma could be calculated and compared with that actually observed.

$$\text{Potency of unknown (as \% of normal)} = \frac{\text{conc. of normal}}{\text{conc. of unknown}} = \frac{0.5\,\%}{2.0\,\%} \times 100\,\% = 25\,\%$$

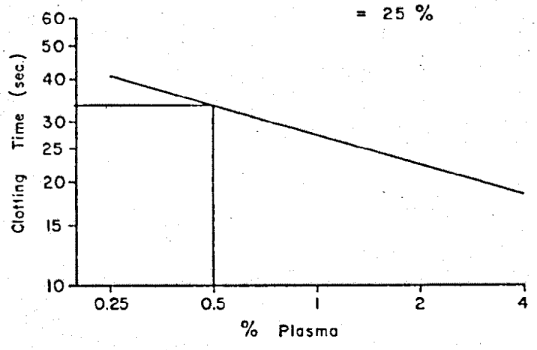

FIG. 2. Illustration of the calculation of the AHG concentration of a plasma sample.
The unknown sample, tested at a concentration of 2.0 per cent, gave a clotting time of 34 seconds, equivalent on the standard curve to 0.5 per cent plasma.

The AHG content of an unknown plasma is measured by comparing its activity with that of the standard plasma. It is diluted, adsorbed and added to the system just as described for the standard plasma except that a single dilution is chosen which is expected to give a clotting time falling on the straight-line portion of the standard curve. It is assayed in duplicate and the results are averaged. The simple calculation which gives the value of the unknown as a percentage of the normal standard is given in the legend to Fig. 2.

Although we have not measured an extensive series of normal subjects, approximately twenty-five such determinations indicate that the AHG content of the plasma of our standard donor is very close to the mean normal value. The AHG content of the normal subjects ranged from 50 per cent to 200 per cent of that of our standard plasma, approximately the same range reported by Langdell, Wagner and Brinkhous (1953), Biggs, Eveling and

Richards (1955), and Pitney (1956). The slope of the curve relating clotting time to plasma concentration is the same for different subjects (within the limits of the error of the method) even though the absolute level varies considerably; this, of course, is a necessary condition for the use of a standard curve.

TABLE I

DATA AND CALCULATIONS FOLLOWING THE TRANSFUSION OF THREE HAEMOPHILIC PATIENTS

*Patient and plasma volume**	GRO-A (2900 *ml.*)			GRO-A (3200 *ml.*)			GRO-A (3200 *ml.*)			GRO-A (2900 *ml.*)		
	Clotting time (sec.)	*AHG (%)*	*Calculated AHG (%)*	*Clotting time (sec.)*	*AHG (%)*	*Calculated AHG (%)*	*Clotting time (sec.)*	*AHG (%)*	*Calculated AHG (%)*	*Clotting time (sec.)*	*AHG (%)*	*Calculated AHG (%)*
Patient before transfusion	48.4	3.4	...	45.0	4.3	...	52.8	2.3	...	50.3	2.9	...
1st unit of plasma†	27.5	46.0	...	29.4	37.0	...	25.0	64.0	...	27.1	48.0	...
Patient following 1st unit of plasma	33.2	12.0	7.4‡	36.6	8.7	7.1	36.0	9.2	7.5	...	...	7.1
2nd unit	24.0	73.0	...	29.5	36.5	...	27.3	47.0	...	28.3	42.0	...
Patient following 2nd unit	30.8	16.0	17.2§	34.7	10.5	10.9	...	...	12.1	34.8	10.5	10.1
3rd unit	...	...	...	30.2	33.0	...	21.4	108	...	31.3	29.5	...
Patient following 3rd unit	...	...	...	33.0	12.5	12.1	29.8	17.8	19.1	33.0	12.5	12.0
4th unit	...	...	...	...	...	...	33.4	24.0	...	...	...	...
Patient following 4th unit	...	...	...	...	...	...	29.6	18.0	18.2	...	...	...

* Based on height and frame size (i.e., slight, medium or heavy build).
† Unit of plasma = 300 ml.
‡ Sample calculation: 2900 (3.4) + 300 (46.0) = 3200 X
9860 + 13,800 = 3200 X
23,660 = 3200 X
7.4% = X
§ Calculation for the second unit is made starting with the observed value after the first unit and the original plasma volume. Samples of the patients' plasma were assayed at 4 per cent dilutions and the units of plasma transfused at 2 per cent dilutions.

Table I gives the results of four transfusion studies in three haemophilic patients and a comparison of the observed and calculated post-transfusion levels. The simple equation for calculating the expected post-transfusion circulating AHG level from the AHG content and volume of the plasma transfused and the AHG content and volume of the patient's plasma is shown at the bottom of Table I. The result is compared with the value obtained when a sample taken from the patient 10 minutes after transfusion is assayed.

Ten transfusion experiments on five different haemophilic patients were carried out, with similar results. All the patients were severely affected and had no demonstrable prothrombin consumption. They were bleeding at the time the transfusions were begun. The correspondence between the observed and calculated levels was good, except after the transfusion of single units of plasma. Under these circumstances the AHG content of the patient's plasma before transfusion is involved in the calculation; and this is necessarily in greater doubt than the higher levels since it lies on the curve's plateau. For units beyond the first one, however,

the difference between the observed and calculated levels was 7 per cent or considerably less, which is no greater than the error of the method.

DISCUSSION

The changes made in the reagents used by Biggs, Eveling and Richards (1955) and by Pitney (1956) were dictated by the following requirements: maximal simplicity of preparation (lyophilization only when absolutely necessary); maximal stability of the activity of each reagent, preferably for a period of several months; ease of replacement of an exhausted reagent with a new batch of the same activity; a concentration of each reagent such that the system is maximally sensitive to changes in AHG level and yet only to such changes.

The three major changes made in the procedure were empirically observed to improve reproducibility: pre-incubation of the thromboplastin components other than adsorbed plasma before addition of the latter; immediate instead of delayed determination of the thromboplastic activity generated, and storage of the working reagents in melting ice. The timing permits the procedure to be carried out by one technician working alone and does not require special pipettes or the simultaneous emptying of two pipettes.

One of the objectives of the modifications made in the assay was a steep assay curve, since the precision of the assay is directly dependent on the slope of the curve. Two further modifications which increase the slope of the standard curve, delay the appearance of the plateau at low concentrations, and lengthen the 'blank' time of the system to 100 seconds have been adopted since this study was concluded. One is the maintenance of a constant citrate level, as the test plasma is diluted, equivalent to that of 4 per cent citrated plasma; the other, which was suggested by Dr. Samuel Rapaport, is the use of aged human (outdated bank) plasma as substrate in place of beef plasma. The ageing permits deterioration of both AHG and Ac-globulin, with the result that the substrate is far less likely to contribute thromboplastin to the system. This is only important in relation to the long clotting times obtained with low AHG concentrations, for in those cases thromboplastin contributed by the substrate causes the curve to deviate from the straight line, as the plasma is diluted, earlier than it otherwise would.

The precision of the assay was determined for two different situations, one using a standard curve determined each day, and the other using an 'established' standard curve. The precision in the latter case is necessarily decreased because it is affected by the particular condition and dilution of the standard reagents made on that day. It would be convenient not to have to determine the standard curve each day, and, in fact, the day-to-day coefficient of variation of the assay is of the same order as Pitney's within-a-day coefficient, suggesting that a long-term reference curve is feasible. However, since setting up the daily curve involves making and testing four dilutions only and requires only 16 minutes to carry out, in practice we construct a fresh curve each day. The fact that there are only small differences between these daily curves provides a valuable check on the standard plasma.

Since AHG has not yet been crystallized or chemically analysed, we can still define it only by its activity in the correction of the haemophilic defect. It follows from this that the only criterion of the validity of the assay is its ability to predict the post-transfusion AHG level in a haemophilic patient. Biggs, Eveling and Richards (1955), using their own assay, recovered considerably less than half the AHG transfused when the circulating level was determined 10 minutes after transfusion. They stated that 'these results suggest that both human and ox

AHG disappear rapidly from the blood of haemophilic patients'. We have made no studies of the survival of ox AHG in the circulation, but human AHG, as measured by our assay, appears to be completely recoverable 10 minutes after transfusion. The successful prediction of the circulating AHG level following transfusion seems to us to argue against the theory that there was any circulating AHG-inhibitor in the five cases of classical haemophilia we studied. A full discussion of this question, however, lies outside the scope of this report.

SUMMARY

Modifications of the reagents and procedures of the thromboplastin-generation test of Biggs and Douglas are described which permit the plasma antihaemophilic globulin (AHG) to be reliably assayed. The coefficient of variation for measurements made on the same day was approximately 7.6 per cent and λ value approximately 0.0366.

The use of the assay in measuring the AHG level of transfused plasma and in predicting the rise in AHG level produced in haemophilic patients by tranfusion is described.

ACKNOWLEDGMENT

We are indebted to Dr. Lincoln Moses, Associate Professor of Statistics, Stanford University, for the statistical analysis of the assay.

REFERENCES

Bell, W. N. and Alton, H. G. (1954). 'A brain extract as a substitute for platelet suspensions in the thromboplastin generation test.' *Nature, Lond.*, **174**, 880.

Biggs, R. (1957). 'Assay of antihaemophilic globulin in treatment of haemophilic patients.' *Lancet*, **ii**, 311.

Biggs, R. and Douglas, A. S. (1953). 'The thromboplastin generation test.' *J. clin. Path.*, **6**, 23.

Biggs, R., Eveling, J. and Richards, G. (1955). 'The assay of antihaemophilic globulin activity.' *Brit. J. Haemat.*, **1**, 20.

Biggs, R. and Macfarlane, R. G. (1957). *Human Blood Coagulation and Its Disorders*, 2nd ed., p. 423. Thomas, Springfield, Illinois.

Davies, C. L. (ed.). (1954). *The Design and Analysis of Industrial Experiments*. Chapter 6. Oliver and Boyd, Edinburgh.

Langdell, R. D., Wagner, R. H. and Brinkhous, K. M. (1953). 'Effect of antihemophilic factor on one-stage clotting tests. A presumptive and a simple one-stage antihemophilic factor assay procedure.' *J. Lab. clin. Med.*, **41**, 637.

Mertz, E. T. and Owen, C. A. (1940). 'Imidazole buffer: its use in blood clotting studies.' *Proc. Soc. exp. Biol., N.Y.*, **43**, 204.

Pitney, W. R. (1956). 'The assay of antihaemophilic globulin (AHG) in plasma.' *Brit. J. Haemat.*, **2**, 250.

Tocantins, L. M. (ed.). (1955). *The Coagulation of Blood: Methods of Study*, p. 124. Grune and Stratton, New York.

Ware, A. G. and Seegers, W. H. (1949). 'Two-stage procedure for the quantitative determination of prothrombin concentration.' *Amer. J. clin. Path.*, **19**, 471.