parabiosis is primarily of benefit because it accelerates recovery of myelopoiesis by the transfer of some cellular or non-cellular material from the non-irradiated partner.

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Assay of Plasma Antihemophilic Activity in Normal, Heterozygous (Hemophilia) and Prothrombinopenic Dogs.* (18755)

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The corrective action of normal plasma on the delayed clotting of hemophilic blood is attributed to its content of antihemophilic factor (AHF). It has been shown(1-3) that the amount of prothrombin consumed in whole hemophilic blood is directly proportional to the amount of AHF added. On the basis of this relationship, an assay procedure for AHF (2,3) was recently developed. In this paper, a series of studies on AHF are reported in which an improved assay procedure was used. The new method is more sensitive than the previous one, and it permits expression of results in per cent of normal control plasma. Studies have been made on the AHF level in normal dogs and in female dogs heterozygous for hemophilia, as well as in animals with hypoprothrombinemia due to liver injury and to dicumarol administration.

Assay of antihemophilic activity. The plasmas being tested for AHF were prepared from citrated whole blood (9 parts blood and 1 part 3.2% sodium citrate) in an angle centrifuge at about 3000 g for 20 minutes, and then dialyzed ("Visking" cellulose casing) with continuous agitation against 0.032% sodium citrate in 0.9% NaCl solution for 2 hours at 4°C. The normal control and test plasmas were diluted varying amounts (1-2 to 1-50) with saline. To 0.1 ml of each dilution of each plasma were added 0.15 ml imidazole buffer pH 7.25, and 1 ml freshly drawn hemophilic dog blood. A single hemophilic dog, either male or female(4), was used for each assay. After a reaction period of 20 minutes (28°C), 0.15 ml 3.2% sodium citrate was added to each tube, and the serum (or plasma) expressed by immediate centrifugation. Residual prothrombin was determined by the two-stage method(5), with added Factor V(6) prepared from dog plasma. The results were expressed in per cent (citrated plasma from the hemophilic dog = 100%). The prothrombin is plotted against the calculated amount of native test or control plasma added to 1 ml native hemophilic plasma. Fig. 1 illustrates the method used for comparing AHF in the test plasma with

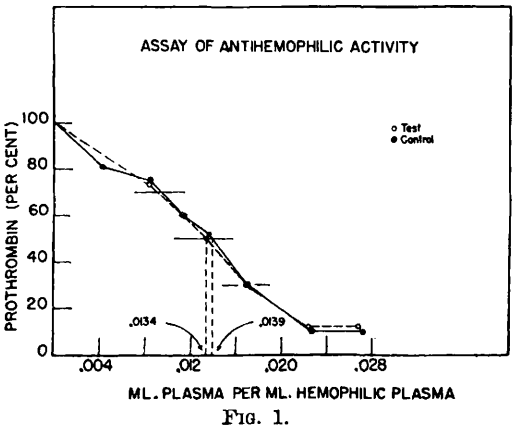

FIG. 1.

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† Markle Scholar in Medical Science.

1. Graham, J. B., Buckwalter, J. A., Hartley, L. J., and Brinkhous, K. M., *J. Exp. Med.*, 1949, v90, 97.

2. Graham, J. B., Penick, G. D., and Brinkhous, K. M., *Fed. Proc.*, 1950, v9, 330.

3. Graham, J. B., Penick, G. D., and Brinkhous, K. M., *Am. J. Physiol.*, 1951, v164, 710.

4. Brinkhous, K. M., and Graham, J. B., *Science*, 1950, v111, 723.

5. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, v66, 801.

6. Owren, P. A., *The Coagulation of Blood*, J. Chr. Gundersen, Oslo, 1947, p. 82 ff.

TABLE I. Comparative Plasma AHF Levels in Normal Dogs and Female Dogs Heterozygous for Hemophilia.

Test dog			Control dog		AHF
Type	No.	Sex	No.	Sex	% of control plasma
Normal	1	♀	2	♂	89
	4	♂	3	♂	93
	5	♀	3	♂	101
	5	♀	3	♂	106
	6	♂	3	♂	92
	6	♂	3	♂	103
Heterozygous	8	♀	3	♂	80
	9	♀	7	♀	108
	10	♀	5	♀	87
	10	♀	5	♀	105
	10	♀	5	♀	101

the normal control plasma. The test plasma in this illustration was from a dog 6 days after prolonged chloroform anesthesia (see Fig. 2A). The amount of each plasma required to give a residual prothrombin of 70, 50 and 30% respectively in the hemophilic substrate was estimated by interpolation. For each prothrombin level the value, ml control plasma/ml test plasma × 100 is determined. This value represents the AHF in the test plasma in per cent of that contained in the control. In the example shown, these values were 107, 104 and 101%, giving an average of 104%.

AHF levels in normal dogs and dogs heterozygous for hemophilia. Table I shows the results obtained in 6 experiments with normal dogs. In each experiment a pair of dogs of either sex was tested for plasma AHF. One animal was arbitrarily designated as the test animal, the other as the control. It will be seen that, under these circumstances, the AHF in the normal test animals varied from 89% to 106%. In another set of similar experiments, non-bleeder females, heterozygous for hemophilia (Table I), were compared with normal males or females. Again, it will be noted that the AHF was nearly the same in the two sets of plasma. The heterozygous females varied from 80 to 108% of the normal controls.

Liver injury. Liver damage was produced by chloroform anesthesia in 6 normal dogs. Each animal was fasted for 24 hours and then subjected to deep anesthesia for 90-120 minutes. Fig. 2A shows the results of one experiment. Frequent determinations of plasma prothrombin (2-stage) and plasma AHF were made. No appreciable change occurred in the AHF level of plasma, although the prothrombin fell rapidly and then gradually returned to normal, as is characteristic of acute liver damage(5). In 2 of the 6 experiments, fresh citrated plasma was obtained at the time the hypoprothrombinemia was most severe, and was transfused into hemophilic dogs. The average results of the 2 experiments are given in Fig. 2B. The degree of improvement of the clotting defect in the hemophilic dogs was determined from the prothrombin utilization rates(7,8); the residual prothrombin values of serum one hour after collecting the hemophilic blood are plotted. The corrective effect was immediate and was still evident about 5 days later. It will be noted that the plasma from the dogs with liver injury was about as effective as normal plasma. The correction *in vivo* by plasma from the chloroform-treated dogs, low in prothrombin and without detectable fibrinogen, confirms the findings with the *in vitro* assay procedure.

Dicumarol. Hypoprothrombinemia was induced by dicumarol in 3 normal dogs. The results of one experiment in which assays for plasma AHF were performed frequently are shown in Fig. 2C. No significant change in antihemophilic activity of the plasma occurred, although the prothrombin fell to a low level. In 2 of the experiments, prothrombinopenic plasma from the dicumarol-treated dogs was transfused into hemophilic dogs. This plasma was as effective in correcting the clotting defect of hemophilic animals as was normal plasma (Fig. 2D). Thus it appears that dicumarol administration likewise has no effect on plasma AHF.

Discussion. The assay procedure outlined furnishes a sensitive and accurate method for determination of antihemophilic activity of

7. Brinkhous, K. M., *Am. J. Med. Sc.*, 1939, v198, 509.

8. Buckwalter, J. A., Blythe, W. B., and Brinkhous, K. M., *Am. J. Physiol.*, 1949, v159, 316.

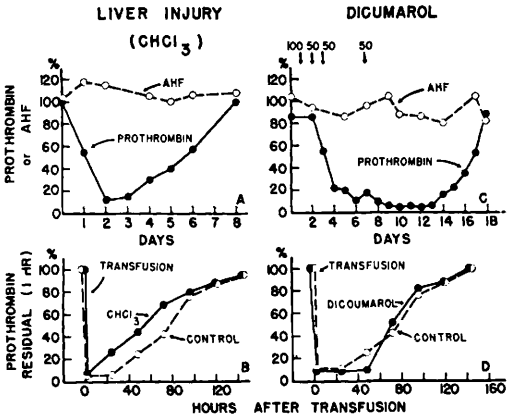

FIG. 2A. $CHCl_3$ anesthesia administered for 105 min. to dog 11 ♂, 19.1 kg, after fasting 24 hr. Normal control plasma (100% prothrombin and AHF values) obtained from Dog 3 ♂, 23.3 kg.

FIG. 2B. Transfusions of hemophilic dogs with plasma from $CHCl_3$-treated and normal dogs. $CHCl_3$ anesthesia for 2 hr and 90 min. respectively to dog 3 ♂, 25.2 kg and dog 12 ♂, 11.3 kg. Citrated plasma from the 2 $CHCl_3$-treated dogs (2 days post-anesthesia, prothrombin $< 5\%$) and from a normal dog (two separate preparations, dog 4 ♂, 22 kg) transfused to hemophilic dogs 13 ♂, 20 kg, 14 ♂, 20 kg, 15 ♂, 21.3 kg and 16 ♂, 20 kg respectively, 2.6 ml plasma per kg body wt. Prothrombin utilization rates of hemophilic whole blood determined before and after transfusion(1).

FIG. 2C. Dicumarol administration to dog 4 ♂, 22 kg. Dosage in mg indicated on chart. Normal control plasma (100% prothrombin and AHF values) obtained from dog 3 ♂, 23.3 kg.

FIG. 2D. Transfusions of hemophilic dogs with plasma from dicumarol-treated and normal dogs, 2.6 ml per kg body wt. One "dicumarol" plasma from dog, Fig. 2C, on 9th day. Other "dicumarol" plasma from dog 1 ♀, 19 kg, given 100 mg dicumarol 1st day, 50 mg 2nd, 3rd, and 4th days. Citrated plasma obtained on 5th day (prothrombin 8%). Hemophilic dogs 15 ♂, 21.3 kg and 17 ♀, 19 kg used as recipients. Normal plasma transfusions as in Fig. 2B.

plasma. However, the procedure is moderately laborious, since as many as 15 to 20 separate two-stage prothrombin tests are required for a single determination of AHF. Although only canine hemophilic blood was used as a substrate in the experiments above, human hemophilic blood has been used by us in similar studies, and it appears to give equally satisfactory results. Under the standard conditions of the AHF assay, an approximately linear relationship was found to exist between the amount of AHF added to hemophilic blood and the amount of prothrombin consumed (Fig. 1). This relationship may provide a basis for the definition of a unit of antihemophilic activity. Tentatively, it is suggested that one unit of AHF be defined as that amount needed to convert 10 units of prothrombin per ml plasma under standard conditions in the assay procedure. Since dog plasma contains about 400 units of prothrombin per ml with the method used, and since about 0.013 ml normal plasma was required to convert 50%, or 200 units, of prothrombin in the assay procedure (Fig. 1), then normal dog plasma would contain roughly 1500 AHF units per ml.

The data in Table I indicate that a relatively steady state of plasma AHF exists in normal dogs, regardless of sex. This is true also for females heterozygous for hemophilia. The normal AHF levels in the heterozygotes appear to rule out the possibility of their identification by merely determining the plasma content of this clotting factor.

The failure of liver damage to reduce the plasma AHF suggests that this factor is not produced in the liver. Likewise, the stability of AHF during dicumarol treatment indicates that it is synthesized by a mechanism much different from that for prothrombin. It is of interest that plasma used for transfusions in the liver injury experiments contained little or no fibrinogen. In one experiment, the blood did not clot, either spontaneously or after addition of thrombin. Yet the plasma AHF was fully active when transfused into a hemophilic dog. This is another example of dissociation of fibrinogen and AHF(3), clotting factors with similar physical properties.

Summary. 1. A method is described for the assay of antihemophilic factor; results may be expressed in per cent of a control plasma or in units. Hemophilic blood, canine or human, is used as substrate. 2. In normal dogs, regardless of sex, and in females heterozygous for hemophilia, a steady state of plasma AHF appears to exist. The antihemophilic activity of heterozygotes is approximately the same as that of normal dogs. 3. The antihemophilic activity of plasma is maintained at normal levels following acute liver injury, as well as after dicumarol administration.

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