The Reaction between Bovine Brain Tissue Factor and Factors VII and X*

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ABSTRACT: Factor VII, essentially free of factor X, but containing prothrombin, was isolated from bovine plasma. When it was incubated with bovine brain tissue factor and calcium ions, a product was formed which activated factor X. The reaction between tissue factor and factor VII was found to be extremely rapid. The reaction product was inhibited by diisopropylphosphorofluoridate and soybean trypsin inhibitor. The coagulant activity generated by tissue factor and factor VII was tightly bound to the tissue particles, and was not eluted from them by chelating agents, detergents, or organic solvents.

Recent studies have demonstrated that tissue extracts derived from lung and brain can activate factor X (Williams, 1964; Nemerson and Spaet, 1964). These investigators suggested that tissue factor acts enzymatically in this reaction. This conclusion was based on the observation that the yield of active factor X was independent of the concentration of tissue extract used, and that the rate of activation was a function of the tissue factor concentration when the system was saturated with factor X. In these experiments, the factor X preparations contained abundant factor VII.

Experiments using serum obtained from patients lacking either factor VII or X have shown that the rate of activation of factor X is also a function of the concentration of factor VII (Hougie, 1959; Straub and Duckert, 1961; Nemerson and Spaet, 1964). The methods used, however, did not determine whether tissue factor first reacts with factor VII to form an intermediate product which then activates factor X, or whether tissue factor directly activates factor X *via* a mechanism which is accelerated by factor VII.

Esnouf and Williams (1962) established that Russell's viper venom specifically activates factor X. This reaction is apparently independent of factor VII (Biggs and Macfarlane, 1962), unlike the tissue factor system which involves the participation of factor VII. If factor VII first reacts with tissue factor and does not merely accelerate the activation of factor X, it should be possible to demonstrate the presence of an intermediate product. This report describes the partial purification of a protein from bovine plasma which generates coagulant activity when treated with tissue extracts,[1] but which cannot be activated by Russell's viper venom (RVV).[2] Data are presented which indicate that when tissue factor reacts with factor VII, an intermediate is formed which enzymatically activates factor X.

Materials and Methods

DEAE-cellulose was Whatman DE 11, purchased from A. H. Thomas. Bio-gel P-100 was obtained from Calbiochem. Sephadex G-100 and G-200 were purchased from Pharmacia Co. Soybean trypsin inhibitor was a twice recrystallized product obtained from Sigma Chemicals. Crystallized bovine albumin was purchased from the same source. Diisopropylphosphorofluoridate (DFP) was obtained in propylene glycol from K & K Laboratories, Inc.

Factor X deficient bovine plasma, prepared according to Denson (1961), was purchased from Diagnostic Reagents Ltd., Oxon, Thame, England. Russell's viper venom was purchased from Burroughs Wellcome, Co. Human fibrinogen was purchased from Squibb & Co. and dissolved in saline. Prior to use it was dialyzed against distilled water for 24 hr. The precipitate that formed was washed with water and dissolved in imidazole-buffered saline (IBS) at a concentration of 5 mg/ml. It was stored at −20°.

IBS (pH 7.35) was prepared according to Mertz and Owen (1940). "Cephalin" was a crude mixture of human brain phosphatides prepared by the method of Bell and Alton (1954). The stock suspension contained 1.52 mg of P/ml. All other chemicals were of reagent grade and purchased from standard sources.

Preparation of Tissue Extracts. A bovine brain was obtained immediately after slaughter, stripped of its membranes, minced, and washed with copious quanti-

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[1] As no attempt has been made to correlate the proteins under study with those lacking in human deficiency states, "factor X" is used to designate the plasma substrate of Russell's viper venom (RVV), and "factor VII" designates the factor which reacts with tissue extracts but not with RVV.

[2] Abbreviations used: RVV, Russell's viper venom; IBS, imidazole-buffered saline; X_a, activated factor X; SBTI, soybean trypsin inhibitor.

ties of tap water. A portion was extracted five times with ten volumes of acetone at room temperature. The remaining powder was homogenized with 20 volumes of IBS containing 2 g of disodium EDTA/100 ml, pH 6.0. The slurry was stirred at room temperature for 30 min and then centrifuged for 2 hr at 37,000*g*. The precipitate was washed with cold EDTA–saline until the absorbance of the supernatant at 280 mμ was less than 0.05. This procedure was selected to remove any factor X adsorbed to the brain (Williams, 1964; Nemerson and Spaet, 1964).

The washed brain suspension was stored at −20°. Immediately prior to use, it was suspended in IBS and dispersed by gentle homogenization in a Teflon and glass homogenizer. This preparation was stable for at least 6 months.

Assays of Coagulant Activity. All assays were performed at 37°. $CaCl_2$ was a 0.025 M solution unless otherwise stated. Factor X and activated X (X_a) were measured as previously described (Nemerson and Spaet, 1964). Prothrombin was assayed in a one-stage system using a substrate of oxalated bovine plasma adsorbed three times with $BaSO_4$ (100 mg/ml) and enriched with 0.01 volume of cephalin. This reagent (0.1 ml) was added to 0.1 ml of factor X reagent (see below) containing at least 25% the activity of a similar quantity of bovine plasma. Immediately before use, 0.1 ml of the material to be assayed was added and the tube warmed to 37°. RVV (0.1 ml, 10 μg/ml) was added, followed by 0.1 ml of $CaCl_2$, and the clotting time was determined. The specificity and reproducibility of this method for prothrombin will be described elsewhere (Y. Nemerson, J. Branch, and A. Zettner, to be published). The prothrombin content of 1 ml of bovine plasma was given the arbituary value of 100 units. A similar value was given to the factor X content of 1 ml of serum.

Factor VII was assayed in a two-stage system based on its ability to enhance the coagulant activity of tissue factor when added to whole-beef plasma. To 0.2 ml of tissue factor (0.38 μg of P/ml) was added 0.2 ml of the material to be assayed and 0.1 ml of $CaCl_2$. At intervals, 0.1 ml of this mixture was added to a tube containing 0.1 ml of $CaCl_2$. Bovine plasma (0.1 ml) containing 0.01 volume of cephalin was added and the clotting time determined.

A three-stage assay was used to evaluate the ability of factor VII to accelerate the activation of factor X by tissue extracts. In the first stage 0.2-ml quantities of factor VII reagent and tissue factor (0.18 μg of P/ml) were incubated for 4 min with 0.1 ml of $CaCl_2$. In the second stage 0.2 ml of this mixture was added to a tube containing 0.2 ml of factor X reagent and 0.1 ml of $CaCl_2$. The amount of X_a formed was then measured in the third stage by adding 0.1 ml of the second stage mixture to 0.1 ml of $CaCl_2$ and 0.1 ml of factor X deficient plasma. The clotting time obtained was related to the concentration of X_a by fully activating an aliquot of the factor X reagent with RVV, diluting the active material, and constructing a curve in which the concentration of X_a was plotted against the clotting time. The factor X reagent contained 8 μg of protein/ml, and clotted the substrate in about 8 sec when fully activated by RVV. The substrate contained 0.01 volume of cephalin.

When activated factor VII was assayed, the same system was employed, but tissue factor was omitted from the first stage. In addition, the assay was run both with and without cephalin (0.01 volume) in the factor X reagent. When cephalin was added to the latter, it was deleted from the substrate plasma.

Preparation of Clotting Factors. Plasma was prepared from bovine blood collected into 0.1 volume of 0.15 M trisodium citrate. Barium citrate was precipitated by the addition of $BaCl_2$ according to the method of Lewis and Ware (1953). The adsorbable clotting factors were eluted at 37° for 10 min with an amount of 0.2 M sodium citrate–citric acid buffer, pH 6.8, equal to one-tenth the starting plasma volume. In lieu of dialysis, barium ion was precipitated by the dropwise addition of 1 M Na_2SO_4 until no further precipitate formed. After removal of the precipitate, 31.3 g of $(NH_4)_2SO_4$/100 ml was added. Precipitation of the proteins was allowed to continue for 1 hr at room temperature, following which the suspension was centrifuged for 20 min at 37,000*g*. The precipitate was collected and dialyzed at 4° for 36 hr against many changes of distilled water, and finally against phosphate buffer, pH 7.0, $I = 0.05$. To each 100 ml of the supernatant from the $(NH_4)_2SO_4$ precipitation was added an additional 10.1 g of the same salt. The precipitate formed at this stage was dialyzed in the same manner.

Factor VII was prepared from the first $(NH_4)_2SO_4$ precipitate. Material obtained from 2–3 l. of plasma containing 50–81 mg of protein was applied to a 20 × 2 cm column containing 15 g of DEAE-cellulose. Prior to use, the adsorbent was washed with 2 M NaCl, pH 6.0, and finally equilibrated with phosphate buffer, pH 7.0, containing 0.15 M NaCl. Elution was accomplished with a linear gradient ranging from 0.15 to 0.40 M in NaCl. Both solutions were in phosphate buffer, pH 7.0, $I = 0.05$. The volume of the eluent was 1600 ml, and the flow rate was 15 ml/hr. Fractions of 15 ml were collected. The fractions containing factor VII were pooled and concentrated by ultrafiltration. The concentrate was dialyzed against the starting buffer to free it of salt and rechromatographed in a similar manner. Portions of the latter material were concentrated as above and applied to a 90 × 2.5 cm column of Bio-gel P-100. The volume applied was 2–3 ml, the flow rate was 18 ml/hr, and 6-ml fractions were collected. The protein was eluted with IBS.

Factor X was prepared from the second $(NH_4)_2SO_4$ precipitate by DEAE-cellulose chromatography in a similar manner except that the gradient ranged from 0.30 to 0.50 M in NaCl. The macromolecular contaminant (Esnouf and Williams, 1962) was removed by filtration on columns of Sephadex G-200. The remaining material (factor X reagent) was stored in small aliquots at −20°. As noted by Papahadjopoulos *et al.* (1964) some spontaneous activation of factor X occurred at this temperature.

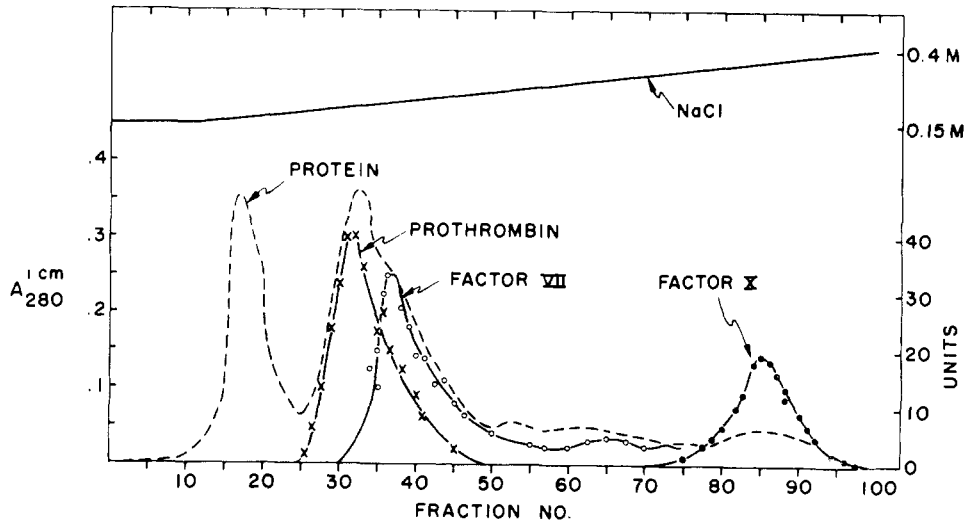

FIGURE 1: Chromatography of $(NH_4)_2SO_4$ fractionated plasma eluate on DEAE-cellulose. Elution was performed with a NaCl gradient in phosphate buffer, pH 7.0. Fractions of 15 ml were collected.

Chemical Methods. Protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Following digestion in perchloric acid, phosphorus was determined by the method of Taussky *et al.* (1953).

Results

Preparation of Factor VII. Factor VII eluted from DEAE-cellulose when the NaCl concentration of the eluent reached about 0.23 M (Figure 1). Prothrombin was eluted slightly ahead of factor VII, but there was considerable overlap. Rechromatography with a flatter gradient failed to resolve these peaks.

Following concentration, the prothrombin–factor VII mixture was filtered through Bio-gel P-100. Slight resolution was obtained, but gross contamination was evident (Figure 2). Prothrombin and factor VII failed to separate on calcium phosphate gel (Mathews *et al.*, 1964), on sucrose gradient electrophoresis (Svensson, quoted by Bloemendahl (1963)), or on carboxymethylcellulose at pH 6.0. As a consequence, the factor VII preparations used were contaminated with prothrombin.

Assay of Factor VII. Factor VII was detected by its ability to accelerate the coagulation of plasma by a standard tissue factor preparation. Because this assay lacks specificity and is influenced by the presence of factor X (and its activation to X_a), only those plasma fractions devoid of factor X, *i.e.*, not activated by RVV, could be assayed for factor VII. A concomitant of this limitation is the inability to estimate the factor VII content of plasma or serum, and, therefore, the degree of purification achieved by the fractionation procedures employed.

The calibration curve for the factor VII assay was prepared by adding varying amounts of factor VII to a standard tissue factor preparation. When this was added to recalcified bovine plasma, a linear relationship between the factor VII concentration and the clotting times was obtained when these values were plotted on logarithmic coordinates (Figure 3). Conversely, when the tissue factor was varied and the factor VII held constant, it was found that the yield of coagulant activity was also limited by the tissue factor concentration (Figure 4).

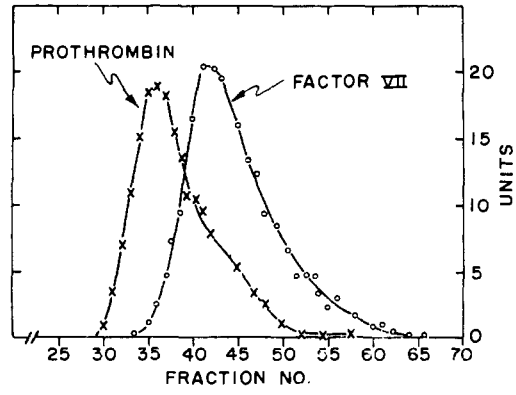

FIGURE 2: Chromatography of rechromatographed DEAE-cellulose fractions on Bio-gel P-100. Fractions of 6 ml were collected.

Because the factor VII preparation contained prothrombin, similar experiments were carried out using a prothrombin preparation[3] (Milstone, 1955) which was shown by independent means to be essentially free of factor VII (Ferguson, 1964). The results shown in Table II indicate that the addition of large amounts of prothrombin to factor VII did not affect the assay, and factor VII was not detected in the prothrombin prepara-

[3] A gift of Dr. J. Haskell Milstone.

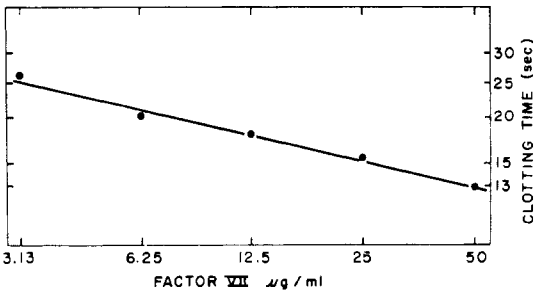

FIGURE 3: Two-stage assay of factor VII. The indicated amounts of factor VII were incubated with tissue factor (0.38 μg of P/ml) and Ca^{2+} (5 mM). Aliquots were added to bovine plasma, and the minimum clotting time obtained for each concentration of factor VII is shown.

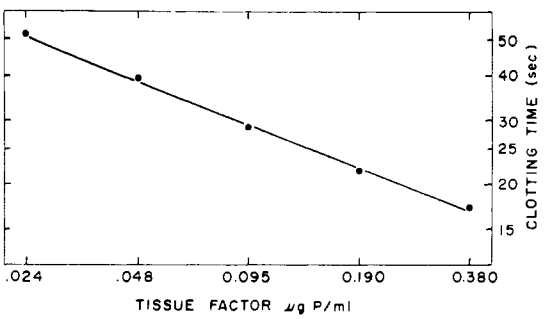

FIGURE 4: Varying amounts of tissue factor were incubated with factor VII (50 μg/ml) and Ca^{2+} (5 mM). Aliquots were added to bovine plasma, and the clotting time obtained for each tissue factor concentration is indicated.

tion. Thus, the presence of prothrombin *per se* does not account for the coagulant product of the tissue factor–factor VII reaction.

Studies on the rate of the reaction between tissue factor and factor VII revealed this to be extremely rapid when tissue factor was in excess, and the factor VII concentration was such that the substrate clotting time was 13–16 sec (50 μg/ml). Under these conditions, maximum coagulant activity was generated within 15 sec. With more dilute preparations (5 μg/ml), the reaction took 1–2 min to reach completion, although it was virtually complete within 15 sec (Table I). To identify the nature of the coagulant activity formed, aliquots of similar incubation mixtures were added to factor X deficient plasma and to fibrinogen (Table I). The clotting times obtained indicate the factor X content to be less than 1 unit/ml, and the thrombin to be less than 0.01 NIH unit/ml. These data indicate, therefore, that neither factor X nor thrombin accounts for the short clotting times obtained using whole plasma as a substrate.

TABLE I: Coagulant Activity Generated by Tissue Factor and Factor VII.[a]

	Time (min)	Substrate Clotting Times (sec)		
		Whole Plasma	X-Deficient Plasma	Fibrinogen
A	0	27.0	108	>3600
	0.25	15.2	98	3600
	1	15.4	94	3000
	5	15.4	96	2400
B	0	34.0	114	3600
	0.25	28.0	109	3200
	1	26.0	113	3200
	2	24.6	117	3200
	5	24.6	110	3000

[a] Aliquots of incubation mixtures consisting of tissue factor (0.38 μg of P/ml) and factor VII containing (A) 50 μg/ml or (B) 5 μg/ml were added at intervals to the indicated substrates. The zero-time value was obtained by mixing the reagents and substrate prior to recalcification. When saline was substituted for factor VII, the clotting time of whole plasma was 40 sec, the X-deficient substrate clotted in 115–125 sec, and no thrombin was detected.

Effect of Calcium. The rapidity of the reaction between tissue factor and factor VII prevented the direct study of the effect of Ca^{2+} on the rate of coagulant activity formed. The amount formed was therefore determined. It was desirable to perform the final assay at a constant Ca^{2+} concentration. When varying amounts of Ca^{2+} were added to tissue factor and factor VII, it was found that following adjustment of the Ca^{2+} concentration to 5 mM, the amount of activity formed was independent of this ion. As noted above the reaction between tissue factor and factor VII is complete within 15 sec, and the adjustment of the Ca^{2+} concentration and the assay could not be completed in this time. Consequently, following addition of Ca^{2+}, the coagulant activity was measured by sedimenting the tissue particles and assaying the sedimented pellet for the tissue factor–factor VII intermediate. The factor VII content of the supernatant solutions was also estimated (Figure 5).

The activity sedimented in the absence of calcium was identical with that sedimented in a control experiment in which no factor VII was added. Despite the fact that the pellets were dispersed in a tissue homogenizer, recoveries were variable. No clear optimum could be demonstrated, but the activity at 10 mM was repeatedly less than at other concentrations studied.

In the absence of Ca^{2+}, the recovery of factor VII in the supernatant was 100%. The minimum factor VII remaining in solution was found at 5 mM Ca^{2+}. The recovery of total coagulant activity was about 60% for each Ca^{2+} concentration studied. This was probably related to the difficulty encountered in dispersing the pellets even with a tissue homogenizer.

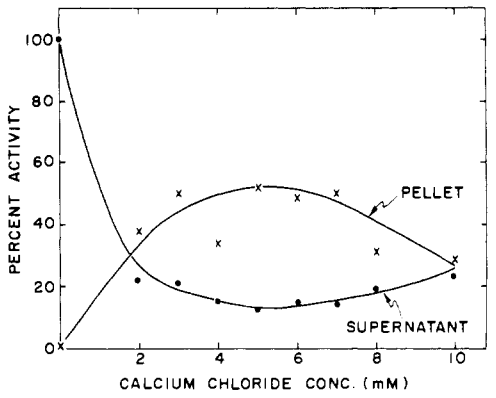

FIGURE 5: Factor VII (50 μg/ml) and tissue factor (0.38 μg of P/ml) were incubated with varying amounts of Ca^{2+}. The mixtures were then centrifuged for 3 hr at 37,000*g*. Following adjustment of the Ca^{2+} concentration to 5 mM, the supernatants were assayed for factor VII by the two-stage assay. The amount of factor VII added was taken as 100%. The pellets were collected and assayed for coagulant activity by adding them to bovine plasma as in the two-stage assay. The coagulant activity generated by these reagents in 5 mM Ca^{2+} was taken as 100% activity. The latter was not centrifuged, but was kept at 4° for 3 hr.

Effect of factor VII on the activation of factor X by tissue factor was studied by adding various amounts of factor VII to a factor X reagent, and measuring the rate of activation of the latter in the presence of an excess of tissue factor. The rate was found to vary with the factor VII concentration (Figure 6A). Linearity was observed over a narrow range (12.5–50 μg/ml). This may be due to contamination of the factor X reagent with factor VII which resulted in rapid activation without added factor VII. The initial rate of activation of factor X is plotted against factor VII concentration in the same figure (6B).

Activation of Factor X by Tissue Factor–Factor VII. Factor VII and tissue factor were reacted in optimum proportions and then diluted in iced IBS. The rate of activation of factor X was then studied at several concentrations of the reaction product (Figure 7). It can be seen that the rate of activation of factor X is proportional to the concentration of the tissue factor–factor VII product over a fourfold range of concentrations. The effect of the concentration of factor X on the velocity of the reaction was also determined (Figure 8). The rate reached a maximum in a manner suggesting that factor X functions as a substrate in this reaction.

The activation of factor X by tissue factor–factor VII proved to be a convenient way to measure this product, and was used in the following experiments.

Effect of Inhibitors. It has previously been shown that neither tissue factor (Nemerson and Spaet, 1964) nor X_a (Spaet and Cintron, 1963) is inhibited by DFP. It was of interest, therefore, to test the effects of this reagent on the tissue factor–factor VII intermediate.

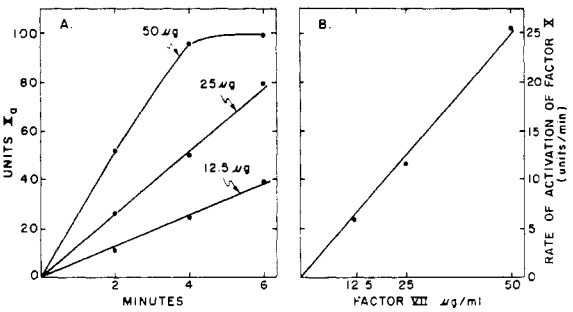

FIGURE 6: Activation of factor X to which has been added various amounts of factor VII. The incubation mixture consisted of 0.2 ml of tissue factor (0.19 μg of P/ml), 0.2 ml of a mixture containing 0.1 ml of factor X (100 units/ml) and 0.1 ml of factor VII containing the indicated amount of protein/ml, and 0.1 ml of 0.025 M $CaCl_2$. (A) Time studies in which the units of X_a generated are plotted against incubation time. (B) Initial rate plotted against the concentration of factor VII added.

Following incubation with 8×10^{-4} M DFP, the intermediate no longer activated factor X (Figure 9). Treatment of factor VII with DFP prior to the reaction with tissue factor failed to inhibit formation of the active product.

Soybean trypsin inhibitor (SBTI) was similarly studied (Figure 10). Total inhibition was achieved by 42 μg/ml. In the control experiment, the same concentration of SBTI was added following the activation of factor X. This amount of SBTI inhibited only a portion of the X_a formed. Neither tissue factor nor factor VII was inhibited. Full activity of the tissue factor was recovered following incubation with 100 μg/ml of SBTI followed by three washes of the tissue particles by centrifugation.

TABLE II: Effect of Prothrombin on the Assay of Factor VII.[a]

Factor VII (% of max)	Clotting Time (sec) Diluent	
	IBS	Prothrombin
100	24.2	...
75	26.4	26.4
50	29.0	29.0
25	35.6	36.0
0	43.0	43.2

[a] A factor VII preparation was diluted in IBS or in a solution containing 250 arbitrary units of prothrombin. A factor VII assay was then performed. For direct comparison, the results are expressed as the substrate clotting times. The factor VII preparation contained 5 units of prothrombin/ml.

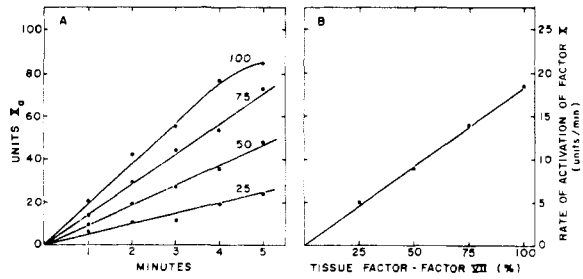

FIGURE 7: Activation of factor X by the tissue factor–factor VII intermediate. The incubation mixture consisted of 0.2 ml of tissue factor (0.38 μg of P/ml), 0.2 ml factor VII (25 μg/ml), and 0.1 ml of 0.025 M $CaCl_2$. The mixture was iced, and 0.1-ml aliquots diluted to the indicated concentration were added to 0.1 ml of factor X reagent (100 units/ml) and 0.1 ml of $CaCl_2$ (0.025 M). (A) Generation of X_a for each concentration of the intermediate is plotted against incubation time. (B) Initial rate of activation of X plotted against the concentration of the intermediate.

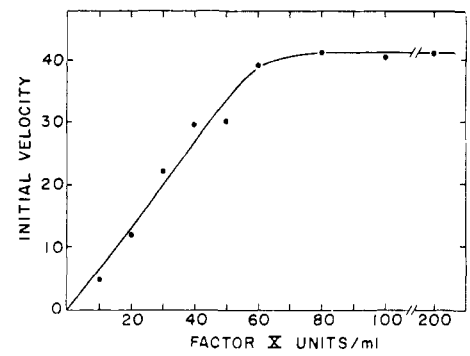

FIGURE 8: The rate of activation of factor X as a function of the concentration of factor X. The conditions are the same as described under Figure 7, except the factor X concentration was varied as indicated.

Factor VII was similarly incubated, and freed of the inhibitor by filtration on Sephadex G-100.

Sedimentation of Tissue Factor–Factor VII Intermediate. Preliminary experiments indicated that all coagulant activity could be sedimented following incubation of tissue factor and factor VII, *i.e.*, the supernatants failed to activate factor X. The effects of various reagents, therefore, were studied for their ability to elute coagulant activity from the tissue particles (Table III). In no instance did the eluate activate factor X. As noted under Methods these assays were run with and without cephalin in the factor X reagent. An interesting finding was the increase in coagulant activity following treatment with butanol. Moreover, subsequent to extraction with this reagent, forces of 105,000*g* for 2 hr were required for complete sedimentation.

TABLE III: Effect of Various Reagents on the Tissue Factor–Factor VII Intermediate.[a]

Reagent	Activation of Factor X by Supernatant	Coagulant Activity of Pellet (%)
IBS	0	100
Citrate 0.2 M	0	88
EDTA 0.2 M	0	90
Butanol 5%	0[b]	≈400[b]
Tween 20 0.1%, 1%	0	82, 68
Desoxycholate 0.1%, 1%	0	57, 29
Urea 6 M	0	0

[a] Incubation mixtures containing tissue factor (0.38 μg of P/ml) and factor VII (50 μg/ml) were stirred at room temperature with the indicated reagents (final concentrations) for 30 min. Following centrifugation at 37,000*g* for 3 hr the supernatants were tested for their ability to activate factor X (see Methods). They were retested after 2, 4, 6, and 24 hr of dialysis against IBS. The pellets were washed three times in IBS and collected by centrifugation. [b] Centrifuged at 105,000*g* for 2 hr (see text).

Discussion

Studies on the activation of factor X by tissue extracts have shown this to be a rapid reaction exhibiting no lag period (Williams, 1964; see also Figure 6). Similar experiments with a soluble fraction of rabbit brain revealed a slower rate, but again, no lag was noted (Nemerson and Spaet, 1964). It was unexpected, therefore, that a reaction would occur between tissue factor and factor VII prior to the activation of factor X. The data presented, however, show that such a reaction does take place. The absence of a demonstrable lag period is explained by the very different reaction velocities; the first reaction, the formation of the tissue factor–factor VII complex, occurs within 15 sec, while the second, the activation of factor X, proceeds over the course of several minutes.

Evidence has been presented in this study that factor VII, prepared from bovine plasma, combines with tissue factor to form a complex with coagulant properties. As one component of this complex is a tissue particle, it is sedimented at relatively low centrifugal forces. Following exposure to a variety of reagents selected because they disrupt several types of bonds, the complex either remained intact or biological activity was destroyed. In no instance was either native factor VII or "active" factor VII recovered. The latter was tested for by incubating the supernatant solutions, obtained by high-speed centrifugation of tissue factor–factor VII preparations, with factor X. Following exposure to detergents, organic solvents, chelating agents, or urea, the supernatants were inert with respect to the activation of factor X (Table III). Urea (6 M) destroyed all activity, so

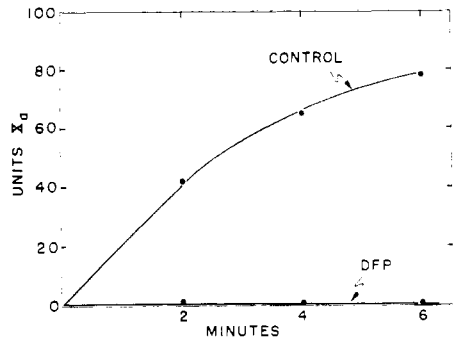

FIGURE 9: Inhibition of tissue factor–factor VII intermediate by DFP. The experimental details were the same as described under Figure 7 except the factor VII concentration was 50 μg/ml. Prior to adding factor X to the intermediate, the latter was incubated with DFP (8×10^{-4} M) for 30 min at room temperature.

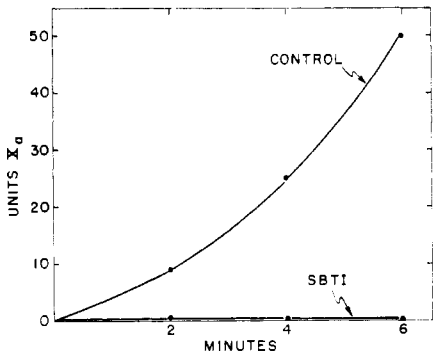

FIGURE 10: Inhibition of tissue factor–factor VII intermediate by SBTI. The intermediate was formed as described under Figure 9. It was then incubated with SBTI 42 μg/ml for 1 min, and then added to factor X. The latter was diluted 1:10 in iced IBS prior to assay. In the control experiment, the diluent contained sufficient SBTI so that the final concentration was 1.68 μg/ml in each case.

the possibility remains that the conditions were not suitable for the detection of "active" factor VII.

It was also shown that the yield of the product of the reaction between tissue factor and factor VII was limited by the concentration of both reagents (Figures 3 and 4). This could mean that factor VII and tissue factor combine in a fixed ratio, yielding a product with properties different from either component. Alternatively, one reagent might act enzymatically on the other. If the latter were true, the limiting reaction, then, might be a nondissociating enzyme–substrate complex. This possibility was indirectly explored in the experiments in which attempts were made to recover "active" factor VII from the tissue factor–factor VII complex. As noted, "active" factor VII was not detected by the methods employed.

In either case, the nature of the bonds between factor VII and tissue factor remains obscure. It is clear that they are very different from those between factor X and tissue factor. The latter, presumably between a lipid component of tissue factor and factor X, are calcium dependent, and readily disrupted by EDTA (Williams, 1964; Nemerson and Spaet, 1964) or butanol (Y. Nemerson, unpublished observation).

That the reaction between tissue factor and factor VII yields a new product is apparent from the effects of inhibitors. Neither of the precursors is inhibited by DFP or SBTI, but the complex was shown to be totally inhibited by these compounds. This observation, plus the kinetic data indicating the complex to behave as an enzyme catalyzing the activation of factor X (Figure 7), suggests the latter reaction might proceed *via* a hydrolytic mechanism.

The factor X used in these experiments was 2000–3000 times purified. It is significant that this reagent was readily activated by tissue factor without added factor VII. This is open to two interpretations: first, the factor X reagent was contaminated with factor VII, or, second, tissue factor directly activates factor X. It does not seem likely that the tissue factor was contaminated with factor VII, as there was no loss of activity when it was exposed to DFP or SBTI, which were shown to inhibit completely the tissue factor–factor VII complex. It seems likely that the factor X reagent contains factor VII as, on DEAE-cellulose chromatography, trace amounts of factor VII were detected in fractions immediately preceeding the elution of factor X.

It was emphasized in the text that the factor VII reagent used contained significant amounts of prothrombin. Although there was negligible thrombin generation when tissue factor and Ca^{2+} were added, the participation of thrombin or prothrombin cannot be excluded. It is apparent, however, that the tissue factor–factor VII complex can form in the absence of functional thrombin as preincubation of these reagents with sufficient DFP to inhibit the thrombin present did not block the formation of the complex. The possibility remains, however, that the thrombin generated either enhanced the activity of the complex or altered the reactivity of the coagulation factors present in the assay.

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Mitochondrial Phosphoriodohistidine. A Possible High Energy Intermediate of Oxidative Phosphorylation*

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ABSTRACT: The possibility that phosphoriodohistidine (PIH) participates in the phosphorylation of adenosine diphosphate (ADP) linked to the oxidation of the reduced mitochondrial electron carriers was investigated. Monoiodohistidine (MIH) and PIH were extracted from beef heart mitochondria and identified by paper chromatography and the P:I ratio. The amount of MIH found was of the same order as the concentration of electron carriers in mitochondria. The rate of phosphorylation of MIH to form PIH was of the same order as the rate of adenosine triphosphate (ATP) production. The phosphorylation of MIH required respiration and was inhibited by arsenate. Arsenate inhibition could be reversed by increasing the P_i concentration. The transphosphorylation reaction of PIH with nucleoside diphosphates was specific for ADP, rather than CDP, UDP, or GDP, and was oligomycin and dinitrophenol sensitive. [^{32}P]PIH in an alcohol extract of mitochondria reacted, nonenzymatically, with ADP to form [^{32}P]ATP. Addition of synthetic MIH to partially uncoupled mitochondria resulted in a decrease in respiration and an increase in phosphorylation, *i.e.*, in an increase in the P:O ratio. A possible mechanism for the reaction of MIH with P_i in the mitochondrion is proposed, and the role of the iodine group is discussed.

The nature of the intermediates responsible for the transfer of energy from the oxidation of the reduced electron carriers to the phosphorylation of adenosine diphosphate (ADP)[1] remains obscure. The most definitive evidence that an intermediate is formed from inorganic phosphate rather than from ADP is contained in the report by Boyer (1958) that the oxygen between the two terminal phosphorus atoms of adenosine triphosphate (ATP) is furnished largely or entirely by ADP. The discovery by Boyer and his associates (Boyer *et al.*, 1962; Boyer, 1963) of a protein-bound phosphohistidine, formed in mitochondria during phosphate participation in oxidative phosphorylation, pointed to the possible participation of histidine in these reactions, even though more recently phosphohistidine has been implicated with succinate thiokinase (Kreil and Boyer, 1964; Mitchell *et al.*, 1964).

The discovery of phosphohistidine led us to consider other phosphorylated imidazole compounds as possible high-energy intermediates. Of the derivatives of histidine known in biological systems, monoiodohistidine (MIH) theoretically seemed to have the biological and chemical requisites. Roche *et al.* (1952) identified MIH as a biological compound in hydrolysates of thyroglobulin extracted from rat thyroid gland. It has been found that thyroxine is rapidly deiodinated when incubated

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[1] The following abbreviations will be used throughout: monoiodohistidine, MIH; monophosphoromonoiodohistidine or phosphoriodohistidine, PIH; adenosine triphosphate, ATP; adenosine diphosphate, ADP.