STUDIES ON THE ACTIVATION OF A SERUM "PROTHROMBIN-CONVERTING FACTOR"

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Recent reports of additional factors operating at the first stage of blood coagulation have stimulated investigators to explore in greater detail the mechanism by which prothrombin is converted to thrombin.

This report describes a new experimental approach to the problem of prothrombin conversion which, it is felt, may help to clear up some of the complexities of this reaction. In the present study, it will be shown that thromboplastin added to human serum is able to activate a substance which will be called a "prothrombin-converting factor." When thromboplastin is added to serum, the activated factor added to plasma then converts prothrombin to thrombin in the absence of ionized calcium. After initial activation of serum by thromboplastin, the "prothrombin-converting factor" undergoes gradual decay until the serum-thromboplastin mixture is no longer reactive. "Prothrombin-converting factor" can then be repeatedly reactivated from the same serum-thromboplastin mixture by addition of more thromboplastin. When serum obtained from patients treated with dicumarol is activated by thromboplastin, a more prolonged action of the released "prothrombin-converting factor" results.

The mechanism by which these observed reactions are brought about is discussed and a modification of current concepts relating to prothrombin conversion is proposed.

Investigators from different laboratories have utilized diverse methods to arrive at the conclusion that there exist in plasma previously unrecognized factors which initiate the conversion of prothrombin to thrombin. When Seegers *et al.* (1) studied purified prothrombin, they observed that a slowed rate of prothrombin conversion occurred after the reaction was initiated by adding thromboplastin and calcium. At first this retarded conversion was thought to be related to denaturation of prothrombin attendant upon chemical procedures necessary for fractionation and purification. Later Ware *et al.* (2, 3) and Fantl and Nance (4) were able to produce acceleration of thrombin formation from this purified prothrombin fraction, when serum was added to the prothrombin, thromboplastin and calcium reaction mixture. The protein fraction responsible for accelerating prothrombin conversion was called accelerator globulin (ac-globulin). Ware *et al.* (3) believed that ac-globulin produced a reaction of co-autocatalysis, wherein thrombin accelerates its own formation through an intermediate action of ac-globulin. A minute amount of thrombin produced early in coagulation of blood, liberates accelerator globulin which then intensifies the rate of thrombin formation from prothrombin.

Milstone (5, 6) reported another plasma globulin fraction, prothrombokinase, which is capable of forming thrombin from prothrombin. Prothrombokinase is activated by calcium to form an active thrombokinase which then converts prothrombin to thrombin. Activation of the thrombokinase follows an autocatalytic reaction, requiring considerable time before maximum thrombokinase activity is reached.

Quick (7, 8) postulated the existence of prothrombin A and a labile factor to explain certain discrepancies occurring in prothrombin determinations made by his method. He found that certain plasmas with a low concentration of prothrombin could be restored to a higher concentration following the addition of de-prothrombinated plasma from a normal individual. These observations suggested that some plasmas might be deficient in prothrombin A or a labile factor.

On the basis of studies made on a patient with an unusual type of prothrombin deficiency that could likewise be restored by addition of de-pro-

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thrombinated plasma from a normal individual, Owren (9) postulated a different interpretation of results. He assumed that normal blood plasma contains an activator substance for prothrombin which he calls Factor VI. Factor VI exists as an inactive pro-enzyme Factor V which can be activated by thromboplastin and calcium in the presence of prothrombin to form Factor VI. Factor VI thereupon causes an enzymatic conversion of prothrombin to thrombin.

With these divergent interpretations and definitions in mind, it has seemed logical in the experiments to be reported, to speak of the factor in serum, which is activated following the addition of thromboplastin, as "prothrombin-converting factor." It may be that the "prothrombin-converting factor" is synonymous with Factor VI of Owren (9), thrombokinase of Milstone (6), prothrombin A and labile factor of Quick (7, 8) and accelerator globulin of Ware *et al.* (3).

EXPERIMENTAL METHODS

Collection of serum: Blood samples were collected from normal individuals and patients hospitalized for miscellaneous diseases. Dry syringes were used to obtain the blood and all collections were made as quickly as possible to avoid incipient coagulation. The plasma was prepared by adding 9.0 cc. of whole blood to 1.0 cc. of 0.1 M sodium oxalate solution. Serum was prepared by allowing whole blood to coagulate in glass tubes with internal dimensions of $\frac{5}{8} \times 6$ inches. Approximately 45 minutes later, the clot was rimmed with a wooden applicator and the serum, after centrifugation, was separated from the clot.

Preparation of thromboplastin: Dried thromboplastin was prepared according to Quick's method (10). Both human and rabbit brain preparations were utilized without any apparent difference in the results. Fresh thromboplastin suspension was made each day by mixing 0.3 gm. of dried brain powder with 5.0 cc. of 0.85 per cent sodium chloride solution. The thromboplastin suspension thus prepared was shaken periodically for 20 minutes at 50° C. after which, gross particles of brain were removed by centrifugation for three minutes at 1,000 R.P.M. Thromboplastin suspension prepared in this manner produced coagulation of recalcified, fresh, oxalated human plasma in 17–20 seconds at room temperature (22° C.).

Method of activating the serum "prothrombin-converting factor": Serum to be tested was allowed to remain at room temperature for two to three hours to allow destruction of thrombin activity to take place. Thrombin activity of serum was measured by adding 0.2 cc. of serum to 0.2 cc. of fresh plasma. Coagulation of the plasma usually did not occur until four or five minutes had elapsed. Serum capable of coagulating plasma in less than four minutes was not used in the tests reported. Many of the sera were also tested for prothrombin activity by adding 0.1 cc. of serum to 0.1 cc. of fibrinogen and 0.1 cc. of thromboplastin suspension. The coagulation time of this mixture was usually greater than 180 seconds indicating that an insignificant amount of prothrombin remained.

Following these preliminary experiments to insure that the serum did not contain substances interfering with the test, activation of the "prothrombin-converting factor" was accomplished in the following manner:

Five-tenths cc. portions of serum were added to 0.1 cc. portions of thromboplastin suspension and thoroughly mixed. Simultaneously a stop-watch was started and at exactly one, three, seven, and 15 minute intervals, 0.1 cc. of the serum-thromboplastin (serum-TP) mixture was withdrawn into a 1.0 cc. pipette, calibrated to the tip, and blown into 0.1 cc. of plasma. The coagulation time of the serum-thromboplastin-plasma mixture was then determined to the nearest second. All of these operations were carried out at room temperature (22° C.). All coagulation tests were made in glass tubes with internal dimensions 12×25 mm. The coagulation time was determined as the point at which the plasma-serum-thromboplastin mixture first showed visible fibrin formation as the glass tube was tilted gently up and down from a diagonal position. A similar dilution of thromboplastin was made in 0.85 per cent sodium chloride solution to which sufficient calcium chloride had been added to produce a molarity of calcium ion corresponding to the ionized calcium of serum (0.0012 M). When this suspension was added to the plasma containing 0.01 M sodium oxalate, no coagulation occurred.

Since no calcium had been added to serum prior to the addition of thromboplastin, it is evident that the coagulating action of the serum-TP mixture could not be related to a direct effect of thromboplastin on the prothrombin of the plasma.

Effect of addition of thromboplastin to serum: The kinetics of the reaction taking place after addition of thromboplastin to serum are presented in Figure 1, which shows the results obtained from activation of sera from three normal individuals on three successive days. One minute after the introduction of 0.1 cc. of thromboplastin suspension to 0.5 cc. of serum, 0.1 cc. of the mixture added to 0.1 cc. of plasma produced coagulation in eight to 13 seconds. After three minutes incubation of the serum-TP mixture, plasma was coagulated in 12–19 seconds. Seven minutes' incubation of the serum resulted in plasma coagulation in 27–41 seconds following the addition of 0.1 cc. of this mixture to 0.1 cc. of plasma; while incubation for 15 minutes resulted in a plasma coagulation time of 50–80 seconds. If the serum-TP mixture was allowed to react several hours, no coagula-

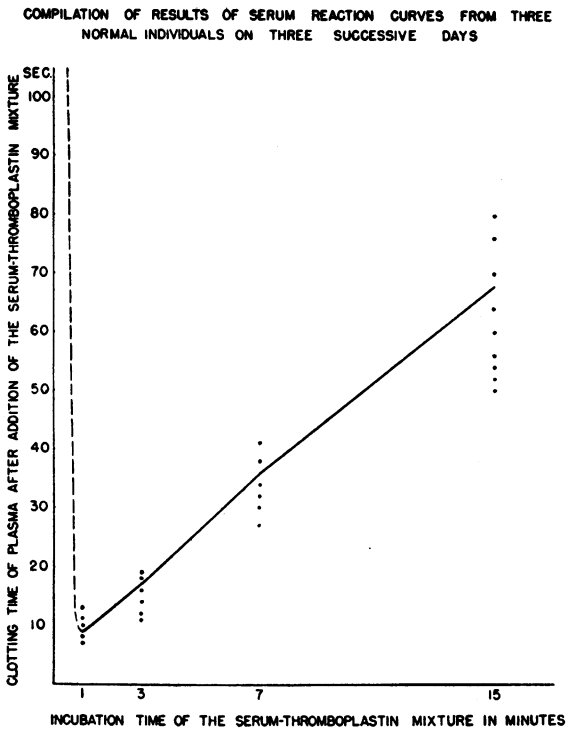

FIG. 1

The coagulation time of oxalated plasma, following the addition of serum-thromboplastin mixture, is indicated on the ordinate. The incubation time of the serum-thromboplastin mixture before it is added to oxalated plasma is indicated on the abscissa. Observe that maximum activation of serum occurs within one minute of the time of adding thromboplastin, following which, there is a slow decay of coagulating power of the serum-thromboplastin mixture.

tion of the plasma occurred when the serum-TP mixture was added to plasma. However, if 0.1 cc. of inactive serum-TP mixture was mixed with 0.1 cc. of plasma and 0.1 cc. of 0.02 M $CaCl_2$ then added, coagulation occurred as rapidly as when an identical amount of thromboplastin alone was added to plasma along with 0.02 M $CaCl_2$. Furthermore, addition of the inactive serum-TP mixture to fresh serum caused activation of a "prothrombin-converting factor" to the same degree as thromboplastin correspondingly diluted in saline and mixed with serum. These experiments indicate that the thromboplastin is still active in the serum-TP mixture, but has apparently been altered in some manner so that activation of the "prothrombin-converting factor" no longer takes place.

If serum is activated by thromboplastin and then the mixture is added to either de-prothrombinated plasma or fibrinogen (Figures 2 and 3), little or no coagulation is produced, indicating that the mechanism of coagulation of the normal plasma cannot be related to a thrombin effect. A mixture of thromboplastin in 0.0012 M $CaCl_2$ in physiological saline which reproduces the molarity of ionized calcium in the serum, does not produce coagulation of plasma containing 0.01 M oxalate, so that one cannot relate the coagulating action of the serum-TP mixture to the thromboplastin and calcium content of the mixture.

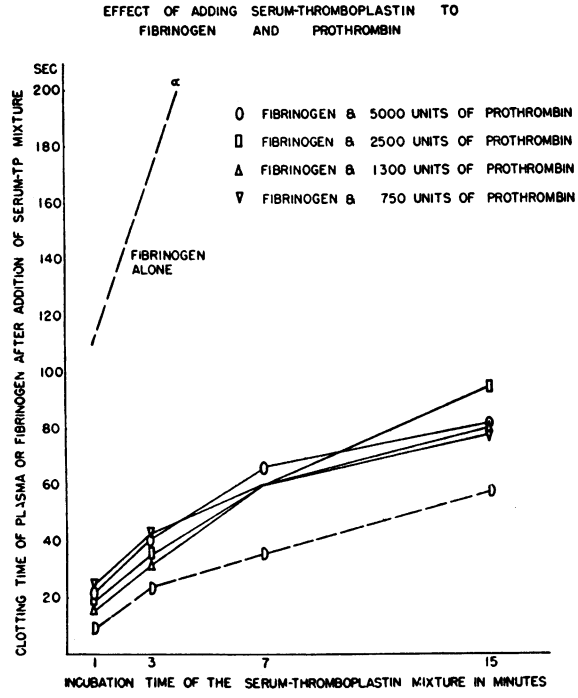

FIG. 2. EFFECT OF ADDING A SERUM-THROMBOPLASTIN MIXTURE TO FIBRINOGEN AND FIBRINOGEN-PURIFIED PROTHROMBIN SOLUTIONS

The ordinate indicates the coagulation time of the various fibrinogen preparations, following addition of serum-thromboplastin. The abscissa indicates the incubation time of the serum-thromboplastin mixture before it is added to the fibrinogen solutions. The uppermost broken line represents the results obtained when fibrinogen alone was used; while the solid lines show the results obtained when varying amounts of prothrombin were added to fibrinogen. The bottom broken line shows the effect of adding the same serum-thromboplastin mixture to fresh oxalated plasma containing a prothrombin concentration 100 per cent of normal.

The maximal coagulating action of the activated serum appears one minute after addition of thromboplastin. At this point plasma is coagulated much more rapidly than one would expect if one added only thromboplastin and calcium to plasma to determine the prothrombin time. From this evidence it can be assumed that addition of thromboplastin to serum initiates a reaction which causes liberation of a substance which in turn is capable of converting prothrombin to thrombin.

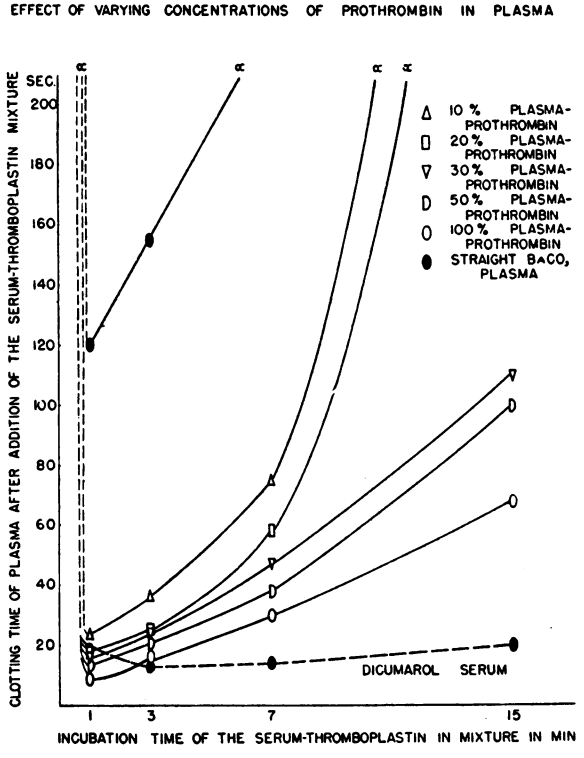

FIG. 3

Prothrombin was adsorbed from plasma with $BaCO_3$. Aliquots of fresh plasma were then added to the $BaCO_3$-treated plasma to produce the desired concentration of prothrombin. The coagulation time of plasma after addition of the serum-thromboplastin mixture (ordinate) was then plotted against the incubation time of the serum-thromboplastin mixture (abscissa). A decreasing concentration of prothrombin in the plasma is associated with a more abrupt decay of the activated serum's power to coagulate plasma. The broken-line curve was obtained by activating serum from a patient treated with dicumarol whose plasma prothrombin concentration was 20 per cent of normal. This serum, activated by thromboplastin, was then added to a $BaCO_3$-treated plasma containing 10 per cent prothrombin. A striking difference in the activation curve is apparent.

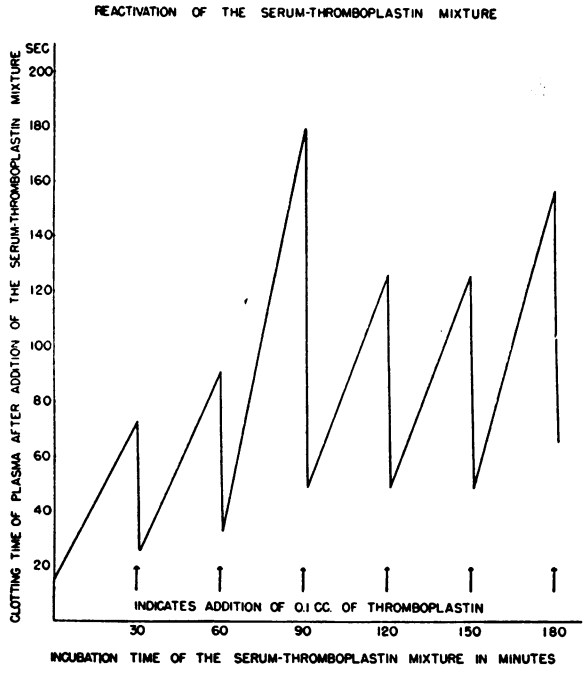

FIG. 4. REACTIVATION OF A SERUM-THROMBOPLASTIN MIXTURE BY INCREMENTS OF THROMBOPLASTIN

One cc. of serum was added to 0.2 cc. of thromboplastin. Thirty minutes later, 0.1 cc. of this mixture was added to 0.1 cc. of oxalated plasma and the coagulation time recorded. An additional 0.1 cc. of thromboplastin was then added to the initial serum-thromboplastin mixture and one minute later, 0.1 cc. of this was withdrawn and added to oxalated plasma. The curve of decay of the serum-thromboplastin coagulating substance is interrupted by addition of fresh thromboplastin. The serum-thromboplastin mixture can be repeatedly reactivated as shown in this graph.

The serum-thromboplastin mixture produces no coagulation of plasma, after a sufficient period of incubation of the serum and thromboplastin has taken place. Reactivation of the serum component can be produced, however, when fresh thromboplastin is added to the inactive serum-TP mixture. After serum and thromboplastin were allowed to react for 30 minutes, it was found that 0.1 cc. of this mixture produced coagulation of 0.1 cc. of plasma in 120 seconds. An additional 0.1 cc. of thromboplastin was then added to the above serum-TP mixture. One minute later 0.1 cc. of the new serum-TP mixture coagulated 0.1 cc. of plasma in 20 seconds, demonstrating that the clotting activity of the serum-TP mixture had been almost completely restored. As shown in Figure 4, alternating decay and reactivation were ob-

served many times. It appears that the activated serum factor capable of producing plasma coagulation is not destroyed but only reversibly inactivated. It can be reactivated repeatedly, merely by adding more thromboplastin to the serum.

Effect of adding activated serum to "artificial plasmas": The clotting activity of a "prothrombin-converting factor" of serum, activated by thromboplastin, was further studied by observing the effect of adding activated serum to fibrinogen solutions.

Human fibrinogen [2] was dissolved in 0.85 per cent sodium chloride solution (pH 7.5) to make a concentration of fibrinogen of 200–300 mg. per cent. It will be observed in Figure 2, that serum-TP mixture coagulated fibrinogen in 110 seconds in contrast to a coagulation time of 10 seconds for plasma (bottom broken line). After incubation for three minutes, the serum-TP mixture produced no coagulation of fibrinogen as compared to a coagulation time of 28 seconds for the plasma.

A preparation of purified prothrombin [2] was then added to the fibrinogen solution as follows: Prothrombin was dissolved in 0.85 per cent sodium chloride solution, adjusted to pH 7.5. Aliquots of this solution, representing a known amount of prothrombin, were then added to the fibrinogen solution. Serum activated by thromboplastin was then added to the different solutions of fibrinogen and prothrombin and the coagulation times were recorded. It will be observed from Figure 2 that the reaction curves produced by adding prothrombin to fibrinogen approximate the same type of curve that is obtained when normal plasma is used.

Plasma was prepared to contain varying amounts of prothrombin by the following method: Fresh plasma was freed of prothrombin by adding it to powdered $BaCO_3$ (method of Fantl and Nance [4]). The plasma and $BaCO_3$ were shaken vigorously and allowed to react for 20 minutes. The plasma was then freed of $BaCO_3$ by rapid centrifugation. The supernatant, clear plasma was removed and tested for prothrombin activity by adding thromboplastin and 0.02 M $CaCl_2$. Complete removal of prothrombin was evident since the adsorbed plasma failed to coagulate. Sufficient fresh plasma was then added to aliquots of the $BaCO_3$-treated plasma to produce a 10 per cent, 20 per cent, 30 per cent, and 50 per cent prothrombin concentration in the plasma utilized in experiments presented in Figure 3. These artificially prepared plasmas were studied in the same manner as fresh plasma to determine the effect of adding a serum-TP mixture. It will be observed that decreasing concentrations of prothrombin in plasma were associated with progressively prolonged coagulation times when the serum-TP mixture was added. This was particularly apparent after the serum-TP had been incubated for seven or 15 minutes. Ten per cent and 20 per cent plasma produced no coagulation after addition of the serum-TP mixture incubated for 15 minutes, whereas 100 per cent plasma was coagulated in 64 seconds by the same mixture. The bottom broken-line curve was obtained by activating serum obtained from a patient treated with dicumarol and then adding this serum-TP mixture to plasma containing 10 per cent prothrombin. The significance of this effect will be discussed later.

On the basis of the above experimental data it is felt that there is sufficient evidence to state that serum can be activated by thromboplastin to produce a "prothrombin-converting substance" which is then capable of causing conversion of prothrombin to thrombin in the virtual absence of ionized calcium. Fibrinogen cannot be converted to fibrin directly by the serum-activated substance. The minimal fibrin formation that does occur when serum-TP mixture is added to fibrinogen is probably related to small amounts of prothrombin contaminating the fibrinogen solution or to small quantities of thrombin or prothrombin residues contaminating the activated serum-TP mixture.

Activation of serum obtained from dicumarol-treated patients: It was found that serum from normal individuals, activated by thromboplastin and incubated as previously described, clotted plasma in a characteristic manner. Most sera from patients with various diseases showed little difference in this respect. In the presence of severe infections, however, a somewhat more flattened reaction curve was produced following thromboplastin activation. For example, the coagulation time of plasma after addition of a serum-TP mixture incubated for 15 minutes was 35 seconds in a typical case as compared with 55 seconds for nor-

[2] The preparations of fibrinogen and purified prothrombin used in these experiments was supplied through the generosity of Dr. W. H. Seegers, for which grateful acknowledgment is made.

mal serum. In these instances it was found that the plasma prothrombin concentration was diminished (Quick method [10]), and in the light of these observations a study was made of sera obtained from patients who were being treated with dicumarol.

The markedly changed pattern of reaction which follows activation of sera by thromboplastin from prothrombin-deficient patients, related to administration of dicumarol, is recorded in Figure 5. Serum D was obtained 12 hours after administration of 300 mg. of dicumarol *per os,* whereas sera C, B, and A, were obtained 36, 60 and 72 hours later. An analysis of the corresponding plasma revealed a reduction of prothrombin concentration from 100 per cent of normal to 33 per cent of normal (Quick's method).

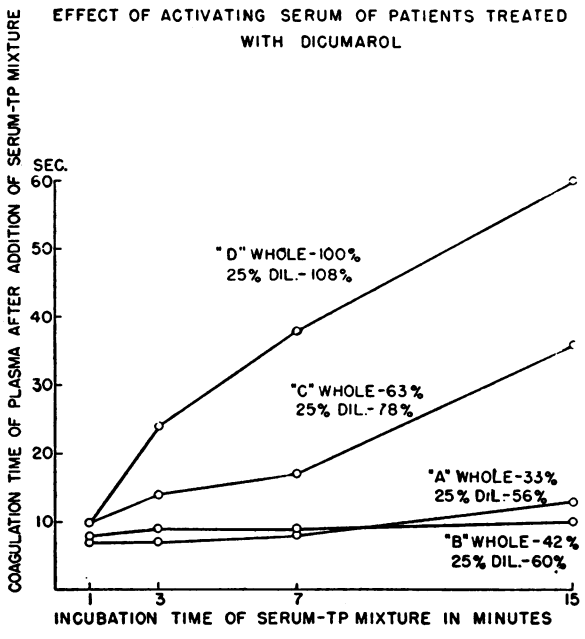

FIG. 5

Sera of patients receiving dicumarol were obtained at varying intervals following administration of 300 mg. of dicumarol. Serum D, whose corresponding plasma prothrombin concentration was 100 per cent of normal, was obtained 12 hours after dicumarol was given. Similarly, serum C (plasma prothrombin 63 per cent of normal) was obtained 36 hours after dicumarol; serum B (plasma prothrombin 42 per cent of normal) and serum A (plasma prothrombin 33 per cent of normal) were obtained 60 and 72 hours after dicumarol *per os.* The activated serum-thromboplastin mixture appears to have a more prolonged action as the corresponding plasma prothrombin concentration falls.

It will be observed that the coagulation time of the serum-TP mixture, added to plasma after incubation for 15 minutes, is 10 seconds for serum B, in contrast to 60 seconds for serum D. Similarly, serum C, with an intermediate reduction of the corresponding plasma prothrombin concentration also showed an intermediate reduction of the plasma-serum-TP coagulation time (36 seconds) at the end of the 15 minute incubation period.

When such serial studies are made of patients who are receiving dicumarol, it is possible to show that there exists a fairly good correlation between the shape of the serum-activation curve and the concentration of prothrombin in the plasma. As the concentration of plasma prothrombin falls, a progressive shortening of the coagulation time of the serum-TP mixture develops when this is tested for coagulating effect on normal plasma after incubation for 15 minutes.

Figure 3, to which reference has previously been made, illustrates in another manner the enhanced effect obtained when serum from dicumarol-treated patients is activated by thromboplastin. It will be observed that coagulation of plasma containing 10 per cent concentration of prothrombin was more rapid, following addition of activated serum from a dicumarol-treated patient, than was coagulation of plasma containing 100 per cent prothrombin following addition of activated normal serum.

Serum from a dicumarol-treated patient was more susceptible of activation when smaller amounts of thromboplastin were utilized. This is clearly shown in Figure 6, where varying concentrations of thromboplastin were employed in activation of the normal serum and serum from dicumarol-treated patients. In this experiment 0.1 cc. of undiluted thromboplastin is designated "whole thromboplastin." Twofold dilutions of thromboplastin suspension were then made in 0.85 per cent sodium chloride solution and 0.1 cc. aliquots of the diluted suspensions were then added to 0.5 cc. amounts of serum to effect activation. The activation curves were then determined in the same manner as described in "methods." In Figure 6, the unbroken lines show results obtained when serum obtained from a dicumarol-treated patient was utilized. The plasma prothrombin concentration in this instance was 20 per cent of normal.

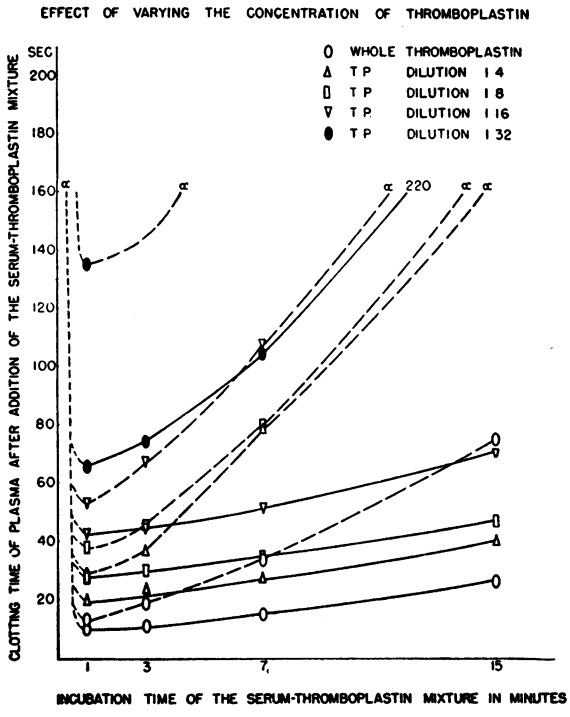

FIG. 6

Effect of varying the concentration of thromboplastin added to serum to activate the coagulation factor. Respective dilutions of thromboplastin are indicated on the graph. One tenth cc. portions of these dilutions were added to serum. After the designated incubation periods (abscissa), 0.1 cc. amounts of this mixture were added to fresh oxalated plasma. There is a lessened coagulating effect when the amount of thromboplastin is decreased. The difference between activated serum from a patient treated with dicumarol (solid line) and normal serum (broken line) is again demonstrated.

The broken-line curves represent the results obtained after activation of serum from a normal individual whose plasma prothrombin concentration was 100 per cent of normal. These data show that serum obtained from the dicumarol-treated patient was much more effectively activated by thromboplastin than the sera of the normal individual.

It is evident that a striking difference exists between sera from fully dicumarolized patients and normal serum, following activation of these sera by thromboplastin. In fact, the data suggest that a converse relationship may exist between concentration of plasma prothrombin and the concentration of "prothrombin-converting factor" which can be activated from the corresponding serum. As the plasma prothrombin concentration falls either more "prothrombin-converting factor" can be activated from the serum or the effect of the "prothrombin-converting factor" is more prolonged.

In view of the results obtained in a study of sera from patients treated with dicumarol, the results obtained in a study of a patient with severe cirrhosis of the liver associated with a prothrombin deficiency, are of particular interest. This patient with cirrhosis was found to have a plasma prothrombin concentration 40 per cent of normal (Quick's method [10]) associated with a coagulation time of 16 minutes (Lee-White method [11]). Vitamin K, administered parenterally, produced no change in the plasma prothrombin concentration. No disturbance of blood platelets or of any other coagulation factor was observed. Despite this, the patient developed severe epistaxis, purpuric skin lesions and at exitus he developed hemorrhage into the gastro-intestinal track. This patient's serum was studied on several occasions according to the method described. It showed a decreased ability to coagulate normal plasma after activation of the serum by thromboplastin as contrasted to normal serum. When serum from the patient with hepatic disease was compared with serum obtained from a patient receiving dicumarol with a reduction of prothrombin to a corresponding level, the difference in the two curves was quite evident. Several other patients with cirrhosis of the liver associated with decreased prothrombin have been studied in this manner. None of these patients had any hemorrhagic phenomena or disturbance of whole blood coagulation. In these patients, no results approximated those seen in sera of dicumarol-treated patients which had corresponding reductions of prothrombin. However, none showed any striking deviation of the reaction curve from that obtained following activation of normal serum and none showed such a striking deviation as the above patient with the prothrombin deficiency associated with disturbance of whole blood coagulation.

It is probable that the patient with hepatic disease associated with purpura, et cetera, had a deficiency of "prothrombin-converting factor" as well as prothrombin itself and that both defects may have contributed to the production of the ob-

served abnormality of coagulation and the purpuric manifestations.

Effect of addition of potassium oxalate to serum before and after thromboplastin activation: The preceding experiments have indicated that once activation of the "prothrombin-converting factor" has been accomplished, the reaction of prothrombin conversion can take place under conditions where the amount of ionized calcium is considerably reduced. When the concentration of oxalate in plasma is 0.01 M, coagulation by activated serum proceeds rapidly. If the molar concentration of plasma is increased to 0.02 M, the coagulation effect of activated serum is retarded and finally, no coagulation results if the concentration of oxalate in plasma is 0.04 M.

Furthermore, it was found that activation of serum by thromboplastin followed by addition of potassium oxalate, resulted in a rapid inhibition of coagulation activity of the serum-TP mixture. This loss of coagulation effect was observed even when the serum-TP mixture to which oxalate was added one minute later, was reacted with partially recalcified plasma. This plasma contained sufficient calcium more than to neutralize the amount of potassium oxalate added to the serum-TP mixture. It appears, therefore, that potassium oxalate is able to reverse the active "prothrombin-converting factor" to an inactive form. This may represent the mechanism by which activated serum-TP mixtures are apparently inhibited in plasma containing high concentrations of potassium oxalate.

TABLE I

Effect of adding potassium oxalate to serum prior to activation by thromboplastin

Final molar concentration of oxalate in the serum*	Coagulation time (sec.) of serum-TP-oxalate mixture added to 0.01 M oxalated plasma	Coagulation time (sec.) of serum-TP-oxalate mixture added to 0.005 M oxalated plasma†
0	26.0	28.0
.0014 M	31.0	31.0
.0021 M	31.0	76.0
.0028 M	36.0	70.0
.0035 M	43.0	83.0
.0037 M	229.0	>300.0
.004 M	>300.0	>300.0

* Concentration obtained by dividing molar concentration of potassium oxalate added by the dilution factor of serum and thromboplastin mixture, *i.e.*,

$$\frac{.02\ M}{7}\ \text{oxalate} = .0028\ M.$$

† 0.005 M oxalated plasma was made by adding 0.05 cc. of 0.1 M $CaCl_2$ to 0.95 cc. of 0.01 M oxalated plasma.

Increasing concentrations of oxalate contained in 0.1 cc. were added to 0.5 cc. of serum. Following this, 0.1 cc. portions of thromboplastin suspension were added to the respective sera and one minute later, 0.1 cc. of this mixture was added to 0.1 cc. of 0.01 M or 0.005 M oxalated plasma. The coagulation time was then recorded.

Table I demonstrates the effect obtained when potassium oxalate was added to serum *prior* to activation by thromboplastin. One-half cc. aliquots of normal human serum were added to 0.1 cc. amounts of a solution which contained varying concentrations of potassium oxalate. Several minutes later, 0.1 cc. of a thromboplastin suspension was added to the serum-oxalate mixture. Exactly one minute later, 0.1 cc. of the serum-TP-oxalate mixture was added to 0.1 cc. of 0.01 M oxalated plasma. It will be observed that coagulation of plasma resulted as long as the molar concentration of potassium oxalate was less than 0.0036 M. When the concentration of serum oxalate exceeded 0.0037 M, the activation of serum by thromboplastin was inhibited.

In order to eliminate the possibility that a cumulative concentration of potassium oxalate in the combined serum-TP and plasma might influence the speed of coagulation, the following control was made. The same series of serum-TP-oxalate mixtures was added to .005 M plasma. This plasma was prepared by adding 0.05 cc. of 0.1 M calcium chloride to 0.95 cc. of 0.01 M oxalated plasma. The amount of oxalate added to serum was more than neutralized by the concentration of calcium in the 0.005 M plasma. At the same time, plasma prepared in this manner did not spontaneously coagulate. It will be observed (Table I) that the coagulation effect of the serum-TP-oxalate mixture was not enhanced by use of partially recalcified plasma. In fact, the speed of coagulation was prolonged over that obtained in plasma which contained a higher concentration of potassium oxalate. The discrepancy of results obtained by use of 0.01 M and 0.005 M oxalated plasma is not clear, but it seems unlikely that any of the coagulation effect can be related to a direct action of thromboplastin *per se* on the plasma prothrombin.

These data are supportive evidence that prothrombin conversion can occur when the ionized calcium concentration is very low. The importance of calcium in coagulation seems to be re-

lated to the stage of activation of "prothrombin-converting factor." In addition, once the "prothrombin-converting factor" has been activated, it can be rapidly inactivated if an excess of potassium oxalate is present in plasma or if potassium oxalate is added directly to the active serum-TP mixture.

Effects of physical agents on serum-thromboplastin activation: It has been shown that serum from normal individuals produces a characteristic predictable reaction curve following activation with thromboplastin (Figure 1). There is a release of "prothrombin-converting factor" followed by gradual decay of activity.

A progressive, slow loss of activatable "prothrombin-converting-factor" occurred when the serum was stored at 0° C. prior to addition of thromboplastin. A rapid loss occurred if serum was kept at 37° C., so that 10–12 hours later, only slight activation of the "prothrombin-converting factor" resulted after addition of thromboplastin.

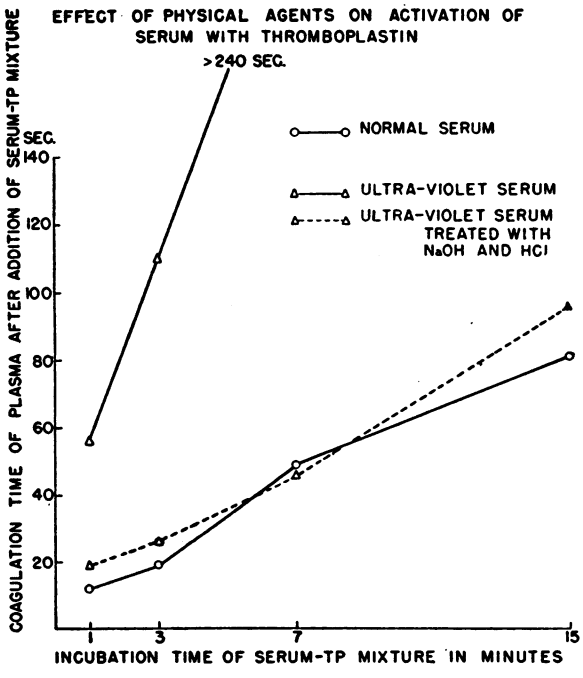

FIG. 7

Effect of change of pH on restoration of activatable serum "prothrombin-converting factor." Ultra-violet irradiation of serum for two hours prior to activation by thromboplastin causes a marked decrease of activatable substance. After this serum was treated with alkali, followed by restoration of pH to 7.3, the activation curve approximates that obtained in the untreated serum.

When serum was irradiated by ultra-violet light, a rapid decrease of the "prothrombin-converting factor," capable of activation by thromboplastin, resulted.

Serum which becomes unreactive by aging or by ultra-violet irradiation can be almost completely restored to activity by treatment with dilute alkali or acid. In experiments illustrated in Figure 7, 0.25 cc. of 0.1 N NaOH was added to 0.5 cc. of inactive serum. Thirty minutes later 0.25 cc. of 0.1 N HCl was added to this mixture to restore the pH to a value between 7.0 and 8.0. When serum was treated in this manner, it was found to have nearly complete restoration of prothrombin-converting activity following addition of thromboplastin.

Storage of serum for as long as one year at refrigerator temperatures does not result in complete loss of the "prothrombin-converting factor." In fact, some of the sera which became alkaline (pH 8.5) after storage, were found to be capable of activation by thromboplastin without preliminary treatment with alkali or acid.

These data indicate that the "prothrombin-converting factor" of serum does not readily deteriorate, but only becomes reversibly inactivated.

Effect of crystalline soybean trypsin-inhibitor on activation of serum by thromboplastin: Since crystalline trypsin-inhibitor substances have been shown capable of delaying blood coagulation by inhibiting prothrombin conversion (12–14) a preparation of soybean trypsin-inhibitor [3] was employed to study the effect of this substance by means of this experimental method. The preparation used in this protocol (Figure 8) produced appreciable prolongation of the plasma prothrombin time when 0.1 mg. was added to 0.1 cc. of plasma. Crystalline soybean trypsin-inhibitor was added in varying amounts to two different sera, one of which was obtained from a normal individual, while the other was obtained from a patient receiving dicumarol. The plasma, in the latter instance, was found to have a prothrombin concentration 28 per cent of normal. Examination of Figure 8 reveals that inhibition of activation of serum by thromboplastin was proportional to the amount of trypsin-inhibitor added to the serum. However, the de-

[3] Crystalline soybean trypsin-inhibitor was supplied through the generosity of Armour and Company, Chemical Research Division, Chicago 9, Illinois.

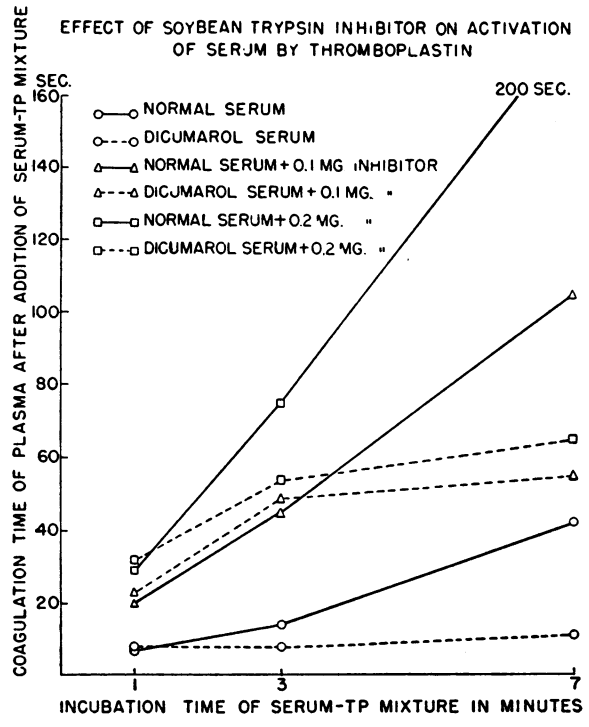

FIG. 8

Soybean trypsin-inhibitor was added to serum (amount designated on the graph) prior to activation by thromboplastin. Two types of sera are shown, the solid lines representing normal sera, whereas the broken lines represent sera obtained from a dicumarol-treated patient. Trypsin-inhibitor reduces the activity of "prothrombin-converting factor," particularly after the serum-thromboplastin mixture with trypsin-inhibitor added has been incubated for seven minutes. In contrast, the "dicumarol serum" was less affected by the trypsin-inhibitor.

cay curve of the "prothrombin-converting factor" was affected less in the dicumarol than in the normal serum. For example, the serum-TP mixture, to which 0.2 mg. of trypsin-inhibitor had been added, was incubated for seven minutes and was found to produce coagulation of plasma in 200 seconds when normal serum was employed, in contrast to a coagulation time of plasma of 65 seconds when serum obtained from the patient treated with dicumarol was used.

Crystalline soybean trypsin-inhibitor was added to plasma in sufficient concentration to reproduce the amount which would be carried over in the above serum-TP mixtures. Serum was then activated by thromboplastin in the usual manner and added to the plasma-trypsin-inhibitor system. It was found that no inhibition of the activated "prothrombin-converting factor" resulted. This observation indicates that soybean trypsin-inhibitor delays coagulation either by inhibition of thromboplastin or by inhibition of *activation* of the serum "prothrombin-converting factor."

DISCUSSION

When plasma coagulates, it is apparent that several constituents are either removed or destroyed as a result of the coagulation process. Prothrombin is removed by being converted to thrombin, which in turn is either destroyed or combined with other plasma components to form "metathrombin" (10). Fibrinogen is converted to fibrin which can then be removed as a clotted mesh. Serum, another end product of coagulation, contains, therefore, only small amounts of prothrombin and thrombin which deteriorate on standing and "metathrombin" which has been reported capable of reconversion to thrombin under suitable experimental conditions (15). At first it seemed logical to suppose that the coagulating effect of serum to which thromboplastin had been added was related to reconversion of "metathrombin" to thrombin. However, from the results of experiments with fibrinogen solutions and deprothrombinated plasmas it became apparent that the coagulating effect of activated serum could not be due to thrombin, but rather represented some factor which initiated coagulation through an intermediate action on prothrombin. It therefore seemed necessary to conclude that serum contains a "prothrombin-converting factor" which can be activated by thromboplastin. During the process of blood coagulation, the "prothrombin-converting factor" present in an inactive state *in vivo* in circulating plasma is activated either by thromboplastic globulin or tissue, or platelet thromboplastin to produce an active "prothrombin-converting factor" which in turn causes conversion of prothrombin to thrombin. Following the initial release of "prothrombin-converting factor" it is then converted to an inactive substance producing no effect on coagulation until more thromboplastin is added to reactivate it.

When serum is utilized to study the activation of the "prothrombin-converting factor" there is no substrate upon which this factor can effect a normal reactive process. It has been possible, therefore, to dissect the first stage of blood coagulation

and study this process more completely. By this method of study one can demonstrate a sudden release of "prothrombin-converting factor" soon after serum has been in contact with a suspension of thromboplastin. Following this, the "prothrombin-converting factor" slowly disappears from the serum-thromboplastin mixture. The degree of release of "prothrombin-converting factor" is directly related to the concentration of thromboplastin added to the serum for activation. Similarly, if one uses plasma which contains a reduced concentration of prothrombin, the clotting time will be correspondingly prolonged following addition of an active serum-TP mixture.

The mechanism by which the inactive serum "prothrombin-converting factor" is activated by thromboplastin is a topic for critical consideration. Ferguson (12), MacFarlane and Pilling (13), and Grob (14) have shown that serum and pancreatic trypsin-inhibitor substances have an appreciable influence on *in vitro* prothrombin conversion. It has been shown above that a crystalline soybean trypsin-inhibitor substance can appreciably alter the reaction curve when this is added to serum which is then activated by thromboplastin. Whether this effect is related to a neutralization of thromboplastin as MacFarlane and Pilling (13) believe, thereby diminishing the activating effect of the thromboplastin for serum, is not clear.

The type of reaction seen in the serum which has been aged or treated with ultra-violet irradiation may be related to a reversible denaturation of the reacting system, so that thromboplastin can no longer activate the "prothrombin-converting factor." When serum is treated by changing the pH, as described, then the "prothrombin-converting factor" again becomes capable of activation by thromboplastin in much the same manner as seen in fresh, normal serum.

On the basis of studies made on serum obtained from patients treated with dicumarol, it is apparent that there is a decided change produced in plasma, irrespective of the effect of dicumarol on the plasma prothrombin concentration. The data presented suggest that dicumarol administration is associated not only with a decreased concentration of prothrombin in the plasma, but also an increase of "prothrombin-converting factor." If this type of balance operates to preserve homeostasis of the coagulative mechanism, in that a reduction of prothrombin would be counter-balanced by an increased concentration of "prothrombin-converting factor," then the enigma of marked prothrombin reduction without a profound disturbance of the coagulation mechanism might be more easily comprehended. A different and adverse situation appeared to be present in the patient with cirrhosis of the liver, who had a moderate reduction of prothrombin with associated hemorrhagic and coagulative disturbance, and at the same time was found to have a decrease in "prothrombin-converting factor."

One must consider furthermore that the altered reactivity of activated serum from dicumarol-treated patients might be associated with a decrease of inhibitor substance, thereby permitting a prolonged effect of the "prothrombin-converting factor."

On the basis of the experimental evidence presented, a revised formula of blood coagulation is suggested.

1. ("Prothrombin-converting factor" − Inhibitor) + (Thromboplastin) + (Ca^{++}) → (Thromboplastin − Inhibitor − Ca^{++}) + ("Prothrombin-converting factor")
2. Prothrombin —("Prothrombin-converting factor")→ Thrombin
3. Fibrinogen —(Thrombin)→ Fibrin

The validity of this equation might be strengthened if it could be determined whether thromboplastin is altered after it is added to serum. It has already been pointed out that thromboplastin remains unchanged if one attempts to determine the activity of thromboplastin by *in vitro* coagulation studies.

In vivo studies by Thomas (16) indicate that thromboplastin is changed when it is incubated with serum. He observed that thromboplastin injected intravenously into mice produced death by causing intra-vascular clotting. If thromboplastin was allowed to react with rabbit serum for several hours, many times a minimal lethal dose of thromboplastin could be injected intravenously without producing any adverse effect on the mice. Despite this, the "inactivated" thromboplastin had normal coagulative properties when *in vitro* studies were made. Thomas (16) treated thromboplastin with serum, following which he centrifuged the thromboplastin suspension free of the serum. When this

preparation was treated with potassium oxalate, it again became reactive to produce intra-vascular coagulation of blood. Similarly, he showed that thromboplastin was not inactivated by adding it to oxalated plasma and would continue to produce death in mice following intra-venous injection. These observations suggested to Thomas that a thromboplastin-inhibitory component of serum combined with thromboplastin in the presence of ionized calcium. This combination was easily dissociable if calcium ion was removed.

If these observations are applied to the equation suggested above to explain blood coagulation, it appears that the mode of activation of the "prothrombin-converting factor" might be related to a combination of thromboplastin with an inhibitor to release the "prothrombin-converting factor." It has been shown that such activation cannot take place unless ionized calcium is present.

It is much more difficult to explain how the serum-TP mixture finally becomes inert, only to be reactivated again by adding more thromboplastin or by adding more fresh serum.

No attempt has been made to correlate the above described "prothrombin-converting factor" with the chloroform-activated factor of serum and plasma, described by Tagnon *et al.* (17, 18). These workers have shown that treatment of serum or plasma with chloroform produces a substance which causes conversion of prothrombin to thrombin in the absence of ionized calcium. In addition, this factor causes lysis of fibrinogen and fibrin. When plasma was coagulated by a serum-TP mixture, according to the method described in this report, no lytic action of the serum-TP mixture was observed. However, Tagnon and co-workers have shown that thromboplastin and prothrombin inhibit the lytic action of the chloroform-activated factor.

It is possible that the chloroform-activated factor may be identical with the thromboplastin-activated factor described in the above experiments. Chloroform may cause a selective destruction of the inhibitor in the postulated "prothrombin-converting factor"-inhibitor complex, thereby permanently releasing a "prothrombin-converting factor."

SUMMARY

1. Preliminary investigations are reported which describe a method of activation of a prothrombin-converting substance ("prothrombin-converting factor") by adding thromboplastin to serum. The activated "prothrombin-converting factor" causes conversion of prothrombin in the virtual absence of calcium; slowly decreases in activity following liberation from serum by thromboplastin; can be reactivated again by adding more thromboplastin to the inactive serum-thromboplastin mixture; and is unable to cause direct conversion of fibrinogen to fibrin.

2. Serum of dicumarol-treated patients, which is activated by thromboplastin, has a more sustained release of "prothrombin-converting factor." The activity of "prothrombin-converting factor," under these circumstances, increases as the level of plasma prothrombin falls. Conversely, a patient with cirrhosis of the liver, associated with an hemorrhagic disorder, was found to have a decrease of "prothrombin-converting factor" as well as a reduction of plasma prothrombin.

3. On the basis of the experimental evidence outlined, a revision of the generally accepted theory of blood coagulation is proposed.[4]

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