

Activation of a Proteolytic System by a Membrane Lipoprotein: Mechanism of Action of Tissue Factor

(blood coagulation/serine protease/thromboplastin/factor VII)

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ABSTRACT One pathway of blood coagulation, the extrinsic system, is initiated by a specific interaction between tissue factor, which is a membrane lipoprotein, and factor VII, one of the plasma coagulation factors. Factor VII was prepared from bovine plasma by adsorption onto and elution from BaSO₄. The eluate was chromatographed on DEAE-Sephadex and purified by preparative disc-gel electrophoresis. Factor VII complexed with purified bovine-brain tissue factor and, when eluted from the complex, factor VII had a greater mobility in acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate, i.e., it had a reduced molecular weight. Factor VII was also cleaved in the presence of orthophenanthroline, an inhibitor of the peptidase activity of tissue factor. Prior treatment of factor VII with diisopropylphosphorofluoridate, however, completely blocked its cleavage and the development of coagulant activity, although factor VII treated with diisopropylphosphorofluoridate complexed equally well as the native protein with tissue factor. Factor VII in whole bovine plasma was also inhibited by the drug. Factor VII labeled with [³²P]diisopropylphosphorofluoridate and radioautographed after electrophoresis in gels showed two major components, only one of which was labeled. We conclude that tissue factor initiates blood coagulation by facilitating a proteolytic attack within the factor VII "complex." The most likely mechanism is proteolysis of one form of factor VII by the diisopropylphosphorofluoridate-sensitive enzyme, although additional intramolecular proteolysis may be involved.

The addition of small amounts of various tissues to blood markedly enhances the rate at which it coagulates. This acceleration is due to an interaction of tissue factor, a specific lipoprotein present in many tissues, and factor VII, a plasma coagulation factor. The protein component of tissue factor was previously purified by conventional techniques after solubilization in deoxycholate (1). In this communication we report the purification of bovine factor VII and preliminary observations on its reaction with tissue factor.

We show that the factor VII preparation contains two major molecular species, one of which is sensitive to diisopropylphosphorofluoridate (DFP). During its reaction with tissue factor, factor VII is converted to a lower molecular weight species, i.e., it is hydrolyzed. The data indicate that the protein that catalyzes this proteolysis is a form of factor VII. Further, we show that the peptidase activity associated with tissue factor (2) is not required for its coagulant action.

MATERIALS AND METHODS

Sodium dodecyl sulfate (SDS) and Tris ("Trizma base") were obtained from Sigma, St. Louis, Mo. DFP and sodium

Abbreviations: DFP, diisopropylphosphorofluoridate; OP, orthophenanthroline (1, 10-phenanthroline); SDS, sodium dodecyl sulfate.

deoxycholate were purchased from Schwarz/Mann, Orangeburg, N.Y. [³²P]DFP was purchased from Amersham/Searle, Arlington Heights, Ill. Orthophenanthroline was obtained from Matheson, Colman and Bell, Norwood, Ohio.

Factor VII. 50 Liters of bovine blood were collected into 1/10 volume of 0.1 M sodium oxalate. Plasma was separated by rapid passage (180 liters/hr) through a continuous flow centrifuge (Westphalia, no. SAOOH205). Barium sulfate (x-ray grade, 50 mg/ml) was added, and after 30 min of stirring the BaSO₄ was collected by continuous centrifugation and washed out of the centrifuge in about 5 liters of 0.45% NaCl in 1 mM sodium citrate. A small amount of 1-octanol was added to prevent foaming. Adsorbed proteins were eluted by stirring in 2 liters of 0.1 M sodium citrate (pH 6.8) for 30 min. The barium sulfate was removed by centrifugation; the supernatant was diluted with 500 ml of water and 500 ml of 15 mM Tris·HCl (pH 7.5). 7 g of dry DEAE-Sephadex A-50 was added, and the mixture was stirred for 20 min. The Sephadex was then collected on a Büchner funnel and washed with 400-ml quantities of 15 mM Tris·HCl, 3 times; 100 mM Tris·HCl, 3 times; and finally 3 times with 0.14 M NaCl in 100 mM Tris·HCl. The slurry was added to a column 5 cm in diameter containing 15 g of DEAE-Sephadex equilibrated with the final buffer. The proteins were eluted with a linear 4-liter gradient of NaCl (0.14–0.45 M) in Tris·HCl buffer (100 mM, pH 7.5) at a flow rate of 120 ml/hr; 25-ml fractions were collected. The fractions containing factor VII were pooled, concentrated by ultrafiltration (Diaflo membrane UM-10), adjusted to pH 6.5 with 0.5 M HCl, and frozen in 100-mg aliquots. For preparative disc-gel electrophoresis, an aliquot was dialyzed into the reservoir buffer (standard system of Davis) (3). The separating gel was 6 cm in height (Canalco gel tube no. PD2/320) and was 6.25% in acrylamide and 0.156% in *N,N'*-methylenebisacrylamide. A 1.5-cm stacking gel (pH 6.8) was cast above the separating gel, and the protein (100 mg in about 10 ml of buffer, 10% sucrose) was layered beneath the buffer. A current of 8 mA was applied, and the gel was eluted at the rate of 100 ml/hr. 4-ml Fractions were collected. The fractions containing factor VII were pooled, concentrated, adjusted to pH 6.0, and frozen.

Tissue Factor. The apoprotein was prepared and purified from bovine brain as described (1). Relipidation was performed with mixed brain lipids at a ratio of 7.5 mg of lipid/mg of protein.

Assays of Coagulation Factors. Charcoal-adsorbed plasma deficient in factors VII and X (Diagnostic Reagents, Ltd., Thame, Oxon, England) was reconstituted with purified

factor X (200 units/ml) (4), thus making factor VII rate-limiting when coagulation was initiated with bovine-brain thromboplastin (crude tissue factor) (5). The assay was performed at 37° by mixing the reconstituted charcoal-adsorbed plasma (0.2 ml) with 0.1 ml of the test material. Coagulation was initiated with 0.2 ml of bovine-thromboplastin containing 25 mM CaCl₂, and the clotting time was determined. The assay was unaffected by factor X, V, or prothrombin, and was thus specific for factor VII. Prothrombin was estimated by the technique of Ware and Seegers (6) and with tiger snake venom (7). Factor X was assayed with Russell's viper venom (8), and factor IX by the method of Biggs and Macfarlane (9). Bovine plasma was used as a standard for all assays and was defined as having 100 units of each factor per ml.

Chemical Methods. Protein was estimated by the method of Lowry *et al.* (10), with bovine-serum albumin as a standard. In the presence of lipid, which interferes with the determination, the samples were diluted in 1% SDS containing 1% Na₂CO₃ and extracted twice with equal volumes of CHCl₃-CH₃OH 2:1. The standards were treated in a similar fashion.

DFP. A stock solution of 0.5 M DFP was made in dry isopropanol. A maximum of 1/100 volume of this was added to the enzyme solutions where indicated. Control experiments containing the same amount of isopropanol were always performed. [³²P]DFP (carrier-free) was dissolved in dry isopropanol to a final concentration of 2 mCi/ml. 2 μCi were used to label factor VII.

RESULTS

The elution pattern obtained from DEAE-Sephadex is illustrated in the upper panel of Fig. 1. Factor VII was found in the descending limb of the peak containing mainly prothrombin. The fractions between the arrows were pooled for preparative disc-gel electrophoresis. The lower panel of Fig. 1 depicts the elution from the disc gel. The insert shows analytical disc gels of the material eluted from DEAE-Sephadex and the preparative disc gel. The disc gel shows factor VII to be free of contaminating proteins. When assayed at 1.0 mg/ml, no prothrombin or factor X was detectable. Two units of factor IX were present per 100 units of factor VII. The specific activity of factor VII was 2500–3000 units/mg, and it was thus about 2000-fold purified. About 15 mg was obtained from each preparative disc gel. The final yield varied from 7 to 12%. The purification is summarized in Table 1.

Formation of the Tissue Factor-Factor Complex. It was previously shown that, in the presence of calcium ions, tissue factor and factor VII formed a complex (11). These experiments were performed with crude, particulate preparations of tissue factor and it is not clear, therefore, whether factor VII was binding to tissue factor itself or to some other component of the tissue particles. Accordingly, purified tissue factor (125 μg of protein) was incubated with factor VII (172 μg) and CaCl₂ (5 mM) in a volume of 0.5 ml. After 30 min at 37°, the mixture was centrifuged at 59,000 × *g* (average) for 30 min. The pellet was taken up in 0.5 ml of 2% SDS and boiled for 2 min. An aliquot was taken for protein determination, and an equal volume of 10 M urea was added to the remainder. The material was analyzed by SDS-polyacrylamide gel electrophoresis (12). Unreacted factor VII and the pellet obtained from the incubation mixture are illustrated

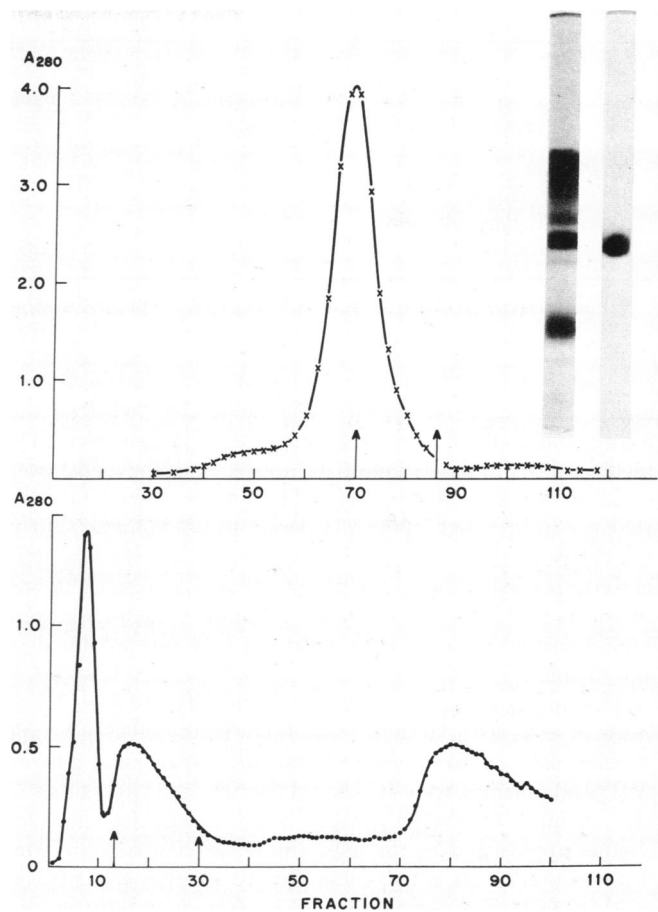


FIG. 1. DEAE-Sephadex chromatography (*upper panel*) and preparative disc-gel electrophoresis (*lower panel*) of factor VII. The material between the *arrows* in each figure contained the bulk of the factor VII. The analytical gels (*insert*) were 7% in acrylamide; on the *left* is shown the proteins eluted from the DEAE-Sephadex and on the *right* is the material eluted from the preparative gel.

in gels 1 and 2, Fig. 2; factor VII bound to the tissue factor, and the factor VII eluted from the pellet is of lower molecular weight. The coagulant activity of the pellet and supernatant was determined after centrifugation. Over 99% of the activity was in the pellet. For this determination, the assay for factor VII without addition of tissue factor was used. It is clear, therefore, that little, if any, coagulant activity is released from tissue factor.

Mechanism of Hydrolysis of Factor VII. Tissue factor was previously reported to be associated with aminopeptidase and endopeptidase activity (2), both of which are completely inhibited by orthophenanthroline (OP). The coagulant activity, however, is not inhibited. The tissue factor-factor VII reaction was allowed to proceed in the presence of 5 mM OP; full coagulant activity developed. The pellet was analyzed as above; the results (gel 3, Fig. 2) are identical to those obtained in the absence of OP. Thus, the peptidase activity of tissue factor cannot catalyze the hydrolysis of factor VII.

The product of the tissue factor-factor VII reaction was previously shown to be DFP-sensitive (11). Williams also reported factor VII alone to be inhibited by DFP (13). Accordingly, factor VII [in Tris·HCl (pH 7.5) 0.1 M] was in-

TABLE 1. Purification of factor VII

Stage	Protein (mg)	Factor VII (units/mg)	Factor VII (total units $\times 10^{-4}$)	Purification	Yield (%)
Plasma	14×10^6	1.4	200	1	100
BaSO ₄ eluate	2.3×10^3	232	5.4	166	27
DEAE-Sephadex eluate	278	928	3.5	663	17.6
Preparative disc*	62	2850	1.7	2036	8.8

* Four preparative disc gel runs were required to process all the material obtained from DEAE-Sephadex chromatography.

incubated with 5 mM DFP for 1 hr at 37° and dialyzed overnight. No coagulant activity was detectable, whereas the isopropanol control was 78% active. The protein was then incubated with tissue factor as above, and the pellet was examined in a similar manner. The results (gel 4) indicate that the inactivated factor VII bound to tissue factor, but it is evident that no hydrolysis occurred. Thus, the catalytic activity resides in the factor VII preparation and not in tissue factor. It is of interest that similar amounts of factor VII were bound to tissue factor whether factor VII was inhibited or not (Table 2).

The time course of hydrolysis of factor VII and the appearance of coagulant activity was followed by SDS gels and specific assays. For these experiments, tissue factor, factor VII, and calcium were incubated for the indicated times at 37°. Aliquots were withdrawn for assay of coagulant activity. Before application to the gels, the reaction was terminated by addition of an equal volume of 10 M urea-2% SDS-10 mM EDTA. The appearance of coagulant activity accompanied the appearance of the cleaved factor VII (Fig. 3).

The rate of this reaction was considerably slower than previously reported (11). In the present experiments, concentrated reagents were used in order to make the product visible on gels. For assay, the reaction mixture was diluted 1:500. When the reagents were diluted to a similar concentration before the reaction and assayed without further dilution, maximal coagulant activity developed within 30 sec. The product, however, was too dilute for analysis by SDS-gel electrophoresis.

DFP Sensitivity of Factor VII. The observed inhibition of factor VII by DFP suggested that the protein was altered to

a DFP-sensitive form during purification. Alternatively, factor VII may circulate in bovine plasma as a DFP-sensitive protein. To test this possibility, DFP (5 mM) was added to fresh plasma collected and processed in cellulose nitrate tubes to prevent glass activation of the clotting mechanism. At intervals, aliquots were withdrawn and coagulation was initiated by addition of tissue factor and calcium. The clotting times progressively lengthened (Fig. 4). At the termination of the experiment, assays were performed for factors VII, X, V, and prothrombin, all the proteins involved in the tissue factor pathway. Factor VII activity was markedly reduced, whereas the activity of the other factors was unaffected (Table 3). Thus, factor VII circulates in bovine blood as a DFP-sensitive protein.

In one experiment, [³²P]DFP was used to label factor VII. The product was subjected to mild acid hydrolysis (2 N HCl, 105°, 8 hr), after which it was passed over a 60-cm column containing Beckman UR 30 resin in 0.2 M citrate (pH 3.28). The radioactivity emerged near the void volume. The radioactive material was then hydrolyzed in 6 N HCl at 105° for 18 hr. The amino acid content of this material was Ser (4 μmol), Asx (1.5 μmol), Gly (2.0 μmol), Glx (1.6 μmol), Ala (0.6 μmol), and Thr (0.5 μmol). These data are consistent with the presence of at least one phosphoserine-containing peptide, since a peptide retaining a net negative charge would elute in this position at pH 3.28.

Heterogeneity of Factor VII. Although factor VII appears homogeneous on acrylamide-gel electrophoresis and on urea-acetic acid gels (not shown), at least three bands are present on SDS gels (see gel 1, Fig. 2). The major protein is apparently factor VII, as it is converted to a lower molecular weight

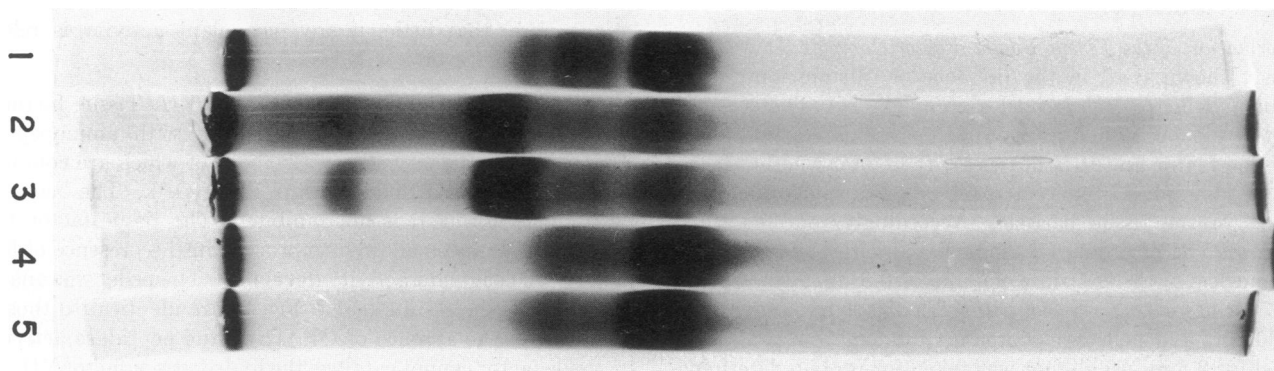


FIG. 2. SDS-acrylamide gel of pellets obtained from the tissue factor-factor VII reaction (1) Native factor VII; (2) factor VII plus tissue factor (the complete system); (3) complete system plus orthophenanthroline; (4) complete system in which factor VII had been previously treated with DFP; (5) as in gel 4 plus orthophenanthroline. These are alkaline gels, 7% in acrylamide and 5 M in urea. Under these conditions (nonreduced), the tissue factor protein does not enter the gels. 65-80 μg of protein was applied to each gel.

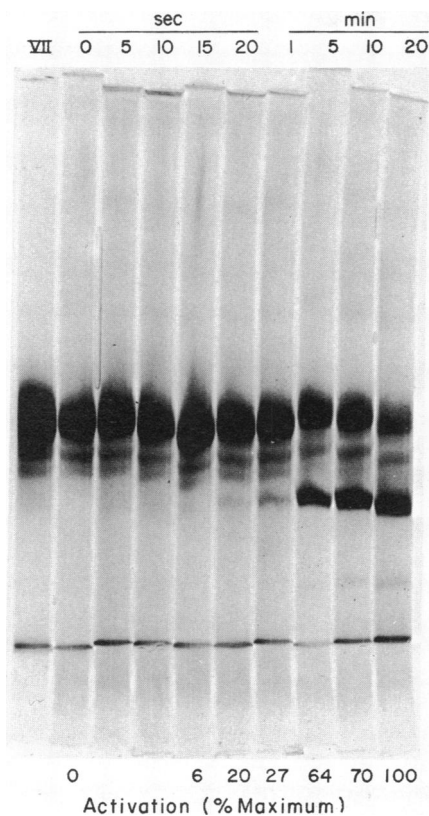


FIG. 3. Time course of the tissue factor-factor VII reaction. Conditions and gel system are the same as for Fig. 2 (complete system). To stop the reaction for assay of coagulant activity, aliquots were withdrawn at the indicated times and diluted 1:500 in 0.38% sodium citrate. For this experiment, maximal activity was defined as the activity generated at 20 min. 75 μ g of protein was applied to each gel.

species in parallel with the appearance of coagulant activity (Fig. 3). To determine whether all the protein reacted with DFP, factor VII was incubated with [³²P]DFP and then applied to SDS gels. The gel was stained, dried to a thin film, and applied to x-ray film. The radioautogram is shown in Fig. 5; only one of the protein bands was labeled by DFP. Thus, factor VII exhibits enzymatic, as well as physical, heterogeneity.† The neutral pH gel system of Weber and Osborn (14) was used for this experiment, as the alkaline system used above resulted in marked loss of radioactivity from both factor VII and trypsin.

DISCUSSION

Tissue factor is a component of the plasma membranes of endothelial and other cells (15); thus, a detailed knowledge of the mechanism of activation of the tissue factor pathway is critical for understanding hemostasis and thrombosis. Preparation of large amounts of factor VII from bovine plasma has allowed an analysis of the primary events of this system. Factor VII had greater mobility on SDS gels after its reaction with tissue factor, almost certainly as a result of proteolysis.

† These results cannot be explained by a subunit hypothesis, as molecular weight determined in an ultracentrifuge showed no change when guanidine and 2-mercaptoethanol were added.

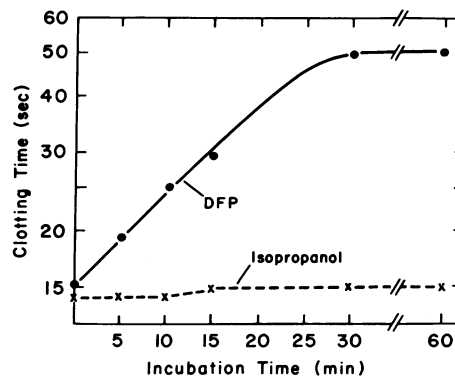


FIG. 4. Inhibition of the tissue factor pathway by DFP. Plasma was made 5 mM in DFP; at intervals, aliquots were withdrawn and coagulation was initiated with crude brain tissue factor and calcium. The clotting times are plotted on a logarithmic scale. Controls for this experiment included isopropanol, 5 mM HCl, and 5 mM NaF, none of which affected the clotting times.

Tissue factor apparently acts by facilitating this proteolysis, but does not catalyze it, as inhibition of the peptidase activity of tissue factor with orthophenanthroline inhibits neither the proteolysis nor the development of coagulant activity.

The factor VII preparation was heterogeneous by SDS-gel electrophoresis. Although it is possible that the preparation is contaminated with extraneous proteins, it is likely that the main components are factor VII. In favor of this view is: (1) there is virtually no contamination with other proteins of the coagulation system; (2) during the reaction with tissue factor, the major protein is hydrolyzed at a rate that parallels the appearance of coagulant activity; and (3) almost all the protein binds to tissue factor. These findings suggest, but do not prove, that factor VII is proteolytically activated; definitive proof of this must await isolation of a coagulant-active fragment of factor VII. It must be acknowledged, however, that even if one of the major components of the preparation is an extraneous protein, tissue factor induces proteolysis within the isolated plasma proteins.

The finding that purified factor VII is DFP-sensitive confirms and extends Williams' observation based on less-pure material (13). DFP-sensitivity is not due to an artifact of purification, as factor VII in fresh bovine plasma is also inhibited by DFP (Table 3, Fig. 4). The enzymatic heterogeneity of the factor VII preparation is indicated in Fig. 5; only one component has bound radioactive DFP.

The presence of two molecular species, both of which participate in the reaction with tissue factor, suggests this reaction involves two steps:

TABLE 2. Binding of factor VII to tissue factor

System	Factor VII (μ g)*		Recovery (%)
	Pellet	Supernatant	
Complete	103	60	94
+ OP	97	75	99
+ DFP	108	60	97
+ OP + DFP	100	69	98

* The quantity of tissue factor protein in the pellet (52 μ g) and in the supernatant (75 μ g) was determined independently and was subtracted from the total protein to give the values for factor VII.

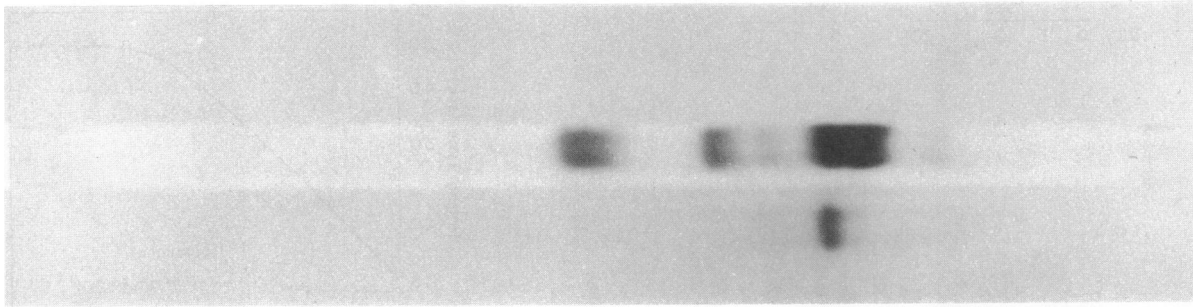


FIG. 5. Radioautogram of factor VII. On the top is the stained SDS gel (neutral system) of factor VII that had been incubated with [32 P]DFP. The x-ray film to which the gel had been applied for 10 days is shown on the bottom. Only the lefthand portion of the major protein band is radioactive.

Tissue factor + "DFP-sensitive VII" \rightarrow Proteolytic Intermediate [1]

"DFP-insensitive VII" $\xrightarrow{\text{proteolytic intermediate}}$ Coagulant-active enzyme [2]

Factor VII has previously been considered to be a single protein that is involved in the conversion of factor X to its enzymatically active form. The data presented show that there may be two distinct proteins involved in this reaction: the DFP-sensitive protein, apparently a serine protease, and another protein, presumably in a zymogen form. The DFP-sensitive protein may be derived from the zymogen by *in vivo* modification. Further data will be required to determine whether this situation pertains or whether two structurally unrelated proteins are involved. It is clear, however, that the DFP-sensitive component of factor VII is required for initiation of the tissue factor pathway of coagulation, as the addition of DFP to bovine plasma inhibits these reactions.

It was previously reported that crude tissue factor and factor VII form a tight complex possessing coagulant activity (11). This communication extends that observation to pure reagents. Recently, Østerud *et al.*, using human materials, demonstrated the release of some coagulant activity from tissue factor when crude, but not purified, factor VII was used (16). This result may represent a species-specific phenomenon, as we have been unable to detect significant amounts

of coagulant activity released from tissue factor in this study, or in a previous study with relatively crude bovine factor VII (11).

It appears likely, in fact, that there are differences between the bovine and human tissue factor pathways. Human factor VII is not inhibited by DFP (data not shown), but this may be a minor difference, as it has been suggested that DFP inhibits human factor VII when the latter is incubated in 25% sodium citrate (17). Thus, it is possible that human factor VII contains a protein similar to the bovine material, but that requires the presence of high ionic strength to be effectively attacked by DFP.

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TABLE 3. Effect of DFP on the proteins of the tissue factor pathway

Factor	Activity (units/ml)			
	Isopropanol		DFP	
	0 hr	30 hr	0 hr	30 hr
VII	96	74	66	1
Prothrombin	94	78	86	78
V	82	54	92	62
X	102	92	90	100

* 1 ml of plasma is defined as having 100 units of each factor.

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