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30th November 1978

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Dear Dr. Lane,

I enclose a copy of the final version of the paper sent to the British Journal of Haematology and you will be glad to know that it has been accepted for publication.

Yours sincerely,

GRO-C

H.H. Gunson, D.Sc., M.D.
Director

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VARIABLES INVOLVED IN CRYOPRECIPITATE PRODUCTION AND THEIR EFFECT ON FACTOR VIII ACTIVITY

Report of a Working Party of the Regional Transfusion Directors Committee

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SUMMARY

The Regional Transfusion Centres in England and Wales have studied the following variables in the preparation of cryoprecipitate: blood group of the donor, choice of anticoagulant (ACD or CPD), conditions of freezing, thawing and centrifugation of the plasma and age of the starting plasma.

Mean factor VIII-activity in cryoprecipitates derived from group A plasma was significantly higher than from group O plasma. CPD may lead to increased potency of factor VIII when freshly collected plasma is used to prepare cryoprecipitates; rapidity of thawing may also be advantageous.

None of the other variables studied produced significant effects on the factor VIII-activity of the cryoprecipitates and particular attention has been drawn to the results of experiments concerned with the age of starting plasma.

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Despite the increasing use of lyophilised factor VIII-concentrates for the treatment of haemophilia A, factor VIII in cryoprecipitates (cryos) prepared from single units of donor plasma still remains an important therapeutic substance. Since it is not practical to assess individual doses of cryo, but it is desirable to obtain as potent a preparation as possible, the Regional Transfusion Directors' Committee, in July 1976, appointed a Working Party with the following terms of reference:

> "To examine the quality of cryoprecipitates produced at Regional Transfusion Centres (RTC's) and the factors affecting it."

After an initial survey of the factor VIII-activity in randomly selected cryos from RTC's had shown considerable variation, each RTC was visited and many differences in the preparation procedure were catalogued.

The present report summarizes the results of investigations into certain variables in cryo production which, it was considered, might affect factor VIII-activity.

MATERIALS AND METHODS

Cryos were prepared from donations collected without difficulty in PVC packs at routine blood collection sessions. Blood was transported to the RTC and stored for a variable period of time at 4°C (see below). After an initial centrifugation the plasma

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was frozen at -70°C usually in alcohol/solid CO_2 mixture. Thawing took place at 4°C overnight (approx. 18h) in all except one RTC where the packs were immersed in a water bath at 8°C for 2.5h. After concentration of the cryo by centrifugation, supernatant plasma was removed to give a residual volume of 5 to 35 ml. All cryos were frozen at -30°C or below immediately after preparation and these conditions were maintained until they reached the Plasma Fractionation Laboratory, Oxford, where all the assays for factor VIII were performed. Unless otherwise specified, a total of 16 cryos (eight group O and eight group A) was prepared to study a given variable, with every effort being made to keep other variables constant.

Preparation of cryos for assay was carried out by thawing at room temperature for 5 min followed by immersion in a water bath at 37°C for 10 min. The outside of the bag was dried and the contents mixed. As much of the redissolved cryo as possible was withdrawn and the solution was transferred to a measuring cylinder. Five ml of 150mM NaCl was injected into the bag as a rinse and transferred to the measuring cylinder. The total volume of solution was recorded before assay. A pool, usually of four cryos, was prepared to reduce the wide variation observed in the factor VIII-activities of different plasma samples (Grant and Biggs, 1967), and except in Section 1, comprised equal numbers from group O and group A plasma

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Factor VIII was assayed by a modification of the two-stage method of Biggs et al (1955). The standard was a freeze-dried plasma, the 5th British Standard for factor VIII (NIBSC code 75/10). Each cryo pool was diluted 1 in 4 in haemophiliac plasma before absorption with aluminium hydroxide. The assays were of balanced design, i.e. the standard and two test preparations each at three dilutions, as follows:

$$S \quad T_1 \quad T_2 \quad T_2 \quad T_1 \quad S$$

Only the assays demonstrated as being valid with respect to both parallelism and linearity of the standard and test responses were included.

Subsequent statistical analyses were carried out on log. potency estimates and the mean values were expressed as the arithmetic mean of the log. potency estimates. Similarly, the standard deviation of the log. potency estimates, expressed in the antilog. form as a percentage, was used as an estimate of variability.

RESULTS

1. Factor VIII-activity of cryoprecipitate derived from group O and group A plasmas

Each RTC selected 12 cryos at random (six O and six A). Pools comprising three cryos of the same blood group were prepared; the results expressed as factor VIII-activity per unit of cryo, are shown in Table 1. Analysis of variance, in which "between

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RTC" and "between blood group" differences in factor VIII-activity were compared with differences between replicate cryo pools, revealed a "between RTC" difference which was highly significant ($p = 0.006$). The difference between cryos derived from group O and group A was highly significant ($p = 0.0001$), group A cryos containing on average 1.28 times the factor VIII-activity of group O cryos.

2. Choice of anticoagulant

Fig. 1 summarizes the mean and one S.D. of the results obtained in the eight experiments comparing the factor VIII-activity in cryos prepared from plasma anticoagulated with ACD (USP, Formula A) and CPD. Four pools of four cryos were assayed in experiments 1, 2, 5 and 7. In the remaining experiments 12 donations of plasma were pooled after centrifugation and were subsequently separated into 200g aliquots. In experiments where the starting plasma was frozen within four hours after collection, the mean factor VIII-activity in cryos derived from CPD plasma was higher than that from ACD plasma, although the difference was significant at the 5% level in experiments 2 and 3 only. Cryos prepared from plasma frozen within 4 to 6h and 18h after collection showed no significant difference in mean factor VIII-activity between the two groups.

3. Age of starting plasma

The results of the eight experiments are illustrated in Fig. 2, in which the mean value and one S.D. are given. In experiments 5 and 7, 200g aliquots of a 12-donation pool of plasma were examined and four pools of four cryos were assayed in experiments 1 to 4, and 6 and 8. The anticoagulant was ACD

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in experiments 1 to 5 and 8, and CPD in experiments 6 and 7; the cryoprecipitate was harvested after a thaw of approximately 18h at 4°C in all experiments except 8, where a thaw of 2.5h at 8°C was carried out. Taking each experiment separately, differences between the mean factor VIII-activities of any two types of cryo could always be explained in terms of variability between replicate pools. The mean starting plasma factor VIII levels were: experiment 5: 0.83, 0.82 and 0.52 and experiment 7: 0.85, 0.71 and 0.69 for "< 4h", "4 to 6h" and "18h" plasmas respectively. The trend in each instance is to a lowered level of factor VIII-activity with increasing age of blood at separation. It is clear from Fig. 2 however, that this decrease in activity is not reflected in the factor VIII-activity of the cryos prepared from the respective plasma pools.

4. Conditions of Centrifugation

Most commonly, centrifugation was carried out in a six place swing-out head at 2075g usually for 20 to 30 min (range 9-30 min). Alternatively angle-heads were used at 4000 to 4800g for shorter periods and one RTC employed 6450g for 5 min. The cryo was usually harvested after centrifugation in the same centrifuge, at the same speed, but for a shorter time.

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Three different sets of whole blood centrifugation conditions were used at one RTC viz: 2075g for 15 min, 4850g for 7 min, and 6450g for 5 min. Each group of 16 cryos was divided into four pools and the factor VIII assayed. Activity per cryo averaged 89.3 iu^{+} 18%, 88.6 iu $^{+}$ 20% and 92.4 iu^{+} 6% respectively in the three groups (differences not significant). In a second experiment, cryos were prepared after an initial centrifugation of 2075g; 16 plasmas were frozen after 9 min centrifugation and 16 after 30 min centrifugation. Subsequent assay of four pools of cryo revealed mean values of 82.0 iu^{+} 26% and 87.8 iu^{+} 6% per cryo; again, no significant difference was apparent. Finally, the effect of double centrifugation of the starting plasma was examined. Nine pools, each comprising 200g of group O and group A plasma were prepared after an initial centrifugation of the whole blood at 3500g for 10 min. Each pool was then divided into two equal parts; the first was frozen at -70°C and the second was subjected to a further centrifugation of 3500g for 10 min. The mean factor VIII-activity of cryo prepared from the single-spun plasma was 96.3 iu^{+} 18% compared with a mean of 91.8 iu^{+} 20% for the double-spun plasma; the difference was not significant.

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5. Time of freezing the plasma

One RTC used the unusually short freezing time of 7 min whereas the usual time was in excess of 15 min. Using a pack containing 200g plasma Dike (1967) showed, by means of a temperature probe inserted into the centre of the plasma, that the temperature at that site did not reach -40°C until 15 min had elapsed. Sixteen cryos were prepared after freezing the starting plasma for 7 min and the same number after a freezing time of 15 min.

The mean values of factor VIII-activity in four pools frozen for 7 min was 67.4 iu± 30% and after 15 min 66.9 iu± 21%; the difference was not significant.

6. Time of thawing of plasma

Cryos prepared by rapid thaw (2.5h at 8°C) were compared with those prepared by a shorter thaw (18h at 4°C). In each group "18h" plasma was used. Fig. 3 shows that the mean factor VIII-activity was higher in rapidly thawed cryos, the difference being significant ($p = 0.05$).

In a further study aliquots of a pool of 12 donations of plasma were frozen within 4h of collection. Cryos were produced by thawing six aliquots at 8°C for 2 to 2.5h and six overnight at 4°C. The mean values (iu per cryo) and S.D. for the two groups were 105± 38% and 95.8± 8% respectively. The difference was not significant but it was noted during this

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experiment that the end of the thawing period was difficult to judge with certainty and the final disappearance of ice crystals occurred very rapidly.

DISCUSSION

In the collaborative study reported it was found that many variations existed in the preparation of cryo in the RTC's in England and Wales. Variability in age of starting plasma is largely unavoidable, being determined by the geography of the Region and the ease with which donations of blood can be returned to the RTC for processing. Other factors, such as the choice of anticoagulant, centrifugation conditions, procedures for freezing and thawing could be standardised if there were significant advantages but with respect to the age of the plasma a striking improvement in quality of the cryo from fresh plasma ("< 4h") would have to be demonstrated to justify the cost of incorporating this procedure.

Investigation of the variables involved in the preparation of cryos was limited by clinical requirements for this product. Hence, in most instances each variable was studied using only 16 cryos. Given the magnitude of the normal variation in factor VIII-activity that was seen to exist, the difference between the mean cryo factor VIII-activity for any two methods would have had to be in the order of 33% to be determined as significant ($p = 0.05$) in any one experiment.

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In comparisons considered to be of particular interest, cryos were prepared from aliquots of a pool of starting plasma in order to eliminate this source of variability. Previous investigations of the variables involved in cryo preparation and their effect on factor VIII-activity, have shown conflicting results. In contrast to the results of Shanberge et al (1972), the present study showed a highly significant increase, on average, of factor VIII-activity in group A compared with group O cryos. Preston and Barr (1964) showed that there was a significantly higher factor VIII-activity in group A plasma compared with group O and the findings in this study may reflect the correlation which has been demonstrated by several workers between factor VIII-levels in a given plasma and the cryo prepared from it (Pennet et al 1976, Bloom et al 1968, Graybeal et al, 1969). In view of this, all other variables were studied using pools of equal numbers of cryos from group O and group A plasmas, a precaution which does not seem to have been taken in other published investigations.

Only the use of CPD anticoagulant and the rapid thawing of the frozen plasma gave results which might suggest that significantly improved factor VIII-yield could result from their use. The use of CPD gives plasma a higher pH than ACD and cryoprecipitation may be pH dependent; Pool (1967), Gilchrist and Ekert (1968).

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On the other hand several workers have failed to demonstrate either pH dependency of cryoprecipitation or improved yields of factor VIII in cryos prepared from CPD plasma compared with those from ACD plasma (Morrison, 1966; Graybeal et al 1969; Shanberge et al, 1972, Burka et al 1975; Slichter et al, 1976 and Vermeer et al, 1976).

In the present study, there was a significantly higher level of factor VIII-activity in two groups of cryos prepared from CPD plasma (4h old at the time of freezing) than in comparable material prepared from ACD plasma; in two other experiments with plasmas of this age and those using plasma stored at 4°C for longer periods prior to the preparation of the cryos, significant differences in factor VIII-activity could not be found. The significant differences found may have been related to higher levels of factor VIII-activity in the CPD starting plasmas, but this could not be proven since the appropriate assays were not carried out.

Advantages from rapid thawing of the frozen plasma in water baths at temperatures of 3°C and 8°C have been described by Brown et al (1967), Masure (1969), Slichter et al (1976), Vermeer et al (1976). Combining rapid thawing with shaking (Burka et al 1975b, Margolis 1976) and with syphoning (Mason, 1978), has also led to increased yields of factor VIII in cryos.

In one experiment carried out in the present study there was a significantly higher level of factor VIII in cryoprecipitates

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subjected to a rapid thaw. A further experiment to confirm these findings was inconclusive, due probably to the difficulties encountered in the exact determination of the period of thawing and the likelihood that factor VIII-activity was lost by dissolution of the cryoprecipitate.

It is clearly important to establish whether the duration of the thawing process is important or whether it is the period of post-thaw which determines the resultant factor VIII-activity as concluded by Kasper et al (1975) and Rock and Tittley (1976). Use of the thaw-syphon technique by Mason (1978) reduces the thaw period considerably with an added advantage of having reduced time of contact between the precipitate and the residual plasma.

Since the slow thawing procedure fits more readily into the pattern of work of most RTC's, possibly more attention should be paid to the prompt harvesting of cryoprecipitate to avoid an extended period of post-thaw. Rapid thawing by immersion of the pack in a water bath carries the danger of bacterial contamination even if an outer wrapping is employed and this assumes greater importance if the plasma is returned to the red cells. Two members of the Working Party (RTW and TJS) are conducting experiments on rapid thawing using warm air and a separate report will be presented.

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The lability of factor VIII in plasma has been known for many years (Penick and Brinkhouse, 1956), and this has traditionally led to the recommendation that blood used for cryo preparation should be processed within 4h of collection. In the eight experiments conducted on the factor VIII-activity of cryos prepared with starting plasma of less than 4h, 4 to 6h and 18h, varying the anticoagulant and the method of thawing failed to show significant differences. Moreover, a fall in the plasma factor VIII-activity during the period of 18h of storage similar to that found by other workers (Preston, 1967, Slichter et al 1976), was not reflected in the factor VIII-activity in the cryos prepared subsequently. This does not conflict necessarily with the previous statement, that factor VIII levels in cryos generally correlate with those of the starting plasma since these observations were made with respect to plasmas of the same age. It may indicate however, that a portion of the factor VIII, which is more labile and does not precipitate well, cannot be recovered in cryos. Confirming the findings of the present study, Smith et al (1978) reported that the amount of factor VIII recovered in an intermediate purity concentrate, prepared by a method involving cryoprecipitation, was independent of the age of plasma at separation from the red cells between 6 and 18h. With respect to the freezing times

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of plasma and conditions of centrifugation the results agree with those found in other studies (Burka et al 1975a, b: Slichter et al, 1976).

Platelet counts were not performed in the present study but from previous experiments (Gunson, 1976) centrifugation under the conditions investigated yielded residual platelet counts in the plasma of 10-35 x 10^9/l. Thus, the conclusion of the above workers that residual platelet counts in the order of 25 x 10^9/l do not affect factor VIII levels in the resultant cryos are substantiated; indeed it is likely that much higher levels can be tolerated without significant effect on factor VIII-activity (Smith et al 1977).

CONCLUSIONS

From the present study precise recommendations cannot be made which will consistently increase the potency of cryoprecipitates. The significant result was the increased factor VIII-activity of cryos from group A plasma.

Of particular interest in blood banking is that within limits the age of the starting plasma does not exert a major effect on the factor VIII-activity of cryos. It does not appear that arrangements need be made to prepare cryos within 4h of blood collection unless this fits the work pattern of the RTC concerned. In common with the observations of previous workers, considerable variation is found in factor VIII-activity in small pools of cryoprecipitate even when care has been taken to control the variables. Correspondingly the potency of an individual dose cannot be predicted with certainty.

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