THE CONDITION OF THE BLOOD IN HEMOPHILIA, THROMBOSIS AND PURPURA *

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During the past two years I have made systematic examinations of the blood in patients suffering from various forms of diseases of the blood, hemorrhagic and thrombotic, with special reference to the variations in the amount of antithrombin and prothrombin. The most significant results have been obtained in cases of hemophilia and in so-called idiopathic or spontaneous thrombosis. The chief object of the present paper is to report the findings in these two latter conditions.

METHODS

The specimens of blood used for examination were obtained in all cases from one of the superficial veins of the arm by means of a graduated syringe (Luer form). The syringe was sterilized previously with boiling water and before inserting the needle into the vein it was filled with sterile salt solution (sodium chlorid 0.9 per cent.) by partially filling the syringe and then expelling the solution so as to leave the needle full. The object of this precaution was to avoid the bubble or two of air in the needle which otherwise interfered with the accuracy of the reading of the amount of blood taken. Usually 8 c.c. of blood were taken for each experiment and as soon as possible the portion to be examined for antithrombin and prothrombin, namely, 4 c.c., was emptied into a centrifugal tube containing 0.5 c.c. of a 1 per cent. solution of sodium oxalate (made up in a 0.9 per cent. solution of sodium chlorid). After mixing by inverting the tube this specimen was centrifugalized at a high speed to obtain a clear plasma. The plasma, which in human blood is always deeply colored, yellowish green, was pipetted off and examined as follows:

Antithrombin.—To determine the relative amount of antithrombin in the oxalated plasma of the patient it was compared always with a similar specimen taken at the same time from a normal person. The two plasmas were heated in a water-bath slowly to just 60 C. At this temperature (53 to 60) the fibrinogen is all precipitated and the thrombin and prothrombin are destroyed, while the antithrombin on the contrary is not affected, or at least is affected as little as possible consistent with the necessity of removing the fibrinogen and thrombin. Only at temperatures above 65 C. does the antithrombin show a distinct diminution in strength.

To test the antithrombin in the heated plasmas they were added in known amounts to varying mixtures of thrombin and fibrinogen. The thrombin was prepared in quantity by a method previously described[1] and was kept in dry condition in watch crystals, each crystal containing the same amount. For each test

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1. Howell: Am. Jour. Physiol., 1910, xxvi, 453. For modification see also same, 1913, xxxii, 264.

the contents of one of these crystals was dissolved in water (4 to 8 c.c.).[2] For fibrinogen solutions I have not found it convenient to use so-called pure fibrinogen prepared by the method of Hammarsten, since it is not possible by this method to obtain solutions of uniform concentration or properties. The repeated precipitations of the fibrinogen denatures it in part. In place of such solutions I have used the oxalated and dialyzed blood plasma of dogs or cats. To get this material a fasting animal was bled into an oxalate solution, nine parts of blood to one part of a 1 per cent. solution of sodium oxalate made up in sodium chlorid, 0.9 per cent. This mixture was centrifugalized, the plasma was pipetted off and the excess of oxalate was removed by dialyzing in collodion tubes for twenty-four hours against a solution of sodium chlorid 0.9 per cent. The plasma thus obtained does not clot spontaneously, but coagulates promptly on the addition of a little thrombin. A large quantity of this material was prepared from one animal and it was dried in watch crystals, 3 to 5 c.c. to a crystal, in a current of cold air from an electrical fan. When needed the contents of one or more of these crystals was dissolved in a known amount of a 0.9 per cent. solution of sodium chlorid. The solution obtained is quite clear. In the tests described below these solutions of dried plasma will be spoken of as fibrinogen solutions. Ten drops of this solution when mixed with 2, 3, 4 and 5 drops of the thrombin solution clotted in from two to five minutes. To determine the antithrombin in the heated oxalated plasmas described above the following mixtures were made:

1. 10 drops fibrinogen sol. + 1 drop heated plasma + 2 drops thrombin solution.
2. 10 drops fibrinogen sol. + 1 drop heated plasma + 3 drops thrombin solution.
3. 10 drops fibrinogen sol. + 1 drop heated plasma + 4 drops thrombin solution.
4. 10 drops fibrinogen sol. + 1 drop heated plasma + 5 drops thrombin solution.

Inasmuch as the effect of antithrombin on thrombin varies greatly with the time allowed for their interaction it is necessary in these experiments to add first the drop of heated plasma to the 2, 3, 4 and 5 drops of thrombin solution and to allow these mixtures to stand for a definite time before adding the solutions of fibrinogen. The time interval adopted in all my experiments for this action of the antithrombin and thrombin was fifteen minutes, and care was taken to see that this interval was identical within a second or two in all comparative tests. At the expiration of the fifteen minutes the fibrinogen solution was added and the time of coagulation of each specimen was observed. The mount of antithrombin contained in one drop of heated normal plasma was sufficient, in the solutions used, to delay the action of two drops of thrombin on the fibrinogen usually for an hour or more, or to prevent clotting altogether. In the specimens containing 3, 4 and 5 drops of thrombin the delay was proportionally less marked, the specimen containing 5 drops of thrombin clotting usually in five minutes. It is easy by this method to determine the relative amounts of antithrombin in different specimens of blood. In bird's plasma or the plasma from a peptonized dog the antithrombin exists in relatively large amounts so that a drop of the heated plasma may inhibit completely the action of such amounts of thrombin as are here considered.

In determining the time of coagulation in the different specimens the tubes containing the mixtures were examined, usually at intervals of five minutes, by gently inclining the tubes. When the thrombin was in distinct excess the contents of the tubes set to a firm clot, but when the thrombin was nearly neutralized by the antithrombin it was difficult or often impossible to ascertain when clotting occurred, since under such circumstances the clot forms in several stages. First, a delicate veil which on agitation shrinks to a small membrane while later a more solid gelatinous clot may form.

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2. The solutions of thrombin should not be filtered since the thrombin is absorbed to some extent by the filter paper.

Specimens in which the thrombin and antithrombin existed in this balanced proportion were useless for comparative purposes, and in such cases it was necessary to rely on the specimens containing more thrombin in which the clotting occurred promptly.

By way of illustration of the method, the results of one of the comparisons made between hemophilic and normal blood may be quoted.

Heated Plasma Drops	Thrombin Drops	Time Interval Minutes	Fibrinogen Drops	Coagulation Minutes
		NORMAL INDIVIDUAL		
1	2	15	10	160
1	3	15	10	10
1	4	15	10	10
1	5	15	10	5
		HEMOPHILIC BOY		
1	2	15	10	*
1	3	15	10	35-40
1	4	15	10	15
1	5	15	10	10

* No clot in seven hours.

This method has been applied to the examination of a very large number of specimens of human blood, normal and pathological. It has been found that while the content in antithrombin of normal blood exhibits certain fluctuations the variations are not large and do not necessarily run parallel to the coagulation time, owing to the fact that this latter time depends upon the balance between the thrombin and the antithrombin. In pathological bloods no significant constant variation in the amount of antithrombin has been detected except possibly in the condition known as idiopathic thrombosis.

Prothrombin.—In order to arrive at an idea of the relative amount or relative efficacy of the prothrombin in the plasmas examined I have adopted the procedure of adding to the oxalated plasma the optimum amount of calcium chlorid, that is to say, the amount that causes the most rapid clotting. This method was suggested by the observation that in a successful peptonized plasma (dog) addition of calcium chlorid does not cause clotting even when the plasma has been diluted previously with water. If, however, such a plasma is first oxalated and then calcium chlorid is added in proper amounts, clotting occurs readily. For some reason, not easily understood at present, the act of oxalating, that is, the decalcifying of the solution, intensifies the activation caused by the subsequent addition of calcium, so that the end result is apparently a greater production of thrombin, sufficient in fact to overcome the antithrombin and cause clotting. The alternative hypothesis that suggests itself, namely, that the process of oxalating may weaken the antithrombin, is easily shown to be erroneous. In some way the decalcification and subsequent calcification serve to bring into active form the maximum supply of prothrombin present in the blood. Whether or not this interpretation of the reaction is correct the simple procedure described has brought out a very constant and striking difference between normal and hemophilic blood. The reaction was carried out as follows: Five drops of oxalated plasma were placed in each of four tubes and to these were added in series, 2, 3, 4 and 5 drops of a 0.5 per cent. solution of calcium chlorid, with the idea that in one of the tubes the optimum concentration of calcium would be obtained. With the amount of plasma used clotting occurred first in the tubes containing 2 or 3 drops of calcium chlorid. With one drop the clotting occurred slowly or not at all, owing to an insufficiency of calcium, while in the tubes with 4 or 5 drops the clotting was somewhat slower than in the tubes with 2 or 3 drops, owing to too great an excess of calcium. Tested by this method the clotting of normal blood plasma is remarkably uniform, varying between nine and twelve minutes. In the plasmas

of hemophilic blood, on the contrary, the time of clotting is greatly prolonged. This fact, indeed, has been noted by Addis,[3] although he does not seem to have used the reaction in any extensive way. I have employed the reaction, as described above, for a large series of bloods to throw light on the amount or efficacy of the prothrombin, when parallel tests have established the fact that there is no variation in the amount of antithrombin in the bloods examined.

RESULTS

Purpura.—At different times in the course of this work specimens of blood have been obtained from patients suffering from various forms of purpura, including one case of severe purpura hemorrhagica. The time of coagulation of these bloods, when obtained by venepuncture, did not vary distinctly from normal, and the content of the plasmas in antithrombin and prothrombin was also within the limits found for normal bloods. It does not seem desirable at present to publish the details of these negative findings, since this paper deals especially with hemophilia and thrombosis. Duke[4] has published some interesting observations on cases of purpura hemorrhagica which show that the coagulation time may be normal while the bleeding time is greatly prolonged. By the latter term he designates the time that a small wound will continue to bleed, as determined by pressing against it a bit of filter paper at definite intervals of time. He has connected the increased bleeding time in purpura with a marked deficiency in platelets, in consequence of which platelet thrombi fail to form in the wounded vessels. My work throws no light on this point, but it does demonstrate that severe purpura hemorrhagica may exist without any detectable abnormality in the factors concerned in the clotting of the blood. The tendency to bleeding in these cases cannot be referred apparently to a deficient power of coagulation.

Hemophilia.—I have had the opportunity of examining with care two cases of undoubted hereditary hemophilia and a third case of a young boy in which no history of inheritance could be obtained. The history of these cases is as follows:

REPORT OF CASES

CASES 1 and 2.—Joseph Y., aged 12 years, and Jesse Y., aged 8 years, sons of Joseph Y. of Baltimore, a Russian Jew. The hemophilic tendency in these children was inherited from the mother. She stated that three of her brothers died from hemorrhage; one at 17 years of age as the result of an operation on the tonsils; one at 6 years from a cut on the face made accidentally by a playmate, and one at the time of circumcision. She states also that a cousin, daughter of her father's brother, has married and has hemophilic boys. An effort to locate this latter family was not successful. In the present family of Joseph Y. there

3. Addis: Jour. Pathol. and Bacteriol., 1911, xv, 436.

4. Duke: Jour. Am. Med. Assn., 1910, lv, 1185; Bull. Johns Hopkins Hosp., 1912, xxiii, 145.

are seven children; three boys, Joseph, 12 years, Jesse, 8 years and Fred, 5 years, all markedly hemophilic, and four girls none of whom have exhibited any tendency to bleed. Great difficulty from bleeding has been experienced at various times with the three boys. All have suffered from serious hematomata due to accidents in playing. Joseph at the time of circumcision is said to have bled for a week. At 4 years of age he was brought to the Johns Hopkins Hospital with hemorrhage caused by biting the tongue while eating an apple. The hemorrhage, at first profuse, lasted nearly a week. It was partially controlled by cauterizing with lunar caustic. Parents gave a history of bleeding spells in the boy from slight injuries on four or five occasions. The coagulation time as then recorded was only six to twelve minutes. The method is not stated but probably the blood was taken by puncture through the skin and the result is therefore not reliable. He has been a patient at the hospital since this time on several occasions in connection with joint troubles. Fred when 3½ years of age was taken to the Hebrew Hospital, Baltimore, on account of a persistent hemorrhage from the tonsils supposed to be due to an accident. Bleeding continued for several days and was controlled finally or ceased finally after an injection of horse serum (8 c.c.)

In the experiments here reported only Joseph and Jesse were used as they were old enough to come to the laboratory and could be used conveniently for the operation of venepuncture.

CASE 3.—A. H. S., 4 years old, patient in the Harriet Lane Home of the Johns Hopkins Hospital. Since his third month difficulty has been experienced with bleeding from small cuts, and injuries of any kind have been followed by "black and blue" areas in the region involved. At the time of admission to the hospital—March 25, 1913—the child was very anemic: red blood-cells, 1,730,000; white blood-cells, 15,300; Hb. 33 per cent.; platelets apparently normal, although no count was made. Large purpuric areas existed on right arm and leg, right shoulder, left tibia and right cheek. Right arm, hand and shoulder much swollen; right leg also swollen. The child remained in the hospital until May 1, 1913, and showed very marked improvement. A blood count made April 15, showed red blood-cells 4,270,000; white blood-cells 13,000 and Hb. 80 per cent. On three occasions human blood serum was injected subcutaneously; on April 12, 10 c.c.; April 14, 14 c.c., and April 24, 15 c.c. Through the kindness of Dr. John Howland I was given an opportunity to examine the blood on two occasions; once, the day after admission to the hospital and once just before his discharge. On each occasion the specimens of blood were obtained for me by Dr. W. L. Moss to whose kindness and skill I have been much indebted throughout this work.

COAGULATION TIME OF THE HEMOPHILIC BLOOD

The coagulation time was determined on specimens of 2 or 4 c.c. of blood obtained directly from the vein by means of the sterilized syringe and expelled at once into wide tubes (diameter 21 mm.), which had been cleaned carefully with a bichromate acid mixture. The clotting time was estimated up to the time that the clot was firm enough to allow the tube to be inverted. Comparisons were always made with specimens of blood taken from normal individuals by the same method. Tested by this method the time of clotting of normal blood varies usually between twenty and forty minutes. The time shown by the hemophilic blood at different periods was as follows:

Nov. 2, 1912.....	Joseph Y.....	Clot apparent in 3 hours, complete in 4 hours.
	Jesse Y.....	Clot apparent in 3 hours, complete in 4 hours.

Dec. 21, 1912.....	Joseph Y.....	Clot apparent in 4 hours, complete in 5 hours.
	Jesse Y.....	Clot apparent in 4 hours, complete in 5 hours.
Jan. 18, 1913.....	Jesse Y.....	Clot apparent in 3 hours, complete in 4 hours.
April 19, 1913.....	Joseph Y.....	Clot apparent in 4 hours, complete in 5 hours.
	Jesse Y.....	Clot apparent in 4 hours, complete in 5 hours.
April 28, 1913.....	Joseph Y.....	Clotted between 2 and 2½ hours.[5]
May 17, 1913.....	Joseph Y.....	Clotted in 3½ hours.
June 14, 1913.....	Joseph Y.....	Clotted in 4 hours.
March 26, 1913.....	A. H. S.....	Clotted between 2½ and 3 hours.
May 1, 1913.....	A. H. S.....	Clotted between 3 and 4 hours.

It is apparent from these data that in the three cases examined there was a marked delay in the spontaneous coagulation of the hemophilic blood, such as has been reported by several other observers. So far as could be determined from the examinations made of the blood of the brothers Y., extending over a period of eight months, there was at no time an approach to normal, and in fact but little indication of any variation. The deficient coagulability was due to some permanent alteration in the properties of the blood. It is noteworthy in the case of A. H. S., that in spite of the fact that he had received during his stay in the hospital three injections of human serum and that his general condition had improved so greatly, the coagulation time, at the end, was as long or longer than when he first came into the hospital. In regard to the coagulation time of blood, it should be emphasized that errors may arise easily if the technic of obtaining the specimens fails in any respect. Constant and reliable results are obtained only in those cases in which the needle of the syringe enters the vein readily on the first puncture. When the vein is missed or transfixed and the lumen is found only after several trials, there is a likelihood that the specimen of blood may contain some tissue-juice, and therefore clot more rapidly than it would alone. This effect was shown very distinctly on one occasion with Jesse Y. On the first attempt the needle missed the vein, and only after several trials was it possible to draw about 2 c.c. of blood. When emptied into a tube this specimen clotted in ten minutes. A specimen of 8 c.c. was then obtained from the vein of the other arm. The technic was good, and a specimen of 2 c.c. placed in a tube clotted in the usual time, namely, between four and five hours.

No difficulty from hemorrhage was experienced in taking blood from the hemophilic patient, provided the vein was not wounded. On two

5. In this case 1 c.c. only was taken for examination, and there is a probability that some tissue juice was mixed with this specimen, since difficulty was experienced in getting into the vein.

occasions when the needle did not enter promptly, and there was probably some laceration of the vein, there developed later a somewhat extensive purpuric area which persisted for a long period.

EFFECT OF THROMBOPLASTIC SUBSTANCE AND OF CALCIUM ON THE CLOTTING OF HEMOPHILIC BLOOD

As stated above, admixture with thromboplastic material of the tissues accelerates greatly the time of clotting. On several occasions this result was obtained with the active principle of the tissue extracts; that is to say, with the phosphatid (kephalin) extracted from brain tissue as described in a previous paper.[6] In one experiment the kephalin was dissolved in a 6 per cent. solution of dextrose and a control was made with a similar solution of dextrose without kephalin. The kephalin caused a marked acceleration in the clotting of both normal and hemophilic blood.

Normal blood: 1 c.c. blood plus 1 c.c. dextrose solution (6 per cent.) clotted in 10 minutes.
1 c.c. blood plus 1 c.c. of dextrose solution containing kephalin clotted in five minutes.

Hemophilic blood (Joseph Y.): 1 c.c. blood plus 1 c.c. dextrose solution clotted between two hours, ten minutes, and three hours, thirty minutes.
1 c.c. blood plus 1 c.c. dextrose solution containing kephalin clotted in ten to eleven minutes.

A similar result was obtained with specimens taken from Jesse Y.

In a second experiment the coagulation time of a mixture of blood with kephalin was compared with that of the undiluted blood. The specimens containing the kephalin were placed in narrow centrifugal tubes which would tend also to shorten the time.

Coagulation time of 2 c.c. blood: Normal, 30 minutes; Joseph Y., four to five hours; Jesse Y., four to five hours.

Coagulation time of 2 c.c. blood plus 1 c.c. dextrose solution containing kephalin: Normal, four to five minutes; Joseph Y., twelve minutes; Jesse Y., eleven minutes.

Similar results were obtained in a third experiment, in which the kephalin was added in aqueous solution, and in which consequently there was considerable hemolysis of the blood.

It is stated that the content in calcium of the hemophilic blood is normal (Nolf[7]), and that addition of calcium chlorid does not hasten the time of coagulation (Morawitz and Lossen[8]). In my experiments it was noted that the oxalated hemophilic blood gave a good deposit of

6. Howell: Am. Jour. Physiol., 1912, xxxi, 1.

7. Nolf: Eine neue Theorie der Blutgerinnung, Ergebn. d inn. Med., 1913, x, 275.

8. Morawitz and Lossen: Deutsch. Arch. f. klin. Med., 1908, xciv, 110.

calcium oxalate on centrifugalization, but the deposit to the eye seemed less than that obtained from similar specimens of normal blood. An attempt was made to determine the amount of calcium in small specimens of the blood (8 c.c.), but the results were unsatisfactory. In a single experiment it was found that addition of calcium chlorid to the hemophilic plasma hastened somewhat the time of coagulation, as follows:

2 c.c. hemophilic plasma (Joseph Y.), coagulation time = four to five hours.

1 c.c. hemophilic plasma + 3 drops $CaCl_2$, ½ per cent., coagulation in two hours, thirty minutes.

1 c.c. hemophilic plasma + 6 drops $CaCl_2$, ½ per cent., coagulation in two hours, ten minutes.

ANTITHROMBIN IN HEMOPHILIC BLOOD

Examinations were made of the amount of antithrombin by the method previously described. In the case of the brothers Y., these examinations were made at irregular intervals during a period of several (eight) months, the blood being compared each time with a specimen of normal blood. In all cases the examination was made as soon as possible after taking the specimen from the vein, since experience had shown that when the blood is kept there is a gradual deterioration in the amount of antithrombin. The results of these comparisons are given in the accompanying table. It will be understood that the procedure was as previously described, that is to say, the clear plasma of the blood was heated to 60 C. and filtered. One drop of this heated plasma was then added to 2, 3, 4 and 5 drops of a thrombin solution, and, after an interval of fifteen minutes, ten drops of the fibrinogen solution (dried plasma) were placed in each tube. Owing to variations in the strengths of the thrombin solutions and fibrinogen solutions used, the different experiments are not comparable among themselves. In each experiment the antithrombin in the hemophilic plasma is to be compared with the normal blood taken at the same time and submitted to the same procedure.

Examination of these results shows that in general the amount of antithrombin in the hemophilic blood was somewhat greater than in the normal specimen with which it was compared. The excess, however, was not large and in some cases was not distinctly shown (experiments of December 21, April 19, April 28, May 17). Since in these latter cases the long coagulation time was still exhibited by the blood, it seems most probable that the characteristic delay in coagulation shown by hemophilic blood cannot be referred to an increase in the absolute amount of antithrombin present in the blood. As will be shown, however, in the next paragraph, there is good reason to believe that in these bloods there is always a relative excess of antithrombin, which is quite sufficient to account for the prolonged period of coagulation.

TABLE 1.—Results of Examination for Antithrombin in Hemophilic Blood

Date	Name	Thrombin in Drops	Time of Coagulation
Oct. 19, 1912.....	Joseph Y. (hemophilic)	2	8 to 15 hours.
		3	45 to 50 minutes.
		4	20 minutes.
		5	15 minutes.
	John S. (normal)	2	2 to 8 hours.
		3	30 to 35 minutes.
		4	10 minutes.
		5	10 minutes.
Nov. 2, 1912.....	Joseph Y. (hemophilic)	2	4 to 18 hours.
		3	45 minutes.
		4	35 minutes.
		5	15 minutes.
	Jesse Y. (hemophilic)	2	45 minutes.
		3	20 minutes.
		4	15 minutes.
		5	10 minutes.
	W. H. H. (normal)	2	20 minutes.
		3	10 minutes.
		4	10 minutes.
		5	5 minutes.
Nov. 16, 1912.....	Joseph Y. (hemophilic)	2	6 to 15 hours.
		3	3 to 6 hours.
		4	30 minutes.
		5	20 minutes.
	Jesse Y. (hemophilic)	2	6 to 15 hours.
		3	3 to 6 hours.
		4	30 minutes.
		5	20 minutes.
	D. R. H. (normal)	2	3 to 6 hours.
		3	30 minutes.
		4	15 to 20 minutes.
		5	10 minutes.
Dec. 21, 1912.....	Joseph Y. (hemophilic)	2	No clot in 24 hours.
		3	Begun in 1 hour.
		4	20 minutes.
		5	10 minutes.
	Jesse Y. (hemophilic)	2	No clot in 24 hours.
		3	Begun in 2 hours.
		4	20 minutes.
		5	15 minutes.
	H., hospital patient (normal) ?	2	45 minutes.
		3	15 minutes.
		4	10 minutes.
		5	10 minutes.
	D. (normal)	2	No clot in 24 hours.
		3	?
		4	40 minutes.
		5	20 minutes.
Jan. 18, 1913.....	Joseph Y. (hemophilic)	2	No clot in 3 hours.
		3	15 minutes.
		4	10 minutes.
		5	5 minutes.
	Jesse Y. (hemophilic)	2	No clot in 3 hours.
		3	20 minutes.
		4	10 minutes.
		5	5 minutes.
	M. (normal)	2	1½ to 2 hours.
		3	10 minutes.
		4	5 minutes.
		5	5 minutes.

TABLE 1.—Results of Examination for Antithrombin in Hemophilic Blood.—(Continued.)

Date	Name	Thrombin in Drops	Time of Coagulation
April 19, 1913....	Joseph Y. (hemophilic)	2	20 to 25 minutes.
		3	10 minutes.
		4	5 minutes.
		5	5 minutes.
	Jesse Y. (hemophilic)	2	20 to 25 minutes.
		3	10 minutes.
		4	5 minutes.
		5	5 minutes.
	John S. (normal)	2	15 to 20 minutes.
		3	10 minutes.
		4	5 minutes.
		5	5 minutes.
April 28, 1913....	Joseph Y. (hemophilic)	2	10 minutes.
		3	5 minutes.
		4	5 minutes.
		5	5 minutes.
	John S. (normal)	2	10 minutes.
		3	5 minutes.
		4	5 minutes.
		5	5 minutes.
May 17, 1913.....	Joseph Y. (hemophilic)	2	10 minutes.
		3	10 minutes.
		4	5 minutes.
		5	3 minutes.
	John S. (normal)	2	10 minutes.
		3	5 minutes.
		4	3 minutes.
		5	3 minutes.
March 26, 1913...	A. H. S. (hemophilic)	2	Small membrane at 25 minutes.
		3	10 minutes.
		4	5 minutes.
		5	5 minutes.
	John S. (normal)	2	Small membrane at 20 minutes.
		3	5 minutes.
		4	5 minutes.
		5	5 minutes.
May 1, 1913.....	A. H. S. (hemophilic)	2	?
		3	15 minutes.
		4	10 minutes.
		5	5 minutes.
	George S. (normal)	2	?
		3	5 minutes.
		4	5 minutes.
		5	5 minutes.

PROTHROMBIN IN HEMOPHILIC BLOOD

The relative amounts of prothrombin in the hemophilic and normal bloods were determined by the simple method, described above, of first oxalating the blood and then adding the optimum amount of calcium chlorid to the clear plasma. This procedure was carried out on a great many specimens of blood, normal and pathological. It may be said in

general that the time of coagulation for normal human blood is quite constant, averaging about ten minutes, with variations from eight or nine to twelve or thirteen minutes. In normal dog's blood the time of coagulation is somewhat shorter, averaging about six minutes, with variations between five and nine or ten minutes. In the hemophilic bloods the results were entirely different, as shown by Table 2.

TABLE 2.—RESULTS OF EXAMINATION FOR PROTHROMBIN IN HEMOPHILIC BLOOD

Date	Name	Calcium Chlorid, 0.5 Per Cent., in Drops	Time of Coagulation (5 Drops of Oxalated Plasma)
March 26, 1913...	A. H. S. (hemophilic)	1	No clot.
		2	90 minutes.
		3	120 to 150 minutes.
		4	120 to 150 minutes.
	John S. (normal)	1	No clot.
		2	9 minutes.
		3	12 minutes.
		4	14 minutes.
May 1, 1913.....	A. H. S. (hemophilic)	2	?
		3	Partly clotted in 3 hrs.
		4	Partly clotted in 3 hrs.
		5	?
	George S. (normal)	2	10 minutes.
		3	14 minutes.
		4	20 minutes.
		5	30 minutes.
April 19, 1913....	Joseph Y. (hemophilic)	2	No clot in 4 hours.
		3	No clot in 4 hours.
		4	No clot in 4 hours.
	Jesse Y. (hemophilic)	2	Clot in 2 to 2½ hours.
		3	Clot in 2 to 2½ hours.
		4	Clot in 2½ hours.
	John S. (normal)	2	10 minutes.
		3	12 minutes.
		4	18 minutes.
April 28, 1913....	Joseph Y. (hemophilic)	2	Clot later than 5 hours.
		3	Clot in 5 hours.
		4	Clot later than 5 hours.
	John S. (normal)	2	11 minutes.
		3	13 minutes.
		4	17 minutes.
May 17, 1913.....	Joseph Y. (hemophilic)	2	Clot in 3 hours to 3 hours, 10 minutes.
		3	Clot in 3 hours to 3 hours, 10 minutes.
		4	Clot later than 3½ hrs.
	John S. (normal)	2	8 minutes.
		3	10 minutes.
		4	12 minutes.

As will be seen from examination of the table, the optimum amount of calcium in the normal was obtained with two drops of the solution

of calcium chlorid. With this amount of calcium the clotting time of the normal plasma was, in the different experiments, 9, 10, 10, 11 and 8 minutes. The clotting time for the hemophilic bloods was 90, 180+, 240+, 120, 300, 180+ minutes.

The much delayed coagulation of hemophilic plasma under these conditions may be accounted for theoretically in several ways. It may be due, in the first place, to the presence of an excessive amount of antithrombin. The observations reported above give but little support to this explanation. The amount of antithrombin in hemophilic plasma may be somewhat greater than that found in normal blood, and according to my results, is never distinctly less than normal; but on the other hand, when it is present in normal amounts, the hemophilic blood still exhibits in a marked way the characteristic delay in coagulation when oxalated and again recalcified. In other words, there is no parallelism between the amount of antithrombin and the delay in coagulation, and we cannot therefore explain the latter phenomenon by reference to variations in the antithrombin. In the second place, the delayed coagulation of the hemophilic blood may be referred, as has been done by Sahli[9] and by Morawitz and Lossen, to a deficiency in thromboplastic substance, or rather to that particular form of thromboplastic substance which Morawitz has designated as thrombokinase. There is no satisfactory method of detecting variations in quantity of the thrombokinase. The authors named have arrived at the conclusion that thrombokinase is lacking in hemophilic blood (and hemophilic tissues), mainly by a process of exclusion of other possible explanations. I believe, and have given experimental reasons for the belief, that such a thing as thrombokinase does not exist. Morawitz's ingenious and attractive hypothesis in regard to this substance has proved to be an obstacle rather than an assistance in the study of the phenomenon of clotting. The reasons given for the existence of a kinase in the intestine with the property of activating trypsin, are convincing, since much in the way of direct proof is easily obtained. Any one may demonstrate that the mucous membrane of the intestine contains an organic substance which effects the rapid activation of trypsin. But no such proof is obtainable for the existence of a thrombokinase. From an experimental standpoint the whole matter is complicated here by the additional hypothesis that the kinase alone cannot activate thrombin, but requires the coactivity of calcium. That calcium is concerned in the activation of prothrombin to thrombin is beyond question, but every proof furnished to demonstrate that calcium alone is concerned in this activation is met by the objection that the kinase also is present in the solutions. Since this kinase has no known properties or reactions except that of assisting calcium in the activation

9. Sahli: Deutsch. Archiv. f. klin. Med., 1910, xcix, 518.

of thrombin, the argument assumes the form of a perfect circle. Kinase is necessary to the formation of thrombin, hence if thrombin is formed a kinase is present. What is usually designated as a thrombokinase in the blood and other tissues is in reality the substance or substances which facilitate the process of clotting, and which have long been known under the terms zymoplastic or thromboplastic substances. According to my work, they consist essentially of a special phosphatid (kephalin?), which accelerates the process of clotting, not by any direct action on prothrombin, after the manner of a kinase, but by its neutralizing effect on the antithrombin. In the general sense of the term it is a thromboplastic substance, but not a thrombokinase. Whether or not this material is lacking in hemophilic plasma, I have not been able to determine in any wholly satisfactory way. Addis[3] reports certain experiments which he believes demonstrate that as regards this factor there is no difference between normal and hemophilic plasma, but his experiments do not seem to me to be conclusive. Sahli, on the other hand, reports experiments on the thromboplastic effect of washed blood corpuscles from normal and from hemophilic blood, which seem to show that the former contain more thromboplastic material than the latter. But this conclusion, if correct, is scarcely applicable to the case under discussion. The thromboplastic substance liberated in the blood after shedding comes, it is assumed usually, from the disintegration of the platelets. When blood is received at once into oxalate solutions, this disintegration is prevented. The platelets are fixed, indeed, permanently fixed. When the blood is centrifugalized they are thrown down unchanged, as may be shown by microscopic examination, and subsequent treatment with water or saline solutions shows that they now possess a rigidity altogether different from their original condition in the normal plasma. It is probable, therefore, that when the normal or hemophilic blood is oxalated immediately and centrifugalized, the clear plasma obtained has not received an accession of thromboplastic substance from disintegrating platelets. Nevertheless under these conditions, addition of calcium to such plasmas is followed by prompt and uniform clotting in the normal plasma and by a much delayed and variable clotting time in the hemophilic plasma. My own explanation of the cause of this difference is similar to that proposed by Addis, namely, that the trouble lies in a variation in the prothrombin. This view is discussed further in the succeeding paragraph. Outside of all theoretical considerations, it is well to emphasize the fact that the simple procedure described above, of oxalating plasma and then recalcifying by adding calcium chlorid, gives a convenient method of detecting hemophilic blood and of distinguishing it from the bloods of other hemorrhagic conditions.

THE CAUSE OF HEMOPHILIA

In the recent admirable monograph on hemophilia by Bulloch and Fildes,[10] the condition is defined as "an inherited tendency in males to bleed." The definition is unsatisfactory, in that it fails to suggest a cause for the condition, and possibly also in that it lays too much stress on the hereditary feature. There is no question, of course, that hemophilia is transmissible by heredity, and that most of the undoubted cases reported exhibit this history, but on the other hand it cannot be denied at present that cases may arise spontaneously. To exclude such cases from the group of hemophilia because there is no evidence of a similar condition in the family records, is not justifiable. In all probability such spontaneous cases may in turn be transmitted according to the law controlling this inheritance. Outside its transmissibility as a sex-limited inheritance, hemophilia is characterized clearly by certain reactions or properties of the blood which distinguish it from other hemorrhagic conditions in which there is also a "tendency to bleed." The main distinguishing feature of the hemophilic condition is the greatly delayed coagulation time of the blood, when the blood is removed from the vessels without coming in contact with the tissues. This peculiarity is demonstrated more easily and certainly, in contrast with other bloods, if the specimen of blood is first oxalated, then centrifugalized to obtain a clear plasma and then recalcified by the addition of a suitable amount of calcium chlorid. If the specimen so treated should show a coagulation time exceeding that of the normal blood (nine to twelve minutes), there would be reason for claiming the presence of a hemophilic condition, and the gravity of the condition would be in proportion to the length of time required for coagulation.

All recent workers agree on this delayed or deficient coagulability as the characteristic feature of hemophilia and attempts to explain the proximate or ultimate cause of the condition start from this point. The nature of the explanations offered varies of course with the theory of coagulation adopted. Morawitz and Lossen and Sahli, adopting the theory of coagulation proposed by Morawitz, have been led to believe that the defect of the hemophilic blood consists in a deficiency or lack of thrombokinase; Weil[11] has suggested that the delayed coagulation may be caused by the presence of some inhibiting body (antithrombin); Wright[12] has attributed the trouble, in part at least, to a deficiency in calcium; Nolf, in accordance with his special theory of coagulation, supposes that there is a quantitative or qualitative defect in the factor of coagulation designated by him as thrombozym. Addis in his series of

10. Bulloch and Fildes: Hemophilia, Eugenic Laboratory Memoirs, University of London, 1911, xii.

11. Weil: Presse méd., 1905, p. 672; Bull. et mem. Soc. méd. d. hôp. de Paris, Oct. 26, 1906.

12. Wright: Brit. Med. Jour., 1893, p. 223.

thoughtful experiments has come to the conclusion that the essential difficulty lies in a change in the properties of the prothrombin. My own work has convinced me that the antithrombin theory of hemophilia is incorrect, in spite of the fact that my experiments were begun in the expectation of supporting this view. There is little or no positive evidence in favor of the view that a deficiency of calcium is the fundamental trouble, and I am persuaded that the explanations offered by Morawitz, Sahli and Nolf have been influenced by an incorrect theory of the process of clotting. Sahli's generalization that in the hemophilic there is a loss of thromboplastic power (thrombokinase), not only in the corpuscles of the blood, but in the tissues at large, is certainly not applicable in all cases. The slow clotting hemophilic blood may coagulate quite promptly if it is brought into contact with the tissue juices of the external tissues. There are at present, so it seems to me, no positive facts supporting this general view of a defect in thromboplastic material in the hemophilic blood. My own experiments have led me to believe that the trouble lies, where it was traced by Addis, in the factor of prothrombin; that is to say, the material from which the thrombin is formed. The experiments described above on the coagulation time of oxalated blood after the addition of calcium can only mean that this prothrombin material is deficient in quantity or altered in character. Addis has adopted the latter view. He believes that the prothrombin of the hemophilic blood is present in normal amounts, but is so altered in properties as to require a longer time than normal for activation to thrombin. This explanation has seemed to me to be improbable, because, in the first place, I have found, as reported in this paper, that addition of thromboplastic (phosphatid) material accelerates greatly the time of clotting of the hemophilic blood. Addis admits this fact, but claims that it is true only when large amounts of this material are used. Assuming that this latter qualification is correct, it does not affect the main conclusion that the prothrombin possesses its normal properties in relation to activation by calcium, since the thromboplastic material is effective only by virtue of the fact that it removes the antagonistic influence of the antithrombin. However, the point that Addis raises can be settled only by isolating the prothrombin from the hemophilic plasma and testing the action of calcium on it in comparison with prothrombin obtained by the same method from normal blood.

I have been able, fortunately, to obtain the prothrombin from blood plasma by the simple method which I have employed already for the isolation of thrombin. The method is as follows: Blood is obtained from a vein of the arm by means of a syringe and is oxalated at once (8 c.c. of blood to 1 c.c. of a 1 per cent. solution of sodium oxalate made up in sodium chlorid solution 0.9 per cent.). The specimen is centrifugalized and the clear plasma is drawn off. To this plasma is added an equal

volume of acetone and the precipitate is filtered off through a small filter. The filter paper is opened, the precipitate is spread to a thin layer and is then dried rapidly in a current of air from an electric fan. The dried filter paper with its adherent precipitate is cut into small pieces and is covered with water (8 or 10 c.c.) and allowed to stand for about half an hour. The extract is then filtered. The opalescent solution contains prothrombin, as can be shown most satisfactorily by its reaction with fibrinogen solutions. The fibrinogen solutions used for this purpose I have prepared from the plasma of cat's blood by a modification[13] of Hammarsten's method of repeated precipitation by half saturation with sodium chlorid. If the fibrinogen is precipitated three times the final solution in 0.9 per cent. sodium chlorid (with a trace of sodium bicarbonate if necessary) gives no coagulation at all on the addition of calcium, but clots readily with thrombin. With such solutions of fibrinogen, or indeed with the less pure solutions obtained by one or two precipitations, the presence of prothrombin in the solutions described above may be demonstrated very readily by experiments, of which the following is an example:

1. Fibrinogen solution, 10 drops + $CaCl_2$, ½ per cent., 1 drop = no clot in twenty-four hours.

2. Fibrinogen solution, 10 drops + prothrombin solution 3 drops = no clot in twenty-four hours.

3. Fibrinogen solution 10 drops + prothrombin solution 3 drops + $CaCl_2$, ½ per cent., 1 drop = clot in 2 to 3 minutes.

It should be stated that fibrinogen solutions prepared as described are not constant in their reactions. In some cases they clot very readily with thrombin; in other cases the clotting requires a longer time, and on standing for twenty-four hours or more the fibrinogen may undergo a modification of some kind so that it loses the property of yielding a clot on the addition of thrombin. In all cases, however, the freshly prepared fibrinogen may be used to demonstrate the presence of the prothrombin, since the solutions of the latter have no effect on the fibrinogen until calcium is added, and then the thrombic action is clearly shown. In order to determine whether or not the prothrombin from the hemophilic blood differs in its reaction to calcium from that of normal blood, I have prepared the prothrombin from one of the hemophilic subjects used in this investigation (Joseph Y.) and compared it with the prothrombin from a normal blood. The following results were obtained:

Experiment, Oct. 19, 1913.—Eight cubic centimeters of blood taken from Joseph Y. and 8 c.c. from a normal (John S.). Each specimen was oxalated and treated with acetone as described above. The aqueous solutions of prothrombin were tested on a freshly prepared fibrinogen solution.

Control Experiments.—Fibrinogen solution, 10 drops + water 5 drops + $CaCl_2$ (½ per cent.) 1 drop = no clot in twenty-four hours.

Fibrinogen solution, 10 drops + prothrombin solution 5 drops = no clot in twenty-four hours.

13. Howell: Am. Jour. Physiol., 1910, xxvi, 461.

Prothrombin from Normal Blood.—Fibrinogen solution 10 drops + prothrombin solution 5 drops + $CaCl_2$, 1 drop = clot in twenty-eight minutes.

Fibrinogen solution 10 drops + prothrombin 3 drops + $CaCl_2$, 1 drop = clot in 50 to 60 minutes.

Prothrombin from Hemophilic Blood.—Fibrinogen solution 10 drops + prothrombin solution 5 drops + $CaCl_2$, 1 drop = clot in twenty minutes.

Fibrinogen solution 10 drops + prothrombin solution 3 drops + $CaCl_2$, 1 drop = clot in fifty minutes.

This experiment, which was repeated several times with similar results, demonstrates quite definitely that the separated prothrombin from hemophilic blood yields active thrombin in the presence of calcium quite as readily as the prothrombin from normal blood. The qualitative difference claimed by Addis does not exist. In explaining the delayed coagulation of the hemophilic blood we must assume, it seems to me, that the prothrombin is present in subnormal amount in the circulating blood. Since the antithrombin, on the contrary, exists in normal or even in sligthly supranormal amounts, the delay in coagulation of the shed blood is readily explained. On the basis of the facts presented in this paper, I would conclude that the essential condition in hemophilic blood which is immediately responsible for its delayed coagulation is a subnormal amount of prothrombin in the circulating blood.

Hemophilia may be defined as a condition, limited to the male, in which the coagulation time of the blood is markedly prolonged in consequence of a deficiency in the amount of the contained prothrombin, with the additional characteristic that the defect is transmissible by heredity in accordance with the so-called law of Nasse.

The ultimate cause of this condition cannot be stated. So far as we know, the prothrombin present in blood-plasma is furnished by the blood-platelets, and it is reasonable to assume that the defect in question is referable to some functional change in these elements.

THROMBOSIS

During the course of this work the opportunity has offered to examine several cases of thrombosis in which there were no obvious mechanical or inflammatory conditions to account for the trouble. The cases are few in number, and unfortunately the examination was not complete in each case, but the results seem to be suggestive.

CASE 4.—Dr. H., just recovering from mild attack of thrombosis involving the saphenous and the abdominal veins. Some soreness and edema over region involved. He had had several similar attacks at intervals of some weeks. Specimen of 8 c.c. of blood taken from an arm vein and compared with similar specimen from a laboratory attendant (John S.).

The results of this test indicate that the blood of the thrombotic patient contained less antithrombin than the normal with which it was compared, and, indeed, less than any of the normals used in any of these experiments.

Coagulation time (4 c.c.): Patient, 29 to 30 minutes.
Normal, 38 to 39 minutes.

ANTITHROMBIN TEST

Heated Plasma Drops	Thrombin Drops	Time Interval Minutes	Fibr. Sol. Drops	Coagulation Minutes
		Normal.		
1	2	15	10	40
1	3	15	10	10
1	4	15	10	5
1	5	15	10	5
		Patient.		
1	2	15	10	10
1	3	15	10	5
1	4	15	10	5
1	5	15	10	5

CASE 5.—Charles G., patient in the Johns Hospkins Hospital, service of Dr. Barker. Aged 52. Onset of attack one month previous to admission; dyspnea on exertion, pain in left side, swelling of feet. Physical findings: dyspnea, hydrothorax, mitral and aortic insufficiency. Blood-pressure, 140. Blood-count, r. b. c., 4,300,000; w. b. c., 7,400; Hb., 90 per cent. The day before the blood was examined the patient developed a marked swelling of the whole left leg, diagnosed as due to thrombus in the left femoral or iliac vein. Some of the superficial veins at left elbow firmly thrombosed.

Specimen of blood, 10 c.c., taken from arm vein and compared with a similar specimen from one of the laboratory attendants (M.).

Coagulation time (2 c.c.): Patient, 8 minutes.
Normal, 41 minutes.

ANTITHROMBIN TEST

Heated Plasma Drops	Thrombin Drops	Time Interval Minutes	Fibr. Sol. Drops	Coagulation Minutes
		Normal		
1	2	15	10	*
1	3	15	10	10†
1	4	15	10	5
1	5	15	10	5
		Patient.		
1	2	15	10	5
1	2	15	10	2
1	2	15	10	2
1	2	15	10	2

* First membrane at twenty minutes.
† Partial.

In this case also the amount of antithrombin was distinctly less than in the normal. In fact the clotting with the thrombotic plasma was so rapid as to indicate the practical absence of antithrombin in its heated plasma.

PROTHROMBIN TEST

Oxalated Plasma Drops	$CaCl_2$, ½ Per Cent. Drops	Coagulation Minutes
	Patient.	
5	2	7
5	3	7
5	4	9
5	5	11
	Normal.	
5	2	10
5	3	14
5	4	17
5	5	19

The more rapid clotting of the oxalated plasma of the thrombotic blood may have been due to a greater amount of prothrombin or to a

diminished amount of antithrombin. The previous test for antithrombin indicates that the latter explanation is probably the correct one, and this conclusion is borne out by a subsequent experiment in which the serum from the patient and from the normal were compared in regard to their strength in thrombin, the precaution being taken of adding to each an equal volume of kephalin-solution to neutralize the antithrombin present. This test gave the following result:

COMPARISON OF STRENGTH IN THROMBIN

Fibrinogen Solution Drops	Serum and Kephalin Drops	Coagulation Minutes
	Patient.	
10	2	12
10	3	6
10	4	5
10	5	3
	Normal.	
10	2	10
10	3	5
10	4	5
10	5	3

According to this test there was practically no difference in the amount of thrombin in the two specimens of serum.

CASE 6.—Mrs. N., private patient of Dr. Thayer. History of two attacks of thrombosis — one six years previously with evidence of thrombosed veins in calf, groin and inner side of leg, one beginning four months before examination and following attack of measles. There were pain and tenderness over left femoral vein, knots in groin and under skin of legs, first on left side, later on right side. In bed for three months. On one occasion when convalescing there was a sudden attack of severe pain in side, with marked dyspnea. A similar attack followed after an interval of some time. Examination of the blood was made four months after beginning of last attack when the patient had recovered and showed no physical signs except slight edema in legs.

Coagulation time (2 c.c.): Mrs. N., 20 to 22 minutes.
Normal: Dr. D., 40 to 43 minutes.

PROTHROMBIN TEST

Five drops plasma + 2 drops $CaCl_2$ (½ per cent.) = clot in nine minutes.
Five drops plasma + 3 drops $CaCl_2$ (½ per cent.) = clot in twelve minutes.

ANTITHROMBIN TEST

This test was made with three dilutions of a new stock of thrombin. In two of these the concentration was too great, so that the tubes clotted more rapidly than was convenient for a decisive comparison. In each case, however, the clotting in the tubes containing the heated plasma from the patient preceded that in the controls, indicating, therefore, less antithrombin in the patient's blood. This difference was shown most distinctly with the third dilution of thrombin used as follows:

Heated Plasma Drops	Thrombin Drops	Time Interval Minutes	Fibrinogen Drops	Coagulation Minutes
		Normal.		
1	3	15	10	15 to 20
		Patient.		
1	4	15	10	15 to 20
1	3	15	10	7
1	4	15	10	5

CASE 7.—In a fourth case of thrombosis examined the results were negative. This patient was a hospital nurse with a history of repeated attacks, diagnosed by Dr. Boggs as due to venous thrombosis. At the time of examination of her blood she had been free from any trouble for several months. The examination made was incomplete, including only the coagulation time, thirty-five minutes, and the prothrombin test, ten minutes. As these times were entirely normal the antithrombin tests were not made, owing to circumstances which could not be controlled. This examination was made May 31. Later in the summer the patient had another severe attack, but was not accessible for examination.

The number of cases reported is small, but the indications from the blood examinations of a diminution in antithrombin, especially in Case 2, are sufficiently positive to warrant a suspicion that herein may lie the important factor responsible for the formation of a thrombus. If the antithrombin constitutes a normal defensive agency against intravascular clotting, a diminution in this substance will bring about a condition in which spontaneous coagulation will be favored, particularly in those parts of the circulation in which the mechanical conditions are such as may lead to a partial stasis of the circulation.

In the hemophilic blood there seems to be a relative excess of antithrombin, owing mainly to an actual diminution in the amount of prothrombin. In the blood of patients showing spontaneous thrombosis, just the opposite condition prevails of a deficiency in antithrombin relative to the amount of prothrombin present.

SUMMARY

1. Methods are described for testing the relative amount of prothrombin and antithrombin in the blood.

2. The application of these methods to hemophilia shows that the blood in this condition is deficient in prothrombin. The antithrombin may be normal or somewhat greater than normal. The characteristic peculiarity of hemophilic blood is its markedly delayed time of coagulation. This peculiarity is explained by the diminution in amount of the prothrombin which results in a relative excess of antithrombin.

3. The detection of a hemophilic condition of the blood is facilitated by first oxalating the blood and then recalcifying with an optimum amount of calcium. Under these conditions the time of clotting exhibited by normal plasma is very constant (nine to twelve minutes). That of hemophilic blood is greatly delayed.

4. In patients suffering from spontaneous thrombosis of the veins there is evidence of a diminution in the antithrombin of the blood, the prothrombin being normal. It is suggested that this deficiency in antithrombin operates as a favoring, perhaps as a determining, factor in the production of the thrombus.

5. In purpura hemorrhagica and other forms of so-called purpura, no evidence was found of any variation from normal in either the antithrombin or the prothrombin.