THE PATHOGENESIS OF HEMOPHILIA

An Experimental Analysis of the Anticephalin Hypothesis in Hemophilic Dogs *

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One of the more puzzling features of modern blood coagulation theory is the pathogenesis of hemophilia with its mutually exclusive "procoagulant-deficit" and "anticephalin-excess" hypotheses. Briefly, in the "procoagulant-deficit" hypothesis the hemophilia gene is expressed biochemically as inadequate production of the antihemophilic factor (AHF), a coagulant factor required for normal hemostasis. The antihemophilic factor is considered to be produced in adequate amounts in normal animals by the action of the normal allele(s) at the hemophilia locus (1). This factor is thought to be necessary at an early stage in clotting for the normal rapid transformation of the proenzyme, prothrombin, into its active form, thrombin (2).

The "anticephalin-excess" hypothesis postulates that the bleeding tendency in hemophilia results from excess of a circulating inhibitor, "anticephalin." This substance is believed to be present also in normal blood at a lower titer acting to prevent intravascular clotting (3–7). An important corollary of this hypothesis is that there is a full complement of coagulant factors in hemophilic blood.

It is fair to state that the "procoagulant-deficit" hypothesis is the orthodox view. It constitutes the basic premise both of conventional transfusion therapy for hemophiliacs and the fractionation of normal plasma for AHF (8). The "anticephalin-excess" hypothesis was enunciated in its present form in 1943 (9, 10). It has become widely known by the accretion of other types of experiments which appear consistent with it (11–17). A complete (at that time) summary of the evidence for this view was made in 1951 (7), and more recently a pictorial representation has been published (18).

Several experiments have been considered important evidence substantiating the anticephalin hypothesis. Four of the more important ones are: I. The accelerated clotting of hemophilic plasma after contact with asbestos. II. The existence of the biphasic clotting curve on dilution of plasma. III. The increased speed of prothrombin utilization in hemophilic blood on dilution. IV. The activation by ether of an "accelerator," presumed to be AHF, in both normal and hemophilic plasma.

It appeared to us that the difference of concept about hemophilia might possibly be resolved by experiments on hemophilic dogs. The original descrip-

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tions of these animals (19,20) emphasized the similarity of their malady to human hemophilia. The experiments thought to imply deficit of a procoagulant in humans have been duplicated numerous times in these animals (19–27), and are summarized for brevity in Table I. However, the experiments which led to the anticephalin idea have not been attempted previously in dogs.

This paper describes an attempt to duplicate in hemophilic dogs the four experiments listed above. The questions for which answers have been sought are: (*a*) Can results similar to those in the hemophilic human be obtained in

TABLE I

Comparison of Human and Canine Hemophilia

Test	Species of hemophiliac	
	Human	Canine
Clotting time	Prolonged	Prolonged
Bleeding "	Normal	Normal
Prothrombin time	"	"
" concentration	"	"
" consumption	Impaired	Impaired
Platelets	Normal	Normal
Partial thromboplastin time	Prolonged	Prolonged
Thromboplastin generation test	Plasma-inactive; serum-active	Plasma-inactive; serum-active
Mode of inheritance	Sex-linked recessive	Sex-linked recessive
Effect of various types of plasma on defective clotting of hemophilic plasma *in vitro*		
Normal human plasma	Corrective	Corrective
" dog "	"	"
Hemophilic human plasma	Not corrective	Not corrective
" dog "	" "	" "

the hemophilic dog?, and (*b*) Does extension of the experiments affect the previous interpretation?

Materials and Methods

Blood was obtained from the jugular vein of normal and hemophilic dogs and from the antecubital vein of normal human subjects. The two syringe technique, in which the first syringe of blood is discarded, was employed in all instances. All blood was collected in siliconed syringes, moistened with saline to prevent ingress of air, and often precooled to 4°C. Following Tocantins' technique (7) the anticoagulant used in many experiments was 38 per cent sodium citrate in the ratio of 0.1 ml. of citrate to 9.9 ml. of blood. The citrate was either mixed gently with the blood in a centrifuge tube, or introduced into the collecting syringe prior to venipuncture. In other experiments blood was drawn into 0.112 M potassium oxalate solution in the ratio of 1 ml. oxalate to 9 ml. blood.

Plasma was prepared from citrated or oxalated blood by centrifugation in an angle centrifuge for from 20 to 60 minutes, at 4°C., with a force ranging from 2200 to 3200 *g*.

Native plasma was obtained from blood collected in chilled siliconed syringes, transferred to precooled siliconed tubes, and centrifuged rapidly at the above force for 10 minutes at 4°C. After centrifugation, the supernatant plasma was removed from the packed cells with siliconed pipettes.

Dilutions of plasma were made in a serial, twofold fashion in siliconed test tubes in which the appropriate saline diluent had been placed previously. After careful mixing, the diluted plasmas were transferred with siliconed pipettes to glass or siliconed clotting tubes.

Cephalin clotting times were performed in many of the experiments. The standard reaction mixture consisted of 0.5 ml. of citrated or native plasma, 0.1 ml. of 1 per cent cephalin and (with citrated plasmas) 0.05 ml. of 0.2 M $CaCl_2$ (7). The cephalin was prepared by the method described by Langdell *et al.* (30) followed by six precipitations with absolute ethanol. The order of addition of reagents depended upon the purpose of the experiment. Usually, the plasma was added to a tube containing cephalin and $CaCl_2$. In other experiments, plasma was added to cephalin, and the $CaCl_2$ introduced later. A stop-watch was started at the completion of the threefold mixture and stopped when gelation occurred.

Prothrombin content of various samples of plasma was assayed by the two stage method of Smith, Warner, and Brinkhous as described by Wagner *et al.* (28).

Prothrombin utilization rate of blood obtained from hemophilic dogs was determined by the method of Buckwalter, Blythe, and Brinkhous as described by Graham *et al.* (29).

The antihemophilic activity (*AHF*) of plasma samples was determined by the partial thromboplastin technique (30).

Fibrinogen levels were measured by clotting a 0.2 ml. sample of citrated plasma with 0.02 ml. of 100 unit per ml. thrombin topical and 0.2 ml. of an equal mixture of 0.02 M $CaCl_2$, 0.017 per cent imidazole buffer at pH 7.25, and 0.9 per cent saline. After 30 minutes, the clot was wound out on a clean glass rod and thoroughly washed once with 0.9 per cent NaCl, twice with acetone, and twice with ether. The rod, with the clot, was transferred to a Nessler tube for digestion with a mixture consisting of 2 ml. 10 N H_2SO_4 (reagent grade) and 0.4 ml. of a saturated solution of nitrogen-free Na_2SO_4. The nitrogen content of the clot was determined colorimetrically (31), and expressed as milligrams per cent of fibrinogen.

Plasma and serum electrolytes were determined by the methods described in the references indicated, *i. e.* sodium and potassium (32), chloride (33), calcium (34), phosphorus (35).

Total osmolarity was determined with the Fiske osmometer (Fiske Associates, Inc., Boston, Massachusetts) by the freezing point method.

Conductivities of solutions were measured, temperature controlled, with an International Instruments conductivity bridge and expressed in mhos.

Ether Extraction of Plasma.—The normal and hemophilic dog plasmas were extracted essentially by Johnson's technic (11) as follows. One lot of each type of plasma was defibrinated by the addition of an equal volume of purified citrate-activated thrombin diluted to 20 units per ml.; the other lot was extracted without thrombin defibrination. In both instances equal volumes of plasma and anesthetic ethyl ether (U.S.P.) were mixed in stoppered Erlenmeyer flasks and shaken in a mechanical shaker for 30 minutes at room temperature. The mixtures were allowed to stand at room temperature in the stoppered flasks for 15 minutes, by which time the ether layer had separated. The ether layer was removed by pipetting, and the procedure repeated twice more. The extracted plasma samples then were placed in a vacuum dessicator under reduced pressure at room temperature. After 2 hours under these conditions the odor of ether in the samples was usually barely detectable. The samples were quickly frozen at −20°C. and stored overnight, analyses being performed the following day on all samples.

Partial thromboplastin times of various plasmas and mixtures of plasmas were determined by the method of Langdell *et al.* (24). The cephalin used was a 0.3 per cent suspension as described by Langdell *et al.* (30).

Thromboplastin generation test (36) was performed on normal and hemophilic dog plasmas treated in a variety of ways. In all experiments the same 0.03 per cent cephalin suspension (30) and the same sample of normal dog serum diluted 1–10 with 0.9 per cent NaCl were used. Oxalated plasmas were adsorbed with 100 mg. $BaSO_4$ per ml. It was found empirically that 0.012 M $CaCl_2$ rather than the usual 0.02 M $CaCl_2$ was optimal for this system.

EXPERIMENTAL

I. Effect of Contact with Asbestos on Hemophilic Dog Plasma

It has been shown that contact between citrated plasma and asbestos markedly shortens the cephalin clotting time of both normal and hemophilic human plasma (3, 7, 38, 39). This change has been thought due to adsorption of plasma anticephalin by the asbestos. It has been remarked by a proponent of the anticephalin hypothesis that since asbestos apparently adsorbs anticephalin, it should be possible to treat a hemophiliac by transfusion of his own plasma after contact with asbestos (7). This inference provided an excellent test of the effect of asbestos on hemophilic plasma.

TABLE II

The Effect of Contact with Asbestos on the Clotting Time of Hemophilic Dog Plasma

Hemophilic dog No.	Cephalin clotting time	
	Before	After
	sec.	*sec.*
50-28	360	180
51-02	330	120
52-05	360	150

Citrated plasma was prepared from hemophilic dog blood obtained from each of three hemophilic dogs using siliconed equipment. The blood, citrated in the ratio of 1:100 with 38 per cent sodium citrate, was centrifuged in siliconed tubes at 4°C. for 20 minutes at a force of 3200 *g*. A cephalin clotting time was performed on the resulting plasma before and after exposure to asbestos fibers (10 mg./ml. for 1 hour at room temperature). The asbestos fibers were removed from the treated plasma by centrifugation at 3200 *g* before testing.

The experiment was performed 3 times on different hemophilic dogs with uniform results. The clotting time of hemophilic dog plasma was sharply reduced after exposure to asbestos (Table II). The effect of transfusion of this adsorbed plasma on the hemophilic defect is shown in Fig. 1. Prothrombin converted very slowly in the blood obtained from the hemophilic dogs prior to transfusion (curve *A*). After transfusion of autologous asbestos-treated hemophilic plasma with its shorter clotting time, the prothrombin utilization rates were essentially unaltered (curve *B*). Two of the same dogs were given the same quantity of untreated citrated normal plasma at a later time. Prothrombin disappearance was greatly accelerated, approaching normal (20), shown by curve *C*.

II. The Biphasic Clotting Curve on Dilution of Plasma

The biphasic clotting curve on dilution of plasma was described in 1943 (9, 10). This phenomenon can be demonstrated without difficulty with both dog and human plasma, normal and hemophilic. In Fig. 2, the characteristic descending limb (clotting time decreasing on dilution) and ascending limb (clotting time increasing after passing through a minimum) are illustrated. In the experiment shown, plasma and Ca^{++} concentrations were varied by dilution

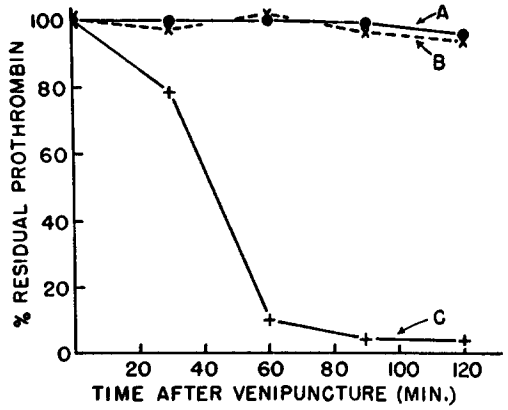

FIG. 1. The asbestos-treated plasmas with the shortened clotting times (Table II) were auto-transfused into the respective hemophilic donors at a dose of 2 ml. per kg. body weight. Prothrombin utilization rates were determined on the recipient dogs immediately before and 15 minutes after these transfusions. Two weeks later *untreated* normal citrated plasma was transfused into two of the same hemophilic dogs at 2 ml. per kg., and similar studies performed at the same intervals before and after transfusion. Values plotted are the means of 3, 3, and 2 experiments respectively, each in duplicate. The pretransfusion prothrombin utilization rates (curve A) were obtained on dogs 50-28, 51-02, 52-05. Asbestos-adsorbed autologous plasma was given to 50-28, 51-02, 52-05. Fifteen minutes later the test was repeated (curve B). Normal plasma was given 2 weeks later to 51-02 and 52-05, and 15 minutes after transfusion the prothrombin utilization rates were determined (curve C).

with 0.85 per cent NaCl. The upper curve was produced by addition of a crude cephalin preparation, 0.3 per cent *w/v*, and optimal $CaCl_2$ to citrated dog plasma serially diluted with 0.85 per cent NaCl. In the lower curve, the plasma was clotted with a purified 1 per cent cephalin preparation. The clotting times were greatly shortened by the stronger cephalin and the biphasic nature of the curve tended to become obscured, but the descending and ascending limbs were still apparent. The acceleration of clotting producing the descending limb of this type of curve has been interpreted as dissociation of an anticephalin-cephalin complex, the dissociation presumably relieving a pre-existing inhibition of the factors tending to clot. The ascending limb, deceleration of clotting, appears clearly to represent dilution of the clotting elements past a critical concentration.

In analyzing these dilution experiments control of ionic strength immediately

became an issue, because the solutions used as anticoagulant and for recalcification were highly concentrated. It was noted first by Tocantins (39,40) and has been our experience uniformly, that the most markedly biphasic curves are produced by highly concentrated citrate and calcium solutions. Obviously ionic strength is greater initially in such plasmas than in conventionally citrated and recalcified plasmas. It seemed possible that ionic strength might be changing appreciably between dilutions on the descending limb of these curves,

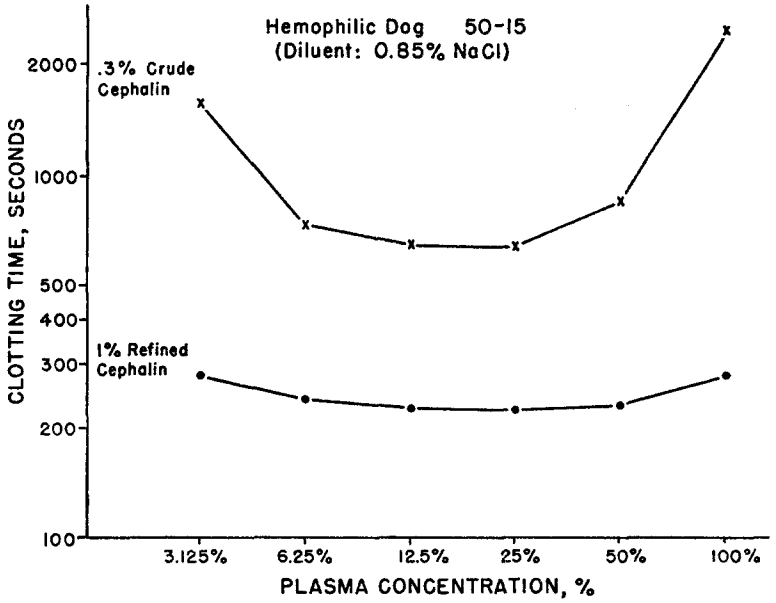

FIG. 2. Plasma was prepared from blood citrated in a ratio of 1:100 with 38 per cent sodium citrate. The blood was centrifuged in siliconed tubes for 1 hour at 3200 *g* at 4°C. to obtain platelet-poor plasma. The upper ¾ of the plasma layer was removed and diluted with 0.85 per cent saline. The plasma was recalcified by adding 0.5 ml. to a test tube containing 0.1 ml. of cephalin, then adding 0.05 ml. of 0.2 M $CaCl_2$. When plasma was diluted with saline the $CaCl_2$ was correspondingly diluted with the same saline solution (37).

and determination of ionic strength in these systems became essential. It was found that direct determination of ionic strength in a complex mixture of salts and colloids such as plasma was not possible. We were advised that the best approximation would be obtained by a calculation based on electrolyte concentrations and employing the fewest assumptions.

In the estimations subsequently made, the calcium and citrate molarities were calculated exactly for all plasmas from the stability equation (since these molarities are multiplied by 4 and 9 respectively) and the following simplifying assumptions made:—

1. That the electrolyte concentrations of our dogs were the same as the mean values found in the literature (41). (Determinations of Na, K, Cl, P, Ca showed that our dogs were normal in this regard).

2. That all electrolytes in plasma except calcium and citrate were completely ionized. (This assumption is obviously not correct since plasma is not a dilute solution and gives a higher value than the true value. The same assumption, however, was made in estimating the ionic strength of all types of plasma).

3. That all the calcium of plasma, bound and free, but none of the magnesium reacted with the citrate. (This simplifies the calculation and has little effect on the result).

4. That secondary ionization of phosphate could be neglected.

5. That the only complex of calcium and citrate which formed was the univalent anion, $CaCitrate^{-}$, formed reversibly from one molecule of Ca^{++} and one molecule of $citrate^{=}$, with a stability constant of 1585 (42, 43).

It should be emphasized that these assumptions give values for ionic strength of both native and citrated plasma which are somewhat higher than the true values. However, the crucial values are the differences in ionic strength between types of plasma and successive dilutions of the same plasma, not the exact ionic strength of a specific plasma.

Using the assumptions mentioned earlier, and taking into consideration dilution of the inert ions, the increase in sodium and chloride on citration and recalcification, and the calcium-citrate equilibrium, it was estimated that the ionic strength after recalcification of citrated plasma from a dog with a 41 per cent hematocrit reading was 0.2079 when the blood was citrated with 38 per cent sodium citrate and recalcified with 0.2 M $CaCl_2$. The ionic strength of native plasma, under the same assumptions, was 0.1522, and that of 0.85 per cent NaCl was 0.1454.[1] Further calculations verified what had been anticipated intuitively.

[1] The actual data on which the ionic strengths were calculated were:—

Ion species	Native plasma		Recalcified-citrated plasma	
	Molarity	$m_i v_i^2$	*Molarity*	$m_i v_i^2$
Na	0.1500	0.1500	0.1933	0.1933
K	0.0044	0.0044	0.0039	0.0039
Mg	0.0009	0.0036	0.0008	0.0032
HCO_3	0.0205	0.0205	0.0183	0.0183
Cl	0.1060	0.1060	0.1310	0.1310
HPO_4	0.0014	0.0056	0.0012	0.0048
SO_4	0.0010	0.0040	0.0009	0.0036
Ca	0.0026	0.0104	0.0037	0.0148
Citrate	—	—	0.0029	0.0261
CaCitrate	—	—	0.0168	0.0168
Sum		0.3045		0.4158
μ		0.1522		0.2079

It is interesting to note that conductivity measurements and freezing point determinations on the two plasmas were consistent with the hypothesis that the recalcified-citrated plasma has an ionic strength greater than native plasma:—

	Recalcified-citrated plasma (R-C)	Native plasma (N)	Ratio R-C/N
Calculated ionic strength, μ	0.2079	0.1522	1.36
Conductivity, *mhos* (25°C.)	0.0129	0.0103	1.25
Osmolarity, milliosmols/kg. H_2O	396	350	1.18

The decrease in ionic strength was greater between successive dilutions of strongly citrated plasma than the decrease between successive dilutions of conventionally citrated plasma or native plasma.

Our tentative hypothesis that the biphasic dilution curve is a result of uncontrolled ionic strength was tested empirically by diluting plasma in three fashions. In one set of experiments an attempt was made to maintain ionic strength constant on dilution; in another ionic strength was decreased or increased very markedly. Under the anticephalin hypothesis, the biphasic curves should persist in all instances. If the descending limb of the curve results

TABLE III

Cephalin Clotting Times of Native Plasma, Normal and Hemophilic, diluted with 0.89 per cent NaCl

Plasma Concentration	Cephalin clotting times			
	Normal plasma		Hemophilic plasma	
	Dog 54-51	Dog 55-05	Dog 52-16	Dog 55-32
per cent	*sec.*	*sec.*	*sec.*	*sec.*
100	105	125	155	165
50	109	133	161	170
25	124	150	202	198
12.5	139	190	218	217
6.25	335	422	364	485
3.125	709	>840	>1080	820

Native plasma was prepared from 2 normal and 2 hemophilic dogs by the 2 syringe technique using chilled siliconed syringes, centrifuge tubes, pipettes, and clotting tubes. Blood was centrifuged at 3200 *g* for 5 minutes at 4°C. Plasma dilutions were made rapidly by serial dilution, and diluted plasma was transferred in 0.5 ml. lots to siliconed tubes containing 0.1 ml. of 1 per cent cephalin.

from decreasing ionic strength, dilution should produce the following effects: (*a*) When ionic strength does not change, the clotting time should remain essentially constant in the first several dilutions, then lengthen; (*b*) When ionic strength is reduced the clotting time should shorten before increasing; (*c*) When ionic strength is increased, the clotting time should lengthen, the extent depending on the rate of change in ionic strength.

In the first set of experiments, an attempt was made to maintain ionic strength constant during dilution. Native dog plasma (without anticoagulant or additional calcium) was diluted with 0.89 per cent NaCl, both having ionic strength calculated to be approximately 0.15. The results are shown in Table III. The dilution curves of both normal and hemophilic dog plasma were "monophasic" when the plasma was diluted with an "iso-ionic" solution.

In the next set of experiments (Fig. 3) the effects of markedly reducing and markedly increasing ionic strength were compared on samples of citrated plasma. This plasma, ionic strength calculated to be approximately 0.21, was diluted with distilled water or with saline solutions at several concentrations. Distilled water exaggerated the biphasic curve. The usual biphasic curve was obtained with 0.85 per cent saline. Saline solutions of ionic strength greater than plasma abolished the biphasic phenomenon, the curve with 2.09 per cent NaCl rising more rapidly than the one with 1.7 per cent NaCl.

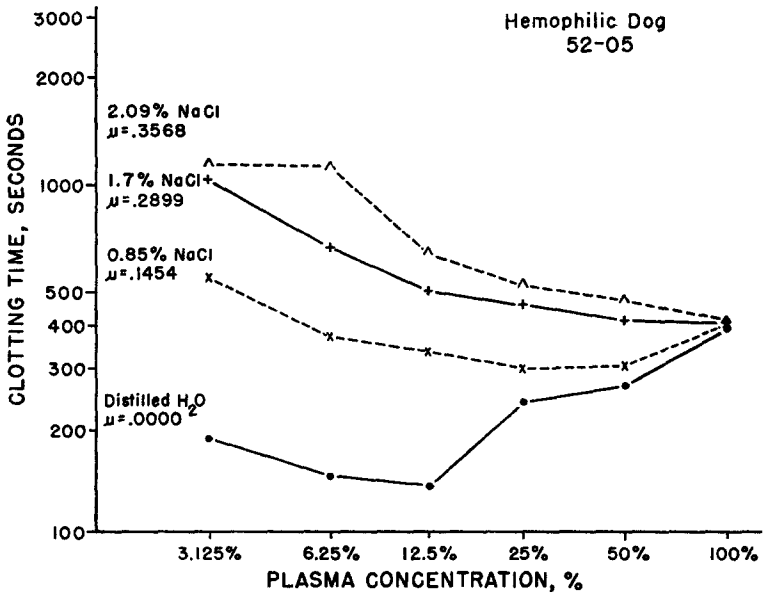

FIG. 3. Citrated plasma was prepared and tested as described in Fig. 2. The plasma was diluted with three saline solutions of widely varying ionic strength and also with distilled water. The clotting time values are the averages of duplicate determinations.

III. Effect of Dilution of Hemophilic Blood on Prothrombin Utilization

It has been reported that the rate of prothrombin utilization in human hemophilic blood increases on dilution, a fact consistent with the anticephalin excess hypothesis (7). In the human experiments used for adducing this fact, prothrombin utilization rates were compared in undiluted hemophilic blood and the same blood diluted 1:2 with 0.85 per cent saline. The 1:2 dilution is ideal for this comparison, because the minimum clotting time of the biphasic dilution curve of citrated plasma usually occurs at this dilution, and prothrombin is still sufficiently concentrated for accurate assays by the two-stage method.

An experiment, similar to the human experiment (7), was performed on undiluted whole hemophilic dog blood and the same blood diluted 1:2 with 0.85 per cent saline. The data from four experiments on different hemophilic dogs are recorded in Fig. 4. This has been accomplished by plotting the residual prothrombin in undiluted blood, at a given incubation time, against the residual

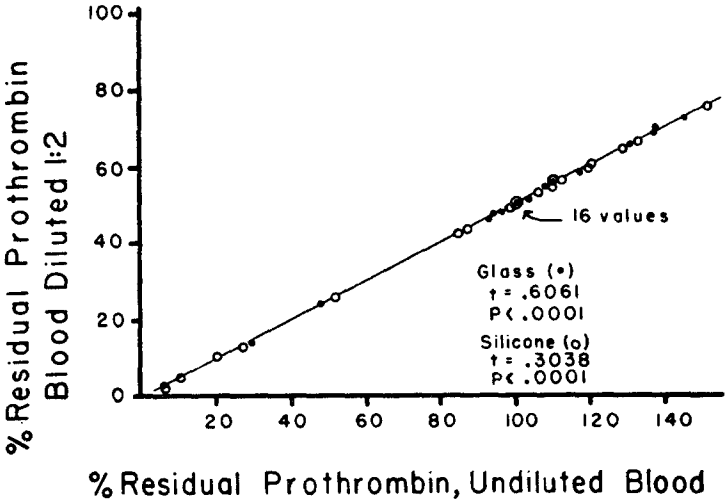

FIG. 4. Undiluted native whole blood and whole blood diluted 1:2 with 0.85 per cent NaCl were obtained from hemophilic dogs with siliconed syringes at the same venipuncture. The diluted blood was obtained by half-filling a siliconed syringe with saline and withdrawing blood to capacity. The blood was mixed evenly by several careful inversions, then transferred in 1 ml. quantities to the clotting tubes. A complete series of both plain glass and siliconed tubes were used in each experiment. At intervals up to 4 hours prothrombin utilization was stopped by the addition of 0.15 ml. of 3.2 per cent sodium citrate to several of the tubes of each type. The citrated blood was centrifuged, and citrated serum obtained. Residual prothrombin was determined by the two-stage method on the samples from each dog, undiluted and diluted, in duplicate, in both siliconed and plain glass tubes. Duplicate values were averaged, and the averages plotted on the chart. The experiment was repeated four times on different hemophilic dogs.

prothrombin in the matched 1:2 dilution from the same dog at the same incubation time. This type of analysis permits comparison of all pairs of values with a single test. The comparison is independent of the period of incubation, the conversion rate at any time, the particular dog, or the type of surface in the clotting tubes. If there is no difference between the rates in the paired samples, all points in Fig. 4 should lie on a line with slope, $m = \frac{1}{2}$. It can be seen that the data approximate this theoretical line rather closely, suggesting that there was no difference between the rates at the two dilutions. With a t test, it was

found that for either plain glass or siliconed glass such a close fit would be expected to occur less than once in 10,000 times by chance if the rates were different.

It will be noted in Fig. 4 that some of the prothrombin values of undiluted blood are greater than 100 per cent and of diluted blood greater than 50 per cent. This phenomenon was observed in all four experiments usually in the 30 and 60 minute samples. Taken literally, it would appear that the prothrombin concentration increased during the early period of the experiment. On the other hand, this increase might have been apparent rather than real. Conceivably it could have resulted from (*a*) deterioration of antithrombin, (*b*) transformation of prothrombin from a less to more active form (44), or (*c*) an incubation mixture for the two-stage prothrombin test not optimal with respect to serum accelerators (25). However, from the standpoint of the comparison of prothrombin disappearance rates in undiluted and diluted blood this puzzling observation appears irrelevant. Both diluted and undiluted blood showed the same degree of the same phenomenon; *i.e.* the points with coordinates greater than (100,50) failed to deviate from the theoretical line.

IV. Some Effects of Ether Treatment of Plasma

It has been observed that the conversion of "purified" prothrombin to thrombin in the presence of calcium, Ac-globulin, and platelets is accelerated by the addition of ordinary plasma, or of plasma extracted with ether (11–15). It has been suggested that the factor accelerating this reaction is the antihemophilic factor (AHF). Hemophilic and normal plasma, after ether extractions, have been found to react similarly in this system. The similarity of effects with the two types of plasma has been attributed to removal by the ether of a lipide "anticephalin," unmasking the antihemophilic factor but not altering it. This interpretation assumes that the two plasmas initially contained the same quantity of AHF and differed only in a greater concentration of "anticephalin" in the hemophilic.

It was decided to determine the AHF activity of ether-treated plasma using hemophilic plasma as substrate rather than purified prothrombin. Several methods were used to test for AHF activity including an *in vitro* AHF assay, transfusion of treated plasmas to hemophilic dogs, the partial thromboplastin times of various mixtures, and the thromboplastin generation test.

It can be seen (Table IV) that ether extraction alone, aside from thrombin, removed fibrinogen from the plasmas. It also reduced the pre-existing AHF activity of the normal plasma to that of hemophilic, *i.e.* both approached zero. By contrast, untreated normal plasma had a striking effect in a hemophilic plasma substrate as represented by a 89 to 100 per cent AHF titer. The controls show that ether extraction had a slight destructive effect on plasma

prothrombin, while thrombin had a rather marked effect. They also show that deterioration of the AHF during the extraction procedure may have been accomplished solely by the ether, although no test was made of the action of thrombin alone.

The finding that antihemophilic activity was not present in ether-extracted plasma as measured by *in vitro* tests was confirmed by *in vivo* experiments. Large volumes of normal and hemophilic dog plasma were extracted by the same technique. Hemophilic dogs were transfused with these extracted plasmas

TABLE IV

Some Effects of Ether-Extraction and Defibrination with Thrombin on the Fibrinogen, AHF and Prothrombin of Normal and Hemophilic Dog Plasma

Test	Normal plasma			Hemophilic plasma			Control plasma	
	Frozen, stored, −20°C.	Ether extracted, 28°C		Frozen, stored, −20°C	Ether-extracted, 28°C.		(28°C., 5 hrs. not defibrinated)	
		Non-defibrination	Defibrination		Non-defibrination	Defibrination	Normal	Hemophilic
Fibrinogen, *mg. per cent*	306	0	0	236	0	0	—	—
One-stage AHF assay, *per cent*	100	<5	<5	<5	<5	<5	89	<5
Two-stage prothrombin, *units*	294	236	108	288	221	129	240	246

Normal and hemophilic plasmas were prepared by drawing 9 volumes of normal and hemophilic dog blood, separately, into syringes containing 1 volume of 0.112 M potassium oxalate. The blood samples were centrifuged at 3200 *g* for 30 minutes at 4°C. The supernatant plasmas were pipetted and a sample frozen and stored at −20°C. for subsequent analysis. The remaining plasma of each type was divided into three lots. One lot was defibrinated and ether-treated, the second was merely ether-treated, and the third was stoppered and allowed to stand on the bench in a glass container during the course of the experiment (5 hours).

at a standard dose of 2 ml. per kg. body weight. Later the same dogs were given transfusions of *untreated* normal plasma at the same dosage. The effects of both types of plasma were measured by prothrombin utilization rates on the animals. As usual, *untreated normal* plasma had a striking effect in accelerating prothrombin conversion in the hemophilic dog (as in curve C of Fig. 1). Consistent with the *in vitro* experiments, the ether-extracted plasma from both genotypes had the same negligible effect as the asbestos-adsorbed hemophilic plasma of curve B, Fig. 1.

The conclusion from the preceding experiments, that ether-extracted plasma lacked antihemophilic activity, appeared inconsistent with the repeated observation (11–15) that such plasma accelerated thrombin formation from

"purified" prothrombin. Another test system was devised with a different ratio of treated plasma to test substrate. The effect of ether-extracted normal and hemophilic dog plasma on an equal volume of untreated hemophilic plasma was studied by the partial thromboplastin time (Table V). It can be seen (*cf.* lines 1, 3, 5, 7) that clot-promoting activity was "generated" in both types of plasma by the ether treatment. It exceeded in fact the accelerating effect of untreated but $BaSO_4$-adsorbed normal plasma (line 4). However, as shown in lines 6 and 8, the activity "generated" was removed by adsorbing the ether-extracted plasmas with $BaSO_4$. In view of the fact that the AHF is *not* adsorbed

TABLE V

*Partial Thromboplastin Times of Hemophilic Plasma When Mixed with Ether-Extracted and $BaSO_4$-Adsorbed Plasmas, Normal and Hemophilic**

Line No.	Hemophilic plasma	Normal plasma				Hemophilic plasma		Partial thromboplastin time
		Untreated	$BaSO_4$-adsorbed	Defibrinated, ether-treated	Defibrinated, ether-treated, $BaSO_4$-adsorbed	Defibrinated, ether-treated	Defibrinated, ether-treated, $BaSO_4$-adsorbed	
								sec.
1	0.10							91
2		0.10						41
3	0.05	0.05						44
4	0.05		0.05					53
5	0.05			0.05				39
6	0.05				0.05			79
7	0.05					0.05		40
8	0.05						0.05	85

* Plasmas were prepared as described in detail in the methods section and the footnote to Table IV.

by $BaSO_4$ (22), this experiment seems to imply that the activity "generated" was not AHF.

The activation by ether of a clotting factor with effects in the partial thromboplastin time was examined also in the thromboplastin generation test (Fig. 5). The plasmas were prepared exactly as in the previous experiments and used as the source of plasma for this test with and without adsorption by $BaSO_4$. In all generation tests except curve A, the normal control, the plasma under study was mixed with an equal part of fresh, $BaSO_4$-adsorbed hemophilic dog plasma. This step was introduced because an adequate supply of factor V has been shown to be necessary for a normal generation test (45, 46). It can be seen that ether-extracted normal plasma was active in this system, but somewhat less active than untreated normal plasma (*cf.* curves B and D). When the extracted normal plasma was adsorbed with $BaSO_4$, it lost most of its generative activity, becoming quasihemophilic (*cf.* curves B, D, and F with

C). Similarly, ether extraction activated a component of hemophilic plasma which showed some activity in the generation test (*cf.* curves C and E). Again, however, it can be seen that the activity "generated" was largely adsorbed by $BaSO_4$ (*cf.* curves C, E, and G).

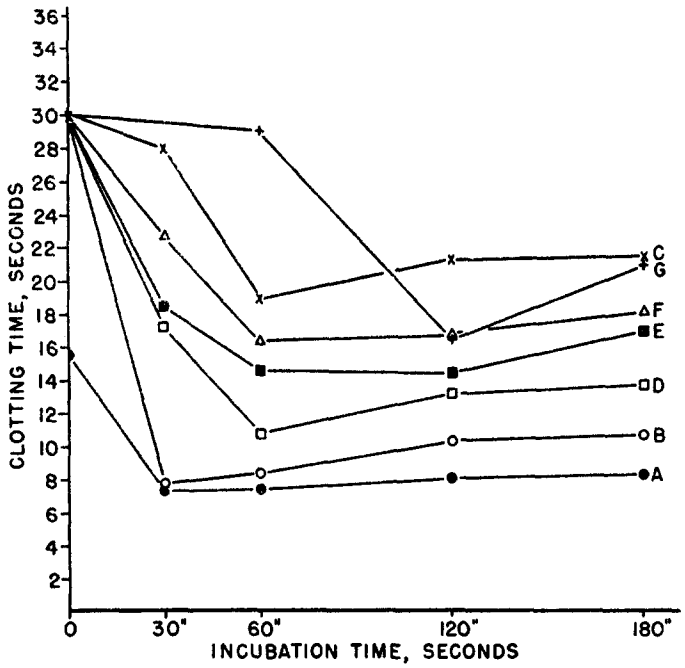

FIG. 5. *Thromboplastin Generation Test.*—All tests were done using the same normal dog serum, diluted 1–10, and the same 0.03 per cent cephalin suspension. Plasma mixtures consisted of equal parts each type as recorded below. The plasmas, diluted 1–4, were as follows: *curve A* (normal, $BaSO_4$-adsorbed), *curve B* (hemophilic, $BaSO_4$-adsorbed + normal, $BaSO_4$-adsorbed), *curve C* (hemophilic, $BaSO_4$-adsorbed), *curve D* (hemophilic, $BaSO_4$-adsorbed + normal, ether-treated), *curve E* (hemophilic, $BaSO_4$-adsorbed + hemophilic, ether-treated), *curve F* (hemophilic, $BaSO_4$-adsorbed + normal, ether-treated, $BaSO_4$-adsorbed), *curve G* (hemophilic, $BaSO_4$-adsorbed + hemophilic, ether-treated, $BaSO_4$-adsorbed). The normal mixture (curve A) when fully activated gave the following clotting times on dilution with saline: undiluted (100 per cent) = 7.4 seconds; 1:2 dilution (50 per cent) = 8.4 seconds; 1:4 dilution (25 per cent) = 14.8 seconds; 1:8 dilution (12.5 per cent) = 29.6 seconds.

DISCUSSION

Great care was taken in these experiments to duplicate the conditions of the original human experiments, because the plasma-clotting mechanism is known to be labile and subject to systematic error. It should be emphasized that the original human experiments have been significantly extended in these dog experiments, particularly by transfusing variously treated plasmas into the hemophilic dog, a type of testing which has not been attempted in humans.

It is important to note that contact with asbestos greatly reduced the *in vitro* clotting time of hemophilic dog plasma but that this plasma was inactive when transfused. This dichotomy suggests that the effect of asbestos has been something other than removal of an inhibitor or an "unmasking" of AHF. Perhaps an accelerator is activated by asbestos, such as the $BaSO_4$-adsorbable serum accelerator(s) known to shorten markedly the clotting time of hemophilic blood *in vitro* without significantly affecting the prothrombin utilization rate (25).

In considering the experiments on the biphasic clotting curve, it should be pointed out that there are serious theoretical objections to a calculation of plasma ionic strength from electrolyte concentrations. Plasma is too concentrated to follow dilute solution theory and contains a variety of colloids. However, if this important variable is considered seriously, there is no better alternative at present. There are also serious objections to conductometric determinations of plasma ionic strength. Not the least of these objections is the theoretical one. Conductivity measures the speed of migration of ions while ionic strength is a measure of the "ion atmosphere," a function of both concentration and squared charge. The fact that ionic strength is commonly estimated conductometrically in the laboratory on the monovalent ions of saline (by determining conductivity, obtaining concentration by comparison with standard solutions, then assuming complete ionization) by no means implies that this is an exact method or can be applied to plasma.

In prospect it was anticipated that the trivalent citrate and bivalent calcium ions would make heavy contributions to the calculated ionic strength of citrated plasma. Actually they were of secondary importance in the calculations, since the stability constant of calcium citrate is fairly high, and the two ions were present in almost equimolar proportions. Roughly 60 per cent of the increase in ionic strength of citrated over native plasma was contributed by the excess sodium and chloride acquired on citration and recalcification.

The experiments on the biphasic clotting curve are consistent with our hypothesis that the biphasic curve results from uncontrolled ionic strength. They show that experimental conditions in which ionic strength may reasonably be expected to remain constant or increase abolish the phenomenon, and that conditions which may reasonably be expected to decrease ionic strength more markedly than 0.85 per cent saline (distilled water) exaggerate the phenomenon. This does not *prove* that variable ionic strength is the cause of the biphasic curve. However, it appears that variation in this parameter is at least as likely an explanation as dissociation of a hypothetical accelerator-inhibitor complex.

If the biphasic phenomenon results from decreasing ionic strength, why does this decrease speed clotting? Lovelock and Porterfield (47) have shown that the ionic strength of plasma (± 0.15) is not optimal for clotting. Their data suggest that, all other things being equal, clotting of plasma is most

rapid at an ionic strength of around 0.03 to 0.05 and that there is a direct relationship between ionic strength and clotting time over a wide range above this point. Thus, when plasma is diluted with a "hypo-ionic" solution, ionic strength decreases toward the optimal. Furthermore, the degree of "sag" in such a dilution curve should be greater with citrated than native plasma when diluted with the same "hypo-ionic" diluent. The change in ionic strength between successive dilutions clearly will be greater in the citrated plasma with its initially higher ionic strength. The British workers have suggested that plasma ionic strength is non-optimal with regard to clotting, because the heavy ion atmosphere increases the charge of the colloids. The increase in charge probably impairs colloidal interactions, perhaps by repulsion. As the ion atmosphere decreases on dilution, repulsion diminishes, the probability of colloidal collision increases, and the clotting reactions proceed more rapidly.

The experiments on the rate of prothrombin utilization during clotting of diluted and undiluted hemophilic blood appear conclusive. The data exclude with high probability the notion that the rate of prothrombin utilization increases on dilution. It should be emphasized that the canine experiments were performed on whole blood without anticoagulation and that very little alteration of ionic strength occurred on dilution, a situation which, as has already been pointed out, abolishes the biphasic curve.

The removal of antihemophilic activity by ether extraction of normal dog plasma at room temperature is not surprising. Ether is known to denature protein under this condition, and the antihemophilic factor is one of the more labile-clotting factors (48). There is no doubt from our experiments that ether removes AHF from normal plasma, if AHF is defined as *that activity of normal plasma, not adsorbed by $BaSO_4$, which accelerates the delayed clotting and prothrombin utilization of hemophilic blood and plasma in the presence of platelets and calcium ions in vitro, and corrects the hemostatic defect when transfused to a hemophiliac.* It is difficult from the literature to ascertain why it had been thought that ether-extracted plasma contained AHF, as this type of plasma appears not to have been assayed previously in a specific manner. It is of interest, however, that we also observed something in the ether-extracted plasma of both genotypes which accelerated clotting and was active in the thromboplastin generation test. The fact that this activity could be adsorbed with $BaSO_4$ appears to exclude AHF as a possibility.

As a result of these experiments in the dog, it is unnecessary to elaborate an "anticephalin" hypothesis to explain the pathogenesis of canine hemophilia. This raises the question whether canine and human hemophilia are different diseases or whether the "anticephalin" hypothesis is also irrelevant in human hemophilia. A categorical answer cannot be given to this question at present, because many of the experiments performed in the dog are dangerous and have not been performed in the human. However, it appears that the "anticephalin" hypothesis is dubious in humans also, because human and canine hemophilia

appear identical (See Table I). Until the two diseases are shown to be different, or incontrovertible evidence supporting the "anticephalin" idea is brought forward, it is logical to conclude that the "anticephalin" hypothesis is not applicable in either species.

SUMMARY AND CONCLUSIONS

It has been possible to duplicate in the hemophilic dog four of the major experiments which have suggested in humans an "anticephalin" hypothesis for the pathogenesis of hemophilia. The experiments in the dog have been considerably extended, as compared with the human experiments, by a variety of techniques.

I. Asbestos was placed in contact with hemophilic dog plasma, and the clotting time became shorter. When transfused, this plasma had no effect on the defective prothrombin utilization of hemophilic dogs, in contrast to untreated normal plasma.

II. The ionic strength of native dog plasma and dog plasma citrated (38 per cent sodium citrate) then recalcified (0.2 M $CaCl_2$) were calculated. The ionic strength of the native plasma was approximately 0.15 while that of the citrated plasma was approximately 0.21.

Conductivity and freezing point determinations on the plasmas described above were consistent with the idea that the ionic strength of the citrated plasma was significantly higher.

The biphasic dilution curve, to which much significance has been attached in arriving at the "anticephalin" hypothesis, can be produced readily in the dog.

Diluting dog plasma with "iso-ionic" or "hyper-ionic" NaCl solution abolished the biphasic phenomenon. Dilution with distilled water exaggerated the biphasic curve. These experiments suggest that the biphasic curve is an artifact of uncontrolled ionic strength.

III. The prothrombin utilization rates of undiluted whole hemophilic dog blood and hemophilic dog blood diluted 1:2 with 0.85 per cent NaCl were found to be the same.

IV. Ether extraction of both normal and hemophilic dog plasma removed fibrinogen and reduced somewhat the concentration of prothrombin. In treated normal plasma AHF was reduced to the level of untreated hemophilic plasma, thus producing a quasi-hemophilic plasma.

Defibrination and ether extraction of both normal and hemophilic dog plasma "generated" clotting activity which shortened the clotting time of hemophilic plasma and was active in the thromboplastin generation test.

The activity "generated" by defibrination and ether extraction of dog plasma was adsorbed by a $BaSO_4$ suspension and shown, therefore, not to be the antihemophilic factor (AHF).

Transfusion of ether-extracted normal or hemophilic dog plasma into hemo-

philic dogs had no effect on the prothrombin utilization rate, unlike untreated normal plasma which had a marked effect.

Thus, four of the main lines of evidence supporting the "anticephalin" hypothesis were duplicated in the dog. However, by extending the experiments it was found that all were explainable on bases other than the presence of "anticephalin." Such an hypothesis is not necessary, therefore, to explain the pathogenesis of canine hemophilia. The apparent identity of hemophilia in the two species suggests that the hypothesis is also not applicable to humans.

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