levels in drug-free leukemic mice (Friedkin *et al.*, 1962a) suggests that selection of a new population of leukemia cells occurs during drug treatment. Mutation to a nonrepressible gene could account for the increased synthesis of the dihydrofolate reductase molecules.

It should be noted in conclusion that altered enzyme formation correlated with the development of amethopterin resistance has been reported by Sirotnak *et al.* (1964) to occur in *Diplococcus pneumoniae*. The dihydrofolate reductase from the mutant cells was strikingly less sensitive to the drug than that of the wild type, as well as differing in heat sensitivity and optimum *p*H.

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Purification and Properties of Bovine Factor X: Molecular Changes During Activation*

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Received August 17, 1964

Factor X of bovine plasma was purified nearly 10,000-fold by combined $BaSO_4$ treatment and DEAE chromatography. Activation of the purified factor X by trypsin or Russell's viper venom resulted in an apparent reduction in molecular size or shape as revealed by chromatography on Sephadex G-100. In the presence of Ca^{2+} a further, pronounced decrease in molecular weight of activated factor X was evident from behavior of the latter component on Sephadex G-100. An examination of the various species of factor X on electrophoresis showed that activation of factor X resulted in a sharp decrease in the net negative charge on the molecule. Activated factor X was capable of activating plasma factor X, whereas 25% citrate, reportedly an activator, did not have any effect on plasma factor X. The affinity of plasma factor X and activated factor X for purified phospholipids was followed through use of gel filtration on Sephadex G-200. In this manner, it was observed that formation of a stable complex between the factor X activity and phospholipids (phosphatidyl serine/phosphatidyl choline, equimolar mixture) was effected only when factor X was activated (by trypsin or Russell's viper venom) and then only in the presence of Ca^{2+}. The net charge on the protein and the phospholipid molecules appear to be of considerable import in these interactions.

Factor X was described by Hougie *et al.* (1957) as a plasma component necessary for the conversion of prothrombin to thrombin. Subsequently, several investigators (Macfarlane, 1961; Esnouf and Williams, 1962; Straub and Duckert, 1961; Williams, 1964; Nemerson and Spaet, 1964; Ferguson *et al.*, 1960; Pechet and Alexander, 1960) have established that factor X can be activated by several physiological and nonphysiological agents prior to its participation in the conversion of prothrombin to thrombin. Interestingly, even though different types of activation systems and sources of factor X were employed in the above studies, it appeared to be general that factor X was changed enzymatically to a more active form. In addition, this activated factor X required the presence of factor V, phospholipids, and Ca^{2+} for its participation in the prothrombin-activating system.

Recently, Spaet and Cintron (1963) and Macfarlane and Ash (1964) have attempted to show that the "intrinsic system" of prothrombin activation also involves an intermediate which could be similar if not identical to activated factor X. This intermediate would appear to be equivalent to the "intermediate"

* This investigation was supported by grants from the American Cancer Society (G-22078) and the Life Insurance Medical Research Fund (G-63-48).

† Inquiries should be addressed to this author. Abbreviations used in this study are: pX, non-activated plasma factor X; X^{Tr}, trypsin activated factor X; X^{R}, Russell's viper venom activated factor X; X^{S}, "spontaneously" activated factor X; II, prothrombin; PS, phosphatidyl serine; PC, phosphatidyl choline; DEAE, diethyl aminoethyl; DFP, diisopropyl phosphofluoridate.

product I, postulated by Bergsagel and Hougie (1956). Furthermore, it appears likely that thrombokinase described by Milstone (1962) and autoprothrombin C described by Kowarzyk and Marciniak (1961) may be similar, if not identical, to activated factor X. All these substances exhibit a functional similarity to activated factor X; namely, the ability to activate prothrombin in the presence of factor V, phospholipids, and Ca^{2+}.

This ability of the various kinds of activated factor X to participate in a common biological reaction is by no means an indication of the structural similarity between the different activated forms. It is entirely possible that activated factor X obtained by the several methods of activation has very different molecular characteristics. Although little specific information is available regarding the mechanism of factor X activation, it is reasonable to assume that it involves proteolytic attack on factor X. Thus, it is feasible to propose that the various activators might attack at different sites on the factor X molecule, with the resultant formation of different molecular species.

This communication describes a study of the activation of bovine plasma factor X by trypsin, Russell's viper venom, and a "spontaneous"-type reaction. Certain molecular characteristics of the products as well as the starting material are described.

Experimental

Materials and Methods.—$BaSO_4$, U.S.P., suitable for X-ray diagnosis, was obtained from Merck and Co. Sephadex G-200, obtained from Pharmacia Co., was passed through a standard sieve to obtain the 200–300 mesh fraction. Sephadex G-100, obtained from Pharmacia Co., was used without further fractionation. DEAE-cellulose was a product of Brown Co. (Lot no. 1272) and was used without additional treatment. Benzamidine hydrochloride was obtained from Aldrich Chemical Co. A stock solution of 8 mg/ml was made up in veronal buffer, *p*H 7.35, and stored at 4°. Further dilutions were made with the same buffer. Trypsin, twice crystallized and containing 50% $MgSO_4$, was a Worthington product. A stock solution, containing 5 mg/ml (excluding $MgSO_4$) in 0.001 N HCl, was prepared. This stock solution, in 1-ml aliquots, was kept frozen at −20°. It was thawed immediately before use and was further diluted with veronal buffer, *p*H 7.35. These diluted solutions were kept on ice and used within one hour. Russell's viper venom was purchased from Burroughs Wellcome Co. under the trade name "Stypven." The entire contents of one ampul (0.5 mg) were dissolved in 5 ml Tris/maleate buffer, *p*H 7.0, and stored at −20° as a stock solution. This solution was thawed and diluted with the same buffer before being used. Thrombin was a commercial preparation obtained from Upjohn Co. and further purified on Sephadex G-200 (Papahadjopoulos *et al.*, 1964).

Tris/maleate buffer was prepared by dissolving equimolar amounts of Tris [tris(hydroxymethyl)aminomethane] and maleic acid in water, each with a final molar concentration of 0.4. The *p*H of the resulting solution was adjusted by the addition of concentrated (4 M) NaOH and kept at 4°. Before use, this solution was diluted to the desired molarity. Veronal buffer (Michaelis) was prepared by a previously described procedure (Streuli, 1959).

"Cephalin" was the phospholipid mixture prepared from human brain according to Bell and Alton (1954). The final suspension in 0.14 M NaCl contained 145 μg of phosphorus per ml. Tissue thromboplastin was a saline extract of human brain and was prepared according to Toohey (1958).

Assays for Coagulation Factors.—One unit of a coagulation factor was defined as the amount present in one ml of fresh, normal human plasma.

Prothrombin was measured according to Koller *et al.* (1951). Factor V was measured according to Kappeler (1955), using aged oxalated human plasma. Factor VII was measured by the ability to correct the abnormal Quick prothrombin time of a severe congenital factor VII-deficient plasma. Factor IX concentrations were determined by the following modification of the partial thromboplastin time which uses kaolin (Proctor and Rapport, 1961): 0.1 ml of each of the following reagents were mixed at 37°: (*a*) kaolin-cephalin mixture (4 g kaolin/100 ml 0.14 M NaCl, mixed with an equal volume of cephalin suspension containing 1.4 μg of phosphorus/ml); (*b*) factor IX–deficient plasma (collected in citrate in siliconized glassware and stored at −20°); (*c*) one-tenth dilution of test plasma (or plasma fraction). The three reagents were incubated at 37° for 5 minutes, then 0.1 ml 0.025 M $CaCl_2$ was added and the clotting time was recorded. Factor X was assayed by the procedure described by Bachmann *et al.* (1958), which utilizes Seitz-filtered bovine plasma, cephalin, and Russell's viper venom. The last two reagents were mixed and kept frozen until required. Activated factor X was measured by the same procedure, but omitting the Russell's viper venom. Protein concentration was determined by the biuret method as modified by Weichselbaum (1946), or by the Folin-Ciocalteau method as modified by Lowry *et al.* (1951), with Armour bovine albumin used as a standard. Phosphorus was determined by the method of King (1932).

Purification of Factor X.—Preparation of the plasma $BaSO_4$ eluate.—Bovine blood was obtained immediately after slaughter and mixed in plastic containers with 0.1 volume of 0.1 M sodium oxalate solution. The plasma obtained by centrifugation at 3000 × *g* for 40 minutes at 4°, usually 2 liters per batch, was mixed with 75 mg solid $BaSO_4$ per ml and stirred for 30 minutes at room temperature. This mixture was centrifuged at 1,500 × *g* for 30 minutes at 4°, the clear supernatant was decanted, and the solid cake of $BaSO_4$ was suspended in one volume of cold (0°) 0.45% NaCl. The suspension was centrifuged at 1500 × g for 30 minutes at 4°, the supernatant wash discarded and the process repeated 3 times. Most of the yellow color was eliminated during these successive washes. Finally, the washed $BaSO_4$ was suspended in 1/10 volume of a solution of 5% sodium citrate (*p*H 5.8). The mixture was stirred mechanically at 0° for 1 hour and subsequently centrifuged at 2000 × *g* at 4° for 30 minutes. The supernatant, with only a slight yellow coloration, was then dialyzed for 24 hours against running tap water (temp. approx. 10–12°). The dialysate was centrifuged briefly to eliminate the small amount of white precipitate formed during dialysis and then stored at −20° until further use. After collection of a number of different batches, they were thawed, pooled, and cleared of a small amount of a white precipitate by centrifugation. The resulting clear solution was lyophilized and the white residue was maintained in covered plastic containers at −20° for later use.

Fractionation on DEAE-cellulose.—The procedure followed was a modification of the method described by Duckert *et al.* (1960). The cellulose, previously washed repeatedly with distilled water and finally with 0.15 M NaCl solution, was packed under gravity into a glass column. Gentle air pressure was used as a final aid in producing a compact bed of absorbent, usually 2 cm by 20 cm. The column was

then equilibrated with distilled water at 4°. The lyophilized powder (see above) was dissolved in distilled water (usually 100 mg protein/ml) and approximately 10 ml was applied to the column. A stepwise elution of the proteins was then accomplished with citrate buffers of increasing molarity from 0.04 to 0.08 M at *p*H 7.0. The flow rate was maintained near 10 ml per hour and the fraction volume was 10 ml. Figure 1 shows the elution pattern of the total protein and of the different clotting factors. Prothrombin and factor VII, with small amounts of thrombin and factor V, were eluted with 0.04 M citrate; the remaining factor VII activity was eluted with 0.06 M citrate, while factor X was eluted last with 0.08 M citrate. Factor IX activity was not detected in any fractions up to and including the factor X peak.[1] The column upon washing with 0.2 M citrate and then with distilled water at 4° was ready for further use. The pooled fractions were then frozen as such or lyophilized and reconstituted to a more concentrated solution in versonal buffer and stored frozen in siliconized tubes at −20°. These concentrated solutions contained 30 units of factor X per ml with a specific activity of 40 units per mg of protein. The recovery of factor X activity from the DEAE chromatography was usually 40 to 60%.

GEL FILTRATION ON SEPHADEX.—G-100 or G-200 were used essentially under the same conditions as described by Flodin and Killander (1962) and Papahadjopoulos *et al.* (1964). The chromatography columns were made from Lucite and measured 2.2. × 51 cm. The gel (total bed volume 200 ml) was completely enclosed within the column, which was fitted with porous polyethylene disks at top and bottom. The sample, usually less than 5 ml, was introduced into the top of the column through a thin polyvinyl tubing. The buffer, containing 0.04 Tris/maleate, *p*H 7.0, and 0.14 M NaCl, was introduced through the same tubing. The column was run at 4°, with a flow rate of approximately 10 ml per hour. The effluent was collected in 3-ml portions. The distribution coefficients were calculated as suggested by Flodin (1961).

Electrophoresis on cellulose acetate strips was performed on a Beckman Model R-101 microzone electrophoresis apparatus, under the following conditions: voltage, 250 v; barbital buffer (ionic strength, 0.075), *p*H 8.6. The total time for each run was 20 minutes, at which time the electrophoresis was stopped and the strips were stained with Ponceau red. The stained strips were then read with a densitometer attachment installed on a Model RB analytical model R-102 microzone scanner.

RESULTS

Activation of Factor X by Different Reagents.—(*a*) ACTIVATION OF FACTOR X BY TRYPSIN.—The same general procedure as described by Yin (1964) was followed here. One ml of purified factor X was mixed with 0.1 ml of trypsin solution (1/400 of the stock solution) and 0.1 ml 0.025 M $CaCl_2$. The mixture was incubated at 0°. At specific intervals 0.1-ml aliquots were removed and diluted with 0.9 ml buffer containing benzamidine-HCl (containing 80 μg per ml) to inhibit further action of trypsin (Mares-Guia and Shaw, 1963). Then a 0.1-ml aliquot of this latter mixture was used for the assay for factor X activation by transferring it to a tube containing 0.1 ml "cephalin" (1/40 dilution of stock suspension), 0.1 ml Seitz-filtered plasma, and 0.1 ml 0.025 M $CaCl_2$, and the clotting time was observed. Figure 2 shows the amount of activation obtained during the incubation of trypsin and factor X at 0°. The clotting times obtained in these assays were converted to arbitrary units of activity and plotted as such. The conversion curve was obtained by plotting the logarithm of the clotting times against the logarithm of dilutions of activated factor X. The dilution which gave a 50-second clotting time was arbitrarily taken as 1 unit and coincided with the amount present in the preparations of factor X before activation. Under these conditions, maximal activity was reached in 20 minutes, whereas at 30 minutes the inactivation was apparent. Higher amounts of trypsin in the incubation mixture, or a higher temperature during incubation, resulted in much faster activation and inactivation rates, respectively.

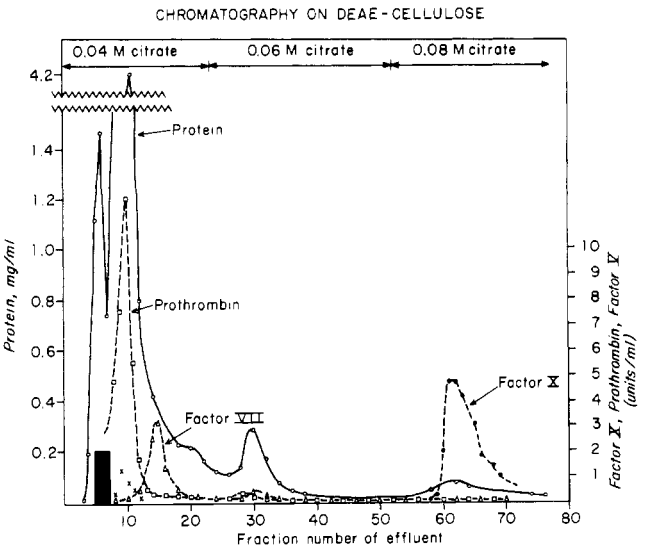

FIG. 1.—Chromatography of bovine plasma $BaSO_4$ eluate on DEAE-cellulose. Elution of the proteins was performed with citrate buffer, *p*H 7.0, by stepwise increase in molarity, from 0.04 M to 0.08 M. Effluent volume in each fraction, 10 ml. Total protein (–), factor X activity (O), factor VII activity (△), prothrombin (□), factor V activity (×), thrombin (shaded area).

(*b*) ACTIVATION OF FACTOR X BY RUSSELL'S VIPER VENOM.—One ml of purified factor X was mixed with 0.1 ml Russell's viper venom (1/20 of stock) and 0.1 ml of 0.025 M $CaCl_2$. The mixture was incubated at 37° and the activation followed as described for trypsin. The curve obtained is shown in Figure 2. Maximal activation was obtained within 10 to 15 minutes and no inactivation similar to that of trypsin was observed. Activation stopped when the temperature of the mixture was brought to 0°.

(*c*) ACTIVATION OF FACTOR X BY TRYPSIN AND RUSSELL'S VIPER VENOM.—The following experiment was performed under the same conditions as those described in (*a*) and (*b*) above. Factor X was either activated first by trypsin and then by Russell's viper venom or vice versa. When the trypsin activation was performed first, aliquots were removed from the activating mixture at designated time intervals (Fig. 3A) and mixed with benzamidine-HCl as previously described. Each of these mixtures was then activated by further addition of Russell's viper venom and Ca^{2+}. The amount of activated factor X was measured in each mixture before further activation and also after the addition of Russell's viper venom at frequent time intervals until maximal activation was reached.

[1] In the method described by Duckert *et al.* (1960), it was shown that factor IX was not eluted from DEAE-cellulose columns with citrate buffers at *p*H 7.0, but was eluted at *p*H 5.8. Hence, factor IX activity would not be eluted under the conditions used here.

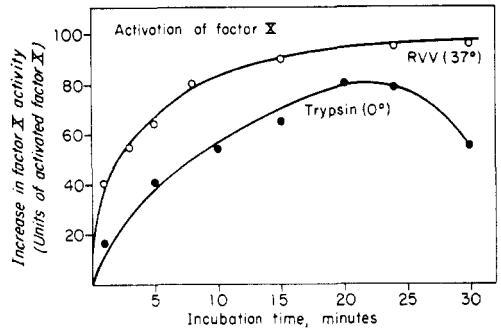

FIG. 2.—Activation of purified factor X by trypsin (closed circles) or by Russell's viper venom (open circles). The amount of activated factor X present before and after activation was obtained from a calibration curve, in which the clotting times obtained with Seitz-filtered plasma, "cephalin," $CaCl_2$, and the factor X mixture were plotted on a log-log scale against dilution of activated factor X. The dilution which gave a 50-second clotting time was arbitrarily taken as one unit.

When the Russell's viper venom activation was performed first, 0.5-ml aliquots were removed from the activating mixture at the time intervals shown in Figure 3B and quickly frozen at −70° to halt further action of the venom. When needed, the aliquots were thawed and 0.1 ml was used for the assay of activated factor X, while 0.4 ml was mixed with 0.1 ml trypsin (1/60 dilution of the stock solution) and 0.1 ml 0.025 M $CaCl_2$ at 0° for further activation. At frequent intervals, 0.1 ml of these mixtures were diluted with 0.9 ml of benzamidine and further assayed for factor X activity. The clotting times obtained from all the assays were converted into units of activated factor X as described in (*a*) above. The amount of activated factor X generated during the first activation by either trypsin or Russell's viper venom was plotted on the right hand side (open bars) of each of the double columns in Figure 3. The amount of activated factor X generated during the second activation was plotted as the difference between the total amount present after both activations and the amount generated only during the first. This difference is plotted on the left hand side (filled bars) in each double column in Figure 3.

The results obtained from both experiments (Fig. 3) make it clear that the initial partial activation of factor X by either trypsin or Russell's viper venom reduced the amount of subsequent activation produced by the second agent. In addition, when activation by either of these agents was completed, no further activation could be brought about by the other. Trypsin appeared to give a higher activation than Russell's viper venom in the particular experiment shown in Figure 3A, but this is not generally true, as already demonstrated in Figure 2.

(*d*) "SPONTANEOUS" ACTIVATION.—The preparation of factor X used for the studies described in parts (*a*), (*b*), and (*c*) above was found to contain considerable amounts of activated factor X after 3 months. The phenomenon was of interest as these fractions had been kept frozen in siliconized tubes at −20° during that time. The amount of activation differed among the different portions into which the original preparation had been divided. Evidently activation was not completed as these fractions could be activated when fresh. Since the physical or chemical agent responsible for this activation is not known, the activation will be referred to as "spontaneous." Interestingly, nonactivated factor X was similarly activated by factor X^S in the presence and absence of Ca^{2+}.

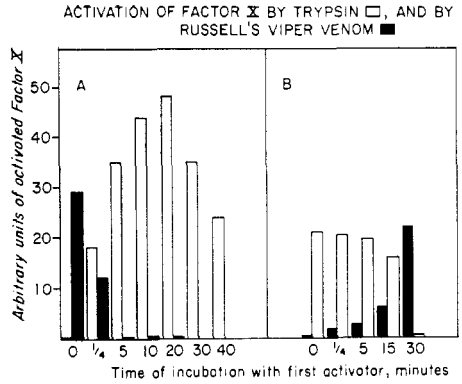

FIG. 3.—Activation of purified factor X by trypsin (open bars) and by Russell's viper venom (shaded bars). Factor X was either (A) activated first by trypsin and then by Russell's viper venom, or (B) activated first by Russell's viper venom and then by trypsin.

TABLE I
ACTIVATION OF PURIFIED FACTOR X BY INCUBATION WITH ACTIVATED FACTOR X[a]

	Incubation Mixture	0.005 M $CaCl_2$ Present	Increase in Activity After Incubation at 37° for 30 min
1.	pX + X^{Tr}	+	20
2.	pX + X^R	+	4[b]
3.	pX + X^{Tr}	−	15
4.	pX + X^{Tr} + benzamidine/HCl	+	4[b]
5.	pX + X^S	+	15
6.	pX + X^S	−	10

[a] The increase in activity was calculated as the ratio of the units per ml present after maximal activation over the units per ml present at zero time. The amount of nonactivated factor X present in the above mixtures was 2 units/ml, and the amount of activator added was 0.5 unit/ml. The abbreviations used here are: pX, for nonactivated plasma factor X; X^{Tr}, trypsin-activated factor X; X^R, Russell's viper venom–activated factor X; X^S, spontaneously activated factor X. [b] The apparent low yield of activated factor X was due to the use of a lower concentration of plasma factor X (pX).

(*e*) ACTIVATION OF FACTOR X BY ACTIVATED FACTOR X.—An increase in factor X activity was observed when nonactivated factor X was incubated with activated factor X (either X^{Tr} or X^R) at 37°. The activated factor X was freed of trypsin or Russell's viper venom (present as activators) by passage through Sephadex G-200. Table 1 gives the amount of activation obtained under different conditions. A slow increase in activity, reaching a plateau at 30 minutes at 37°, was observed with both trypsin (line 1) and Russell's viper venom activated factor X (line 2). The presence of calcium ions did not seem necessary (line 3), although it resulted in slightly higher activation. The possibility of residual trypsin activity was eliminated when the activation was also observed in the presence of benzamidine HCl (line 4).[2]

(*f*) ACTIVATION BY 25% CITRATE.—The citrate activation experiments were performed as described by

[2] Additional supporting evidence that the trypsin-activated factor X contained no active trypsin centers on the fact that any traces of trypsin not neutralized by benzamidine HCl would have destroyed all the factor X activity. All our preparations of activated factor X exhibited remarkable stability over long periods of time.

TABLE II
EFFECT OF 25% CITRATE ON FACTOR X, PROTHROMBIN, AND VARIOUS MIXTURES[a]

Mixture	Activities Present in the Mixtures (Units/ml)					
	Before Citrate			After Citrate		
	Thrombin	Prothrombin	Factor X	Thrombin	Prothrombin	Factor X
1. pX + buffer			0.4			0.08
2. pX + thrombin[b]	0.03		0.15	0.01		0.10
3. pX + X^S			3.5			1.0
4. pX + X^T			1.0			4.0
5. pX + $BaSO_4$-eluate	0.02		3.0	3.0		10
6. $BaSO_4$-eluate + buffer	0.01	1.5	0.8	3	20	60
7. Prothrombin + buffer	0.00	0.4	0.00	0.01	0.35	0.08
8. Prothrombin + thrombin	0.008	0.4	0.00	0.015	0.30	0.15
9. pX + prothrombin		0.06	0.3	0.45	0.5	1.2

[a] The activities present in each mixture were measured immediately before placing into the dialysis bags and again when the bags were opened after dialysis against 25% citrate (48 hours) and against distilled water (24 hours). The temperature during dialysis was kept at 22°. The abbreviations used here are: pX, nonactivated plasma factor X; X^{Tr}, trypsin-activated factor X; X^R, Russell's viper venom–activated factor X; X^S, "spontaneously" activated factor X; $BaSO_4$-eluate, the proteins of oxalated bovine plasma adsorbed on $BaSO_4$ and then eluted with 5% citrate and dialyzed against distilled water.
[b] The thrombin level in this fraction was controlled so that only a trace was present and this was reflected in the clotting time of fibrinogen of 118 sec. An aliquot of the same fraction was further diluted (1/10) for the usual factor X assay, which clotted the substrate in 35 seconds. After citrate activation, the thrombin-fibrinogen time was 240 seconds and the factor X assay (1/10 dilution of fraction) gave a clotting time of 40 seconds.

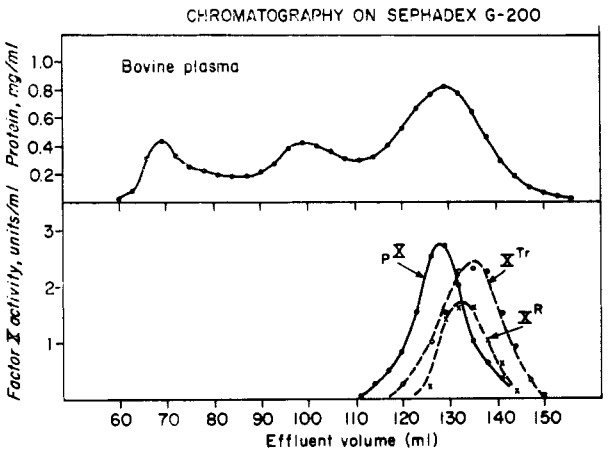

FIG. 4.—Chromatography of bovine plasma fractions on Sephadex G-200. The elution was performed with buffer containing 0.04 M Tris/maleate at *p*H 7.0 and 0.14 M NaCl. Total column volume, 200 ml. Upper curve, $BaSO_4$-treated bovine plasma; lower curve, nonactivated plasma factor X (●—●—●), trypsin-activated factor X (O—O—O), Russell's viper venom–activated factor X (X—X—X).

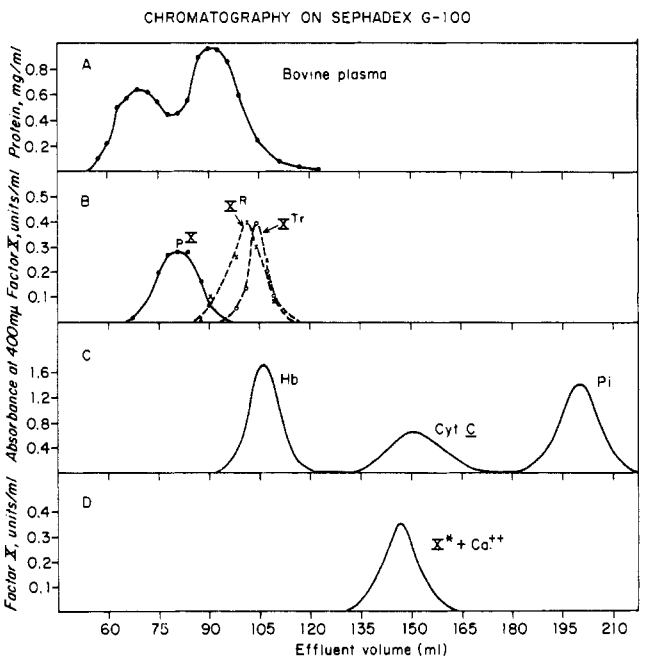

FIG. 5.—Chromatography of bovine plasma fraction and other proteins on Sephadex G-100. The elution was performed with buffer containing 0.04 M Tris/maleate at *p*H 7.0 and 0.14 M NaCl. Total column volume, 200 ml. A, $BaSO_4$-treated bovine plasma; B, nonactivated plasma factor X (●—●—●), trypsin-activated factor X (O- - -O), Russell's viper venom–activated factor X (X- - -X); C, hemoglobin (peak at 106 ml), cytochrome c (peak at 151 ml), and inorganic phosphorus (peak at 200 ml); D, activated factor X in the presence of Ca^{2+}, eluted with the above buffer containing, in addition, 0.005 M $CaCl_2$.

Spaet and Cintron (1963). The protein fractions were assayed for the presence of thrombin, prothrombin, and factor X before and after treatment. The results are illustrated in Table II. No increase in factor X activity was observed when purified factor X was treated by 25% citrate either alone (line 1) or in the presence of thrombin (line 2) or spontaneously activated factor X (line 3). There was considerable activation in the presence of trypsin-activated factor X (line 4), freed of trypsin as described above, and a much larger increase in both thrombin and factor X activity in the presence of the crude plasma $BaSO_4$ eluate (line 5). Nevertheless, the same amount of activation was observed with the crude plasma eluate alone (line 6). There was a small amount of thrombin and activated factor X generated during the citrate treatment (line 7) of "purified" prothrombin. This was slightly enhanced when a small amount of thrombin was mixed with prothrombin during the treatment (line 8). However, when nonactivated factor X was mixed with prothrombin, the generation of thrombin and activated factor X during the treatment with citrate (line 9) was considerably more than that achieved by either of the reagents when treated separately.

Molecular Characteristics of Factor X Before and After Activation.—(*a*) SEPHADEX G-200.—Factor X was chromatographed on Sephadex columns either before activation (pX) or after activation by trypsin (X^{Tr}) or by Russell's viper venom (X^R). The conditions of chromatography were described above. The elution

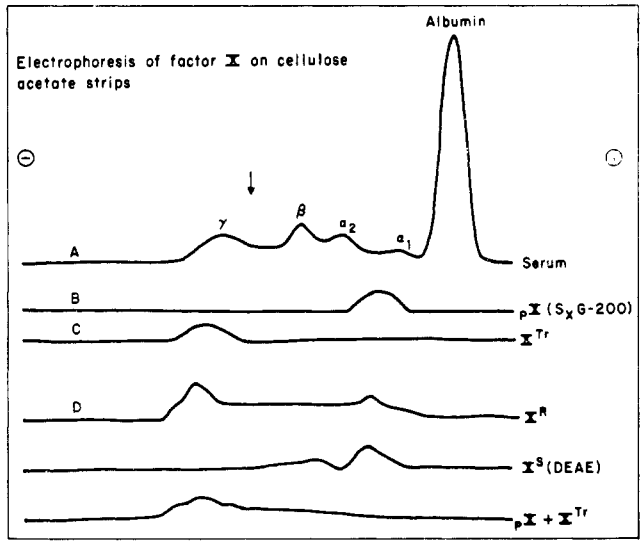

FIG. 6.—Electrophoretic mobility of factor X preparations. Cellulose acetate strips were used as supporting medium, with barbital buffer, *p*H 8.6, 0.075 ionic strength. The tracings shown here were obtained with a densitometer after the strips were stained with Ponceau S: A, whole human serum; B, factor X, after DEAE-cellulose and Sephadex G-200 purification; C, purified factor X after activation by trypsin; D, purified factor X after activation by Russell's viper venom; E, factor X after purification on DEAE-cellulose, showing "spontaneous" activation; F, mixture of trypsin-activated factor X and non-activated factor X. The arrow indicates the point of application of the individual samples.

patterns are shown in Figure 4. When pX was applied on the column, the peak of factor X activity was recovered after an effluent volume of 128 ml. The elution pattern of $BaSO_4$-treated bovine plasma proteins is also shown in Figure 4. On the basis of these results, it can be seen that pX resembles proteins of a size similar to that of albumin. A small but repeatable delay of 6 to 10 ml occurred when the activated material was chromatographed. The elution of pX was not affected when Ca^{2+} or benzamidine-HCl was added to the sample of factor X, before chromatography, in amounts similar to those present during trypsin activation. The delay observed with the activated material was indicative of a small change of molecular size during the activation process, and therefore it was thought that chromatography on Sephadex G-100 might result in a better resolution.

(*b*) SEPHADEX G-100.—Fractions containing pX, X^{Tr}, X^R, or X^S were chromatographed individually on columns of Sephadex G-100. The conditions of chromatography as described in Methods were the same in all cases. The results are presented in Figure 5B. The difference in the elution pattern between the activated and nonactivated forms of factor X was now more pronounced on G-100 than on Sephadex G-200. Although X^{Tr} and X^R each showed similar elution patterns (distribution coefficients approximately 0.29), they were delayed considerably in relation to pX or X^S (distribution coefficient, 0.13). Figure 5A presents the elution pattern of $BaSO_4$-treated bovine plasma proteins, and Figure 5C presents the resolution obtained when hemoglobin, cytochrome *c*, and inorganic phosphate were passed through the same column. These latter components were used as "markers" for a better evaluation of the behavior of factor X on Sephadex G-100. It can be seen that the "delay" produced during the activation of factor X represents a difference similar to that between plasma albumin (distribution coefficient 0 19) and hemoglobin (distribution coefficient 0.31).

(*c*) EFFECT OF Ca^{2+} ON THE PROPERTIES OF ACTIVATED FACTOR X.—A remarkable change in the properties of activated factor X was observed when 0.005 M $CaCl_2$ was included in the eluting buffer during chromatography. Figure 5D shows the elution pattern of factor X activity in the presence of Ca^{2+} (distribution coefficient 0.59). The same change in elution behavior was observed with X^{Tr}, X^R, or X^S, but not when pX was used under the same conditions. This apparent delay in the elution of factor X activity observed in the presence of Ca^{2+} resembles the elution characteristics of cytochrome *c*, (distribution coefficient 0.64; Fig. 5C) indicating a considerable change in molecular size. The reversibility of this change was tested by rechromatography of the material eluted in 5D with a distribution coefficient of 0.59 in the absence of Ca^{2+}. Under these conditions, the elution of factor X activity was the same as that indicated in Figure 5A for X^{Tr}, indicating a reversal to the original molecular size.

(*d*) ELECTROPHORESIS OF FACTOR X BEFORE AND AFTER ACTIVATION.—Although the activation of factor X had only a small effect on its molecular size in the absence of Ca^{2+} (Fig. 4 and 5), a much more profound influence was noted in the electrophoretic properties of the molecule. Figure 6 shows the electrophoretic pattern of purified factor X preparations before and after activation. It can be seen that the nonactivated material obtained after Sephadex chromatography showed a single peak migrating close to α_1 globulins (Fig. 6B). The same material after activation by trypsin and chromatography on Sephadex G-200 (Tris-maleate buffer, no added Ca^{2+}) showed a single electrophoretic peak exhibiting a drastically reduced negative charge and moving parallel to the γ-globulins (Fig. 6C). The Russell's viper venom–activated factor X showed two major peaks, one with a negative charge similar to that of the nonactivated material and another showing the charge comparable to that of the trypsin-activated material (Fig. 6D). The spontaneously activated material showed two peaks, both negatively charged.

(*e*) BINDING PROPERTIES OF FACTOR X.—The ability of the different species of factor X to form complexes with the lipid materials taking part in the blood coagulation system was tested as follows: Factor X preparations were mixed with isolated phospholipids (equimolar mixture of PS and PC) or brain saline extracts, both in the presence or absence of Ca^{2+}. The mixtures were incubated for 4 minutes at 37° and then chromatographed on Sephadex G-200 as described. The results are summarized in Figure 7. When factor X, activated or not, was chromatographed alone, the peak of activity was recovered in effluent volume of 62 ml (Fig. 7A, dotted line). When the chromatography was preceded by a short incubation of factor X (again activated or not) with a mixture of emulsified phospholipids (50 μg/ml) without added Ca^{2+}, the factor X activity was recovered again as shown in Figure 7A (effluent volume, 62 ml) the phospholipids having been eluted earlier as indicated by the recovery of all organic phosphorus material (dashed line, effluent volume, 28 ml).

The presence of Ca^{2+} (0.005 M) was found to change the elution pattern of the above mixture. This effect is demonstrated in Figure 7B, which shows that activated factor X activity was recovered in the fractions containing the organic phosphorus. Significantly, this "complexing" between factor X and phospholipids was observed only with activated factor X, and then only when $CaCl_2$ was also present in the eluting buffer. When Ca^{2+} was not present in the eluting buffer, even

if present in the incubation mixture, the two components were eluted separately, as shown in Figure 7A, indicating reversibility of the complex upon removal of Ca^{2+} during chromatography. Plasma factor X (pX) when mixed with phospholipids even in the presence of Ca^{2+} gave the same elution pattern as in Figure 7A.

When a brain tissue (saline) extract was chromatographed on Sephadex G-200, the results shown in Figure 7C were obtained. Essentially, three major components were isolated, the first of which contained organic phosphorus, protein, and "tissue thromboplastin" activity, as measured by the one-stage Quick prothrombin time test. The second peak contained protein and the third contained organic phosphorus, with no "tissue thromboplastin" activity in either of these two fractions.

The addition of X^{Tr}, without added Ca^{2+}, to the tissue extract produced little change in the elution pattern with factor X activity eluted at an effluent volume of 62 ml. However, when Ca^{2+} was included in the incubation mixture and in the buffer during chromatography, the two activities (brain tissue thromboplastin and factor X activity) were eluted together as shown in Figure 7B (peak at effluent volume of 28 ml).

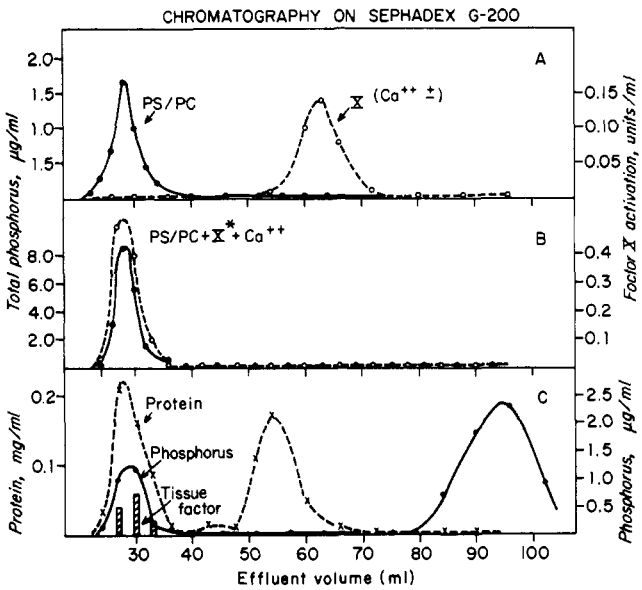

FIG. 7.—Chromatography of nonactivated and activated factor X on Sephadex G-200 in the presence of lipids. The elution was performed with buffer containing 0.04 M Tris/maleate, *p*H 7.0, 0.14 M NaCl, and, in certain instances, 0.005 M $CaCl_2$. Total column volume, 100 ml. *A*, mixture of equimolar amounts of phosphatidyl serine (PS) and phosphatidyl choline (PC), ●——●, and purified, nonactivated factor X (pX), in presence and absence of Ca^{2+} (pX alone is eluted at the same effluent volume of 62 ml), O---O; *B*, mixture of equimolar amounts of PS and PC, ●——●, and trypsin-activated factor X (Xtr), O---O; in the presence of Ca^{2+} (in absence of Ca^{2+}, the elution pattern was similar to that obtained in *A*); *C*, elution pattern of brain saline extract: total protein, ×---×; total phosphorus, ●——●; tissue factor ("thromboplastin" activity), shaded bars.

Discussion

In the present study, factor X of bovine plasma has been isolated in a highly purified form through a combination of $BaSO_4$ treatment and DEAE-cellulose chromatography. An approximately 10,000-fold purification was achieved from a starting level of 3.6×10^{-3} units of factor X per mg protein to a final value of 40 units of factor X per mg protein. This purification procedure removes all of the other known clotting activities from factor X. Of interest is the fact that prothrombin and factor VII, present in the crude plasma samples, were isolated in the same fraction on DEAE-cellulose chromatography but could be separated from each other by rechromatography on DEAE-cellulose. Factor X activity was quite stable for several months either in the lyophilized state at −25° or frozen in solution. Upon storage of the frozen sample, a "spontaneous" activation occurred, but the origin of this reaction is not known. Certain features of this spontaneous activation are discussed below.

It is now apparent that factor X activity as isolated from plasma can be converted to a more active or activated form. This activation of factor X seems to be an important step in the series of reactions leading to the activation of prothrombin. Although the physiological mechanism of its activation is not known yet, it is probably in line with the present concepts of the mechanism of consecutive activations as suggested by Ratnoff and Davie (1962) and by Macfarlane (1964). In the present report, the activation of factor X was accomplished with the use of Russell's viper venom and trypsin, reagents not involved in the physiological process of blood coagulation. The activation by these reagents was compared, and the molecular changes produced during the activation were studied.

The activation of factor X by Russell's viper venom has been well documented as an enzymatic attack on the factor X molecule by a proteolytic (or esterolytic) enzyme (Esnouf and Williams, 1962). The ability of trypsin to activate factor X has been observed mainly by Ferguson's (1960, 1963) and Alexander's (1958, 1962) groups, who noted a stimulatory effect of factor VII in this system. Recently Yin (1964) reported on the activation of purified human factor X by trypsin and concluded that there was an enzyme-substrate relationship between trypsin and factor X. In this latter work Yin indicated that factor VII was not required for the activation of factor X by trypsin. The preparations of nonactivated factor X used in this current study contained less than 1% of factor VII. Consequently, it will be assumed for lack of evidence to the contrary that trypsin attacks factor X directly. The comparative effects of trypsin and Russell's viper venom on the activation of factor X are illustrated in Figures 2 and 3. Activation by trypsin was seen to proceed easily at 0° and was accompanied by simultaneous inactivation, presumably as a result of extensive proteolytic action on the factor X molecule. The product obtained by activation with Russell's viper venom was considerably more stable in the presence of its activator. Nevertheless, both activations tended to result in changes that involve similar "functional" site(s) of the factor X molecule. This latter point is illustrated in Figure 3, where activation by one of the enzymes progressively reduced the potential for further activation by the other.

The activation of factor X probably results in a change in molecular size or shape, as suggested by results given in Figure 4. In their investigations on the use of Sephadex G-200 for the fractionation of plasma proteins, Flodin and Killander (1962) reported the separation of three groups of proteins according to molecular size. A typical separation of bovine plasma is shown for comparison in Figure 4A. As shown in Figure 4B, factor X was eluted slightly earlier than the albumin peak, indicating a somewhat higher molecular weight. This observation would agree reasonably well with that of Esnouf and Williams, who proposed a molecular weight of 80,000. A change in molecular weight or size of factor X on activation

was demonstrated more convincingly through the use of chromatography on Sephadex G-100 (Fig. 5). In these experiments, the nonactivated and activated forms were separated completely, with the activated material traveling slower than albumin. The relative importance of the effect of simple exclusion and the effect of steric and frictional interaction for Sephadex G-100 and G-200 has been studied recently by Ackers (1964). It is reasonable to conclude that partial proteolysis of plasma factor X on activation has altered its elution properties on Sephadex G-100 and has yielded a factor X molecule with smaller molecular weight or a reduced frictional coefficient (less oblong shape). The functional result of such a change would be that an "active site" of the factor X molecule is now available for further reactions with other coagulation factors.

Activation by 25% sodium citrate has been described by Seegers *et al.* (1950) as a means by which purified prothrombin could be activated to thrombin without the addition of any of the known biological activators. Interestingly, Alexander (1958) and Streuli (1959) have reported that prothrombin separated from certain contaminating clotting factors could not be converted to thrombin by 25% citrate. On the other hand Marciniak and Seegers (1962) have recently shown that the 25% citrate activation of prothrombin produces another component (autoprothrombin C), as well as thrombin. Spaet and Cintron (1963) have described the activation of their purified factor X from rabbit serum which contained factor VII by 25% citrate and reached the conclusion that the product was similar to the product of Russell's viper venom activation or to the autoprothrombin C. The experimental results obtained in the present study (Table II) show that purified factor X was not activated by 25% citrate. It was activated, however, when a small amount of activated factor X or "purified" prothrombin was mixed with it prior to treatment with citrate. Prothrombin alone was only slightly activated not only to thrombin but to "factor X" as well. This activation was increased somewhat in the presence of small amounts of thrombin and considerably more in the presence of plasma factor X. A much higher level of activation was observed when a crude plasma $BaSO_4$ eluate was employed. Thus, it appears that purified factor X is not activated by 25% citrate but rather that the prothrombin molecule, which is augmented by the presence of some accessory factors from plasma, is the susceptible component. Factor X probably is one of the accessory factors. It is worth noting in this respect that the further activation of plasma factor X in the presence of previously activated factor X was also observed in the absence of citrate (Table 1).

The pronounced effect of calcium ions on the molecular size of activated factor X (Fig. 5D) may be closely related to the mechanism of the participation of factor X in blood coagulation. This change of the elution pattern on Sephadex could be due either to a change of the shape of the molecule to a more compact form, or simply to a decrease in the molecular weight, or both. If a decrease in molecular weight is the case, the observed change could represent the disaggregation of the activated factor X molecule into four or more subunits (Ackers, 1964).

Electrophoretic studies with factor X have been reported earlier (Fisch, 1959; Straub, 1960; Denson, 1958). In these studies factor X was characterized as a protein traveling close to the α-globulins. Esnouf and Williams (1962) reported that purified factor X from bovine plasma migrated as a single peak, with a net negative charge at *p*H values of 6.15 and 7.35. The same material, after activation by Russell's viper venom, showed an additional peak whose relative position was not identified. Our present observations show that purified factor X migrated as a single band similar in mobility to the α_1 globulins of whole human serum (Fig. 6). Upon activation by Russell's viper venom, or more clearly upon activation by trypsin, a definite change in the electrophoretic mobility of the main protein peak was observed. The major portion of the protein in the activated preparations migrated parallel to the γ-globulins, indicating a sharp decrease in the net negative charge on the molecule as compared to the nonactivated material. It would be reasonable to speculate that this alteration in charge was the result of a proteolytic attack by either trypsin or Russell's viper venom, which removed a negatively charged peptide(s) from the original molecule.

The affinity of activated factor X for phospholipids or tissue lipoproteins, as shown in Figure 7, could very possibly be connected with the alteration of the charge of factor X molecule, as reported above. The affinity of factor X for the lipid materials involved in blood coagulation has been studied earlier by a number of investigators, exclusively by centrifugation (Flyn and Coon, 1953; Straub and Duckert, 1961; Williams, 1964; Bergsagel and Hougie, 1956; Spaet and Cintron, 1963). In the present study, the affinity of factor X before and after activation, with or without calcium, was studied through the use of gel filtration. The formation of a stable complex was observed only when factor X was activated (by either trypsin, Russell's viper venom or "spontaneously") and then only in the presence of Ca^{2+}. Both the phospholipid emulsion used in this study, which carries a negative surface charge (Papahadjopoulos *et al.*, 1962), and tissue extract, which presumably contains a lipoprotein structure, behaved in a similar way insofar as the complexing ability toward activated factor X is concerned. The present studies also showed that the removal of Ca^{2+} separates the factor X lipid complex to one component containing the lipid and another containing the factor X activity.

The knowledge gained in these experiments on the molecular changes occurring during factor X activation aid to a considerable extent our understanding of the mechanism of the interaction of factor X with lipids. The change of the electrical charge carried by factor X molecule from negative to neutral or positive facilitates the interaction of the activated species with the negatively charged phospholipid particles. Nevertheless, the reduction of the charge could not be the only prerequisite for the interactions, since the spontaneously activated factor X, which still has a negative charge, can interact. The necessity for Ca^{2+} during the interaction can be integrated with the change of molecular size of factor X occurring in the presence of the metal ions. It is possible in this respect that in the presence of Ca^{2+} activated factor X breaks down to subunits and some new sites in the interior of the original factor X molecule are then exposed. Consequently, these new sites could exhibit an affinity for the negatively charged phospholipid particles. These sites could be the hydrophobic side-chains of amino acids such as valine or isoleucine or, in general, groups that possess some affinity for the phospholipid interphase.

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Specific Inhibitors and the Chemistry of Fibrin Polymerization

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Received July 16, 1964; revised September 22, 1964

Our earlier proposal that the polymerization of fibrin occurs by a transpeptidating (synonymously called transamidating) mechanism predicts the existence of two types of specific inhibitors of the reaction. Amine compounds would compete with the donor end-amino (glycyl) residues of the attacking fibrin, whereas compounds with carbonylamide functions would compete with the acceptor groups of the attacked fibrin particle. Specific inhibitors of both varieties were shown to exist. The former can be typified by glycine ethyl ester, the latter by carbobenzoxy-L-asparagine amide. Glycine ethyl ester was actually shown to be incorporated into fibrin if present during the polymerization reaction. Chemical and biological implications of the selective inhibitors of fibrin polymerization are discussed.

The terminal phase of blood clotting in vertebrates consists of the following major steps which, in essence, represent the interaction of three blood proteins: fibrinogen, thrombin, and the fibrin-stabilizing factor (FSF).

$$\text{fibrinogen} \xrightarrow{\text{thrombin}} \text{fibrin} + \text{fibrinopeptide}^1 \quad (1)$$

$$n\ \text{fibrin} \underset{\text{e.g., 30\% urea}}{\overset{Ca^{2+}}{\rightleftharpoons}} \text{fibrin aggregates} \quad (2)$$

$$\text{FSF} \xrightarrow[Ca^{2+}]{\text{thrombin}} \text{FSF}^* \quad (3)$$

$$n\ \text{fibrin (or fibrin aggregates)} \xrightarrow[Ca^{2+}]{\text{FSF}^*} \text{fibrin polymer} \quad (4)$$

Step 1 describes the limited proteolysis of fibrinogen by thrombin (Lorand, 1951, 1952). The removal of fibrinopeptide[1] from the parent protein drastically alters the solubility of the product (fibrin) so that at neutral *p*H ($\mu \sim 0.15$; 20°) the latter will aggregate into a gel (step 2). This gel, however, can be reversibly dispersed (Lorand, 1948, 1950; Laki and Lorand, 1948; Mihalyi, 1950; Lorand and Middlebrook, 1952; Donnelly *et al.*, 1955) in a number of solvents such as 30% urea, 1% monochloroacetic acid, or 10% sodium bromide. In contrast, the gel produced during the

[1] After the characterization of fibrinopeptide (Lorand, 1951, 1952) another peptide, called peptide B or fibrinopeptide B, was recovered from the clot liquor (Bettelheim and Bailey, 1952). It is, however, released at a much slower rate, and its removal from the protein is definitely not a prerequisite for the reversible gelation of fibrin in step 2 (Blomback and Vestermark, 1958). It is not known whether fibrinopeptide B must be released from fibrin prior to the polymerization reaction in step 4.