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Activation of Bovine Factor XII (Hageman Factor) by Plasma Kallikrein†

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ABSTRACT: Bovine factor XII is a single-chain plasma protein that is involved in the early or contact phase of blood coagulation. In the reactions leading to fibrin formation, it is converted to factor XII_a, a serine protease composed of a heavy chain and a light chain held together by a disulfide bond(s). The activation of factor XII is catalyzed by plasma kallikrein which cleaves a single internal arginyl–valine peptide bond in the precursor protein. The activation reaction also requires the presence of a lipid component, such as sulfatide, ganglioside, or stearic acid. Kaolin, in the presence of high molecular weight kininogen, or dextran sulfate can provide a nonphysiological contact surface in this reaction. Other materials from bovine sources, including brain galactoceramide, sphingomyelin, phosphatidylcholine, cephalin, aorta proteoglycan, cornea keratan sulfate, vitreous humor hyaluronic acid, submaxillary mucin, and calf skin collagen, were inactive in the activation of factor XII by kallikrein. Factor XI_a and plasmin also activated factor XII in the presence of sulfatide but were only 40 and 20% as active as kallikrein, respectively. Other clotting enzymes, including factor IX_a, factor X_a, or thrombin, showed no activity in this reaction. The effect of sulfatide on reducing the clotting time of plasma suggests that some lipids, such as sulfatide, may be of importance in the initiation of the coagulation process, while others, such as phospholipids, play an important role in the intermediate phase of blood clotting.

Factor XII (Hageman factor)[1] is a glycoprotein (M_r 74 000) circulating in plasma in a zymogen form. It participates in the early or contact phase of blood coagulation, fibrinolysis, and kinin formation when these reactions are initiated by surface activation [see review by Kaplan (1978)]. Contact activation occurs on a negatively charged surface, such as glass, kaolin, Celite, or ellagic acid (Ratnoff & Rosenblum, 1958; Biggs et al., 1958; Nossel, 1964). Four plasma proteins, including factor XII, factor XI, prekallikrein, and high molecular weight kininogen (HMW kininogen),[2] are involved in these reactions. Present evidence suggests that factor XII is first converted to an active form and this initiates the various reactions leading to blood coagulation, clot lysis, and kinin formation. Recently, a factor XII dependent pathway for the renin–angiotensin system has also been described (Derkx et al., 1979).

The initiation of blood coagulation has been studied mainly with human preparations, and thus far three different types of mechanisms have been proposed: (1) activation of factor XII by limited proteolysis in a fluid phase by proteolytic enzymes such as kallikrein, plasmin, or factor XI_a, (2) activation of factor XII by limited proteolysis by the same enzymes on a negatively charged surface, and (3) activation of factor XII on a negatively charged surface leading to a conformational change in the protein and the formation of enzymatic activity. In the last mechanism, no fragmentation or cleavage of peptide bonds in factor XII is thought to occur.

Studies on the activation of factor XII were initially performed in a fluid phase using kallikrein, plasmin, or trypsin. In this system, the fragmentation of human factor XII into three peptide chains was reported (Kaplan & Austen, 1971; Cochrane et al., 1973; Revak et al., 1974). Later, it was found that the activation of human factor XII by limited proteolysis was far more efficient in the presence of a surface such as kaolin (Liu et al., 1977; Maier et al., 1977; Wiggins et al., 1977; Griffin, 1978). In addition, the surface activation was stimulated by the presence of HMW kininogen. Under these conditions, human factor XII was cleaved at one or two peptide bonds, resulting in the formation of factor XII_a containing three polypeptide chains with molecular weights of 40 000,

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] Abbreviations used: HMW kininogen, high molecular weight kininogen; TAME, N^{α}-(*p*-tosyl)-L-arginine methyl ester.

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28 000, and 12 000 (Revak & Cochrane, 1976) or two polypeptide chains with molecular weights of 48 000–52 000 and 28 000 (Griffin & Cochrane, 1976; Revak et al., 1977).

The activation of factor XII in the absence of proteolysis was suggested by Cochrane et al. (1973) and McMillin et al. (1974). Recently, amidase and factor XI activating activities were reported for human factor XII exposed to ellagic acid–Sepharose (Ratnoff & Saito, 1979a,b). A specific substrate-induced activation of bovine factor XII in the absence of proteolysis has also been observed (Kurachi et al., 1980).

In the present studies, we report the activation of bovine factor XII by plasma kallikrein in the presence of dextran sulfate, sulfatide, or kaolin and HMW kininogen. These data suggest that the activation of factor XII on the surface of glycolipid or sulfatide may be a possible physiological mechanism for contact activation and the initiation of blood coagulation, fibrinolysis, and kinin formation.

Experimental Section

Materials. Bovine factor XII and factor XII_a were prepared according to methods previously described (Fujikawa et al., 1977a,b). The specific clotting activities for factor XII and factor XII_a were 178 and 280 units/mg, respectively. Bovine plasma prekallikrein with a specific clotting activity of 60 units/mg was purified by the method of Heimark & Davie (1979). Kallikrein was prepared by the activation of bovine plasma prekallikrein with a catalytic amount of bovine factor XII_a and had a specific activity of 8.2 μmol of Bz-Pro-Phe-Arg-*p*-nitroanilide hydrolyzed per mg per min at 37 °C. Bovine factor XI, with a specific clotting activity of 280 units/mg, was isolated by the method of Kurachi et al. (1980). Factor XI_a, prepared by activation with bovine factor XII_a in the presence of dextran sulfate, had a specific activity of 17 μmol of TAME hydrolyzed per mg per min at 20 °C (Kurachi et al., 1980). These protein preparations, including factor XII, factor XII_a, prekallikrein, kallikrein, factor XI, and factor XI_a, were found to be homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. However, factor XII was found to be contaminated with 1.8% factor XII_a as determined by TAME esterase activity.

Bovine HMW kininogen was prepared from 2 L of plasma by the method of Komiya et al. (1974) with the following modifications. Prekallikrein was mainly separated from HMW kininogen in an initial step employing batchwise adsorption on 600 mL of DEAE-Sephadex, as developed by Komiya et al. (1974). A small, but significant, amount of prekallikrein, however, always contaminated the HMW kininogen fraction from this step. The HMW kininogen fraction from the DEAE-Sephadex column was dialyzed against 0.1 M Tris-HCl, pH 7.5, containing 0.2 M NH_4Cl. After dialysis, urea was added at a final concentration of 3 M and the volume was adjusted to twice the original volume by the addition of water. This sample was applied to a column containing 250 mL of DEAE-Sephacel which had been equilibrated previously with 3 M urea in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NH_4Cl. HMW kininogen was eluted from this column with a linear gradient composed of 500 mL of 0.05 M Tris-HCl buffer, pH 7.5, containing 3 M urea and 0.1 M NH_4Cl, and 500 mL of 0.2 M acetate buffer, pH 6.2, containing 3 M urea and 0.6 M NH_4Cl. HMW kininogen activity was monitored by its clotting activity by using HMW kininogen deficient plasma. The HMW kininogen from the DEAE-Sephacel column was further purified by CM-Sephadex column chromatography according to the method of Komiya et al. (1974). Finally, the CM-Sephadex fraction was passed through a small column of trasylol–Sepharose to remove the last traces of kallikrein activity. The final preparation of HMW kininogen migrated as a single band on sodium dodecyl sulfate–polyacrylamide gels and contained less than 0.3% prekallikrein and/or kallikrein. The specific clotting activity of HMW kininogen was 11–12 units/mg. Bovine plasminogen was prepared by the method of Deutsch & Mertz (1970), and human urokinase was purchased from Weastern Co., Seattle, WA. Bovine plasmin was prepared by activating plasminogen in the presence of urokinase. The specific activity of the plasmin was 1.73 μmol of TAME hydrolyzed per mg per min at 20 °C under the conditions described under Methods.

Bovine brain sulfatide, bovine submaxillary mucin, whale cartilage keratan sulfate, shark cartilage keratan sulfate, bovine cornea keratan sulfate, and glucose 6-*O*-sulfate (Seikagaku Kogyo Co., Tokyo, Japan) were kindly provided by Drs. S. Hakomori and M. Fukuda, Fred Hutchinson Cancer Research Center, Seattle, WA. Bovine aorta proteoglycan and porcine dermatan sulfate were kindly given by Drs. G. Berenson and B. Radhakrishnamurthy of Louisiana State University, New Orleans, LA. Pure rat skin collagen types I and III were gifts from Dr. P. Bornstein of our department. Crude porcine heparin sulfate was provided by Dr. L.-O. Andersson, Kabi, Stockholm, Sweden. Bovine brain sphingomyelin, bovine brain galactoceramides, bovine brain gangliosides, shark cartilage chondroitin sulfate, heparin sulfate, bovine vitreous humor hyaluronic acid, DL-sphingosine sulfate, stearic acid, rabbit brain cephalin, and dextran (M_r 500 000) were purchased from Sigma Chemical Co., St. Louis, MO. Heparin sulfate was also obtained from Weastern Co., Seattle, WA. Soluble calf skin collagen was purchased from Worthington Biochemical Co., Freehold, NJ. Bovine phosphatidylcholine was obtained from Applied Science Laboratories, State College of Pennsylvania.

Dextran sulfate (M_r ~500 000) was obtained from Pharmacia Fine Chemicals, Piscataway, NY. Low molecular weight dextran sulfate (M_r ~3600) from the Kowa Co., Nagoya, Japan, was generously provided by Dr. U. Hedner in our laboratory. N^{α}-(*p*-Tosyl)-L-arginine [^{3}H]methyl ester hydrochloride was a product of Amersham/Searle, Arlington Heights, IL, and a stock solution of 0.1 mmol/(5 μCi mL) was prepared by dilution with nonlabeled TAME in dimethylformamide. Trasylol (Bayer) was generously provided by Dr. H. Kato, Kyushu University, Fukuoka, Japan. Russell's viper venom inhibitor, purified by the method of Iwanaga et al. (1976), and bovine brain cephalin were kindly provided by Dr. W. Kisiel in our laboratory. Human HMW kininogen deficient, prekallikrein-deficient, and factor XII deficient plasmas were purchased from George King Biomedicals, Salem, NH. Bovine factor XI deficient plasma was kindly given by Dr. G. Kociba of The Ohio State University.

The lipids were dissolved in a solvent of chloroform–methanol (1:1 by volume) and stored at −20 °C. Before use, an aliquot was transferred into a siliconized glass tube and the organic solvent was evaporated under a stream of nitrogen gas. The reaction buffer solution was then added to the dry lipid material, and uniform suspensions were prepared by the aid of a vortex mixer.

Methods. The protein concentrations of the purified bovine plasma proteins were determined by absorbance by employing $E_{280}^{1\%} = 14.2$ for factor XII (Fujikawa et al., 1977a), 10.9 for prekallikrein (Heimark & Davie, 1979), 12.6 for factor XI (Koide et al., 1977), and 7.4 for HMW kininogen (Komiya et al., 1974). The same absorbance values were assumed for the activated proteins.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Weber & Osborn (1969)

using 7.5% gels. Samples (3–5 μg of protein) were incubated at 60 °C for 10–20 min with 2% sodium dodecyl sulfate and 0.1 M phosphate buffer, pH 7.0, in the presence or absence of 5% 2-mercaptoethanol. Electrophoresis was carried out at 8 mA/gel for 4–5 h, and protein bands were stained with 0.25% Coomassie brilliant blue for at least 2 h and destained electrophoretically.

Amino-terminal analysis of factor XII_a was carried out as follows. Three milligrams of factor XII was incubated with 3 μg of kallikrein in the presence of 2 mg of sulfatide in 0.73 mL of 0.1 M Tris-HCl buffer, pH 7.6. After 2-h incubation at 37 °C, the reaction mixture was lyophilized and the sulfatide was removed by two extractions with chloroform (3 mL). The protein was then subjected to amino-terminal sequence analysis in a Beckman Model 890C sequenator. Operation of the instrument and methods employed were the adaptations of Hermodson et al. (1972) of the original procedure of Edman & Begg (1967). Phenylthiohydantoin amino acids were identified by high-pressure liquid chromatography (Bridgen et al., 1976).

The clotting assay with various deficient plasmas was performed as previously described (Fujikawa et al., 1977b), except plasma and surface materials were preincubated at room temperature for 5 min before the addition of the phospholipid–$CaCl_2$ solution.

TAME esterase activity of factor XII_a was determined by the method of Roffman et al. (1970) using tritium-labeled TAME. During the activation of factor XII, aliquots were removed and the reaction was stopped by the addition of a large excess of trasylol or Russell's viper venom inhibitor.[3] Subsequently, 10 μL of the stock tritium-labeled TAME solution (1 μmol containing 42000 cpm) was added to this mixture (usually 60–70 μL). An aliquot (55–60 μL) was transferred into 8 mL of toluene scintillant solution, and the appearance of radioactivity in the organic phase was determined in a Beckman scintillation counter, SL-100C. A control sample with substrate alone was incubated under the same conditions, and the radioactivity due to spontaneous hydrolysis of the substrate was substracted from the enzymatic reactions. The activity of factor XII_a was expressed as micromoles of TAME hydrolyzed per milligram per minute at 20 °C.

Results

A time curve for the activation of bovine factor XII by plasma kallikrein is shown in Figure 1. The activation reaction was followed by determining the increase of factor XII_a as measured by its esterase activity employing TAME as the substrate. In the presence of kaolin and HMW kininogen, the activation reaction was rapid and leveled off in about 5 min with an enzyme to substrate weight ratio of 1:100 (open circles). The optimum concentrations of kaolin and HMW kininogen were found to be 5 mg and 135 μg/mL, respectively, to activate 60 μg of factor XII. A sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of an aliquot from the 5-min incubation mixture showed that approximately 50% of the factor XII was converted to factor XII_a under these conditions. In the absence of HMW kininogen (solid circles), the initial rate of the activation reaction was about 4.7 times slower than that observed in the presence of HMW kininogen. The amount of factor XII_a esterase activity generated after

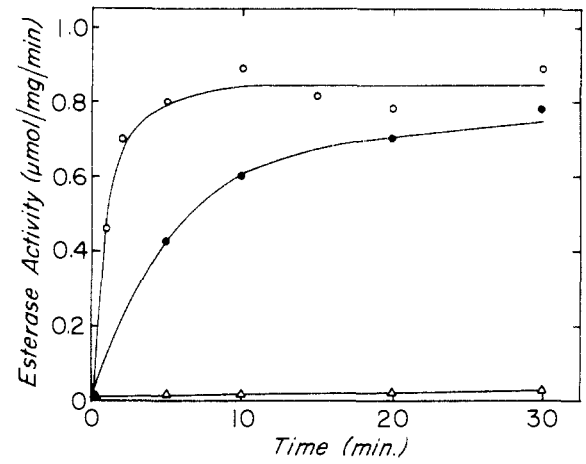

FIGURE 1: Time curve for the activation of factor XII by kallikrein in the presence of kaolin and HMW kininogen. The reaction mixture contained 75.6 μg of factor XII substrate, 0.75 μg of kallikrein, 250 μmol of Tris-HCl buffer, pH 7.6, 6.25 mg of kaolin, and 172 μg of HMW kininogen in a final volume of 1.25 mL. The reaction was initiated by the addition of kallikrein and incubated at 37 °C. Aliquots (150 μL) were removed at various times and added to 10 μL of trasylol solution (10 μg of trasylol). An aliquot (50 μL) of this sample was used for the determination of factor XII_a by TAME esterase activity as described under Methods. (○) Factor XII and kallikrein plus kaolin and HMW kininogen; (●) factor XII and kallikrein plus kaolin; (△) factor XII and kallikrein plus HMW kininogen.

30 min, however, was nearly the same as that formed in the presence of HMW kininogen. In the absence of kaolin (open triangles), very little factor XII_a was generated. Also, no activation of factor XII was observed by factor XI_a or plasmin in the absence of a surface. These latter results differ from those of other investigators who reported that the activation of human factor XII is readily catalyzed by kallikrein, factor XI_a, plasmin, or trypsin in the absence of a surface (Kaplan & Austen, 1971; Cochrane et al., 1973; Revak et al., 1974).

Dextran sulfate has been reported to initiate fibrinolysis in human plasma (Astrup & Rosa, 1974). The addition of dextran sulfate to plasma also initiated a factor XII dependent kallikrein activity (Kluft, 1978). Accordingly, it was of interest to study whether or not this polymer would participate in the contact activation of factor XII in a purified system. A time curve for the activation of factor XII in the presence of high molecular weight dextran sulfate (M_r ~500000) is shown in Figure 2. Factor XII (75.6 μg/1.25 mL) was readily activated by 0.75 μg of kallikrein in the presence of 12.5 μg of dextran sulfate. Under these conditions, the reaction was essentially complete within 2 min (open circles). In the presence of low molecular weight dextran sulfate (M_r ~3600) or dextran (M_r 500000), the activation reaction was negligible (open triangles).

The maximum esterase activity generated in these experiments was 2.0 μmol of TAME hydrolyzed per mg per min (Figure 2). This value is nearly identical with the specific activity of a purified preparation of factor XII_a. This indicates that the activation reaction went to completion and the activity was stable during the entire incubation period. A sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of aliquots removed during the course of the activation of factor XII in the presence of 12.5 μg of dextran sulfate is shown in Figure 3. The panel on the left shows the samples subjected to electrophoresis in the absence of reducing agent. It is evident from these experiments that little, if any, change in the size of factor XII (M_r 74000) has occurred during the activation reaction. The panel on the right shows the gel pattern of the samples reduced with 2-mercaptoethanol. It

[3] This inhibitor has been reported to inhibit plasma and pancreatic kallikrein, trypsin, α-chymotrypsin, and plasmin (Iwanaga et al., 1976). Recently, it was found that this inhibitor also inactivates bovine factor XI_a but not bovine factor XII_a (K. Kurachi, unpublished data).

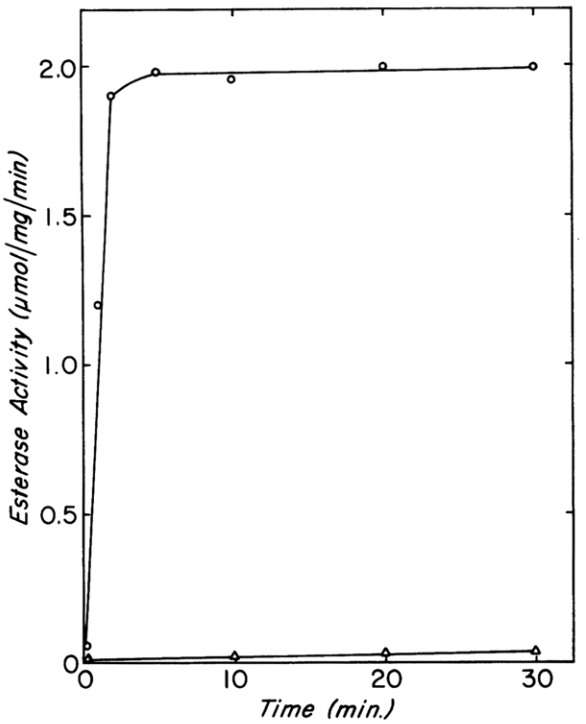

FIGURE 2: Time curve for the activation of factor XII by kallikrein in the presence of dextran sulfate. The reaction mixture contained 75.6 μg of factor XII, 0.75 μg of kallikrein, 250 μmol of Tris-HCl buffer, pH 7.6, and 12.5 μg of dextran sulfate in a final volume of 1.25 mL. The incubation temperature was 37 °C. Aliquots (150 μL) were withdrawn at various times and the reaction was stopped by the addition of 10 μL of trasylol solution. An aliquot (50 μL) of this sample was used for the assay of factor XII_a by its TAME esterase activity, and the remaining portion was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (○) Factor XII and kallikrein plus dextran sulfate; (△) factor XII and kallikrein only or factor XII and kallikrein plus either 12.5 μg of dextran or 12.5 μg of low molecular weight dextran sulfate.

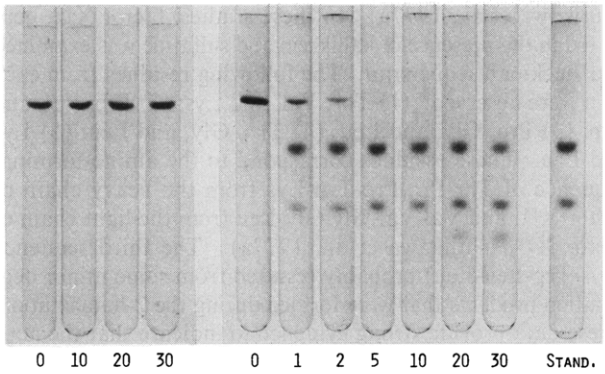

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of samples removed during the activation of factor XII by kallikrein in the presence of dextran sulfate (12.5 μg/1.25 mL). Samples were prepared as described in the legend to Figure 2. The left panel is for the gels of the nonreduced samples (2.5 μg of protein), and the right panel is for the gels of the reduced samples (3.5 μg of protein). Electrophoresis was carried out at 8 mA/gel for 4–5 h as described under Methods. The anode was at the bottom of the gels.

is evident from these gels that the single-chain factor XII (upper band) has disappeared within the first 2 min of the activation reaction and was converted to two new major bands of lower molecular weight. The migration of these two new bands corresponds exactly with those of a standard factor XII_a preparation which was reduced with 2-mercaptoethanol (labeled standard in Figure 3). The slow-moving band corresponds to the heavy chain (M_r 46 000) of factor XII_a and the fast-moving band corresponds to the light chain (M_r 28 000) of factor XII_a. The appearance of these two chains occurred in parallel with the generation of factor XII_a esterase activity. A third band, which is a minor one, was observed in the reduced gels after 20 min of incubation. This band, which migrated in front of the light chain, was not identified. Its appearance was not related to the generation or loss of factor XII_a activity. Accordingly, this fragment, which remains bound to either the heavy or light chain of factor XII_a, results from a minor degradative reaction. These experiments are consistent with our previous studies which indicated that a specific internal arginyl–valine peptide bond is cleaved by a serine protease during the purification of bovine factor XII (Fujikawa et al., 1977a).

Table I: Effect of Various Bovine Lipids on the Activation of Factor XII by Kallikrein[a]

lipids added	amt (μg)	factor XII_a esterase act. (μmol of TAME hydrolyzed per mg per min)
sulfatide	10	1.43
	1	1.33
ganglioside	10	0.19
	1	0.11
stearic acid	10	0.14
	1	0.24
galactoceramide	10	0.03
	1	0.03
phosphatidylcholine	10	0.03
	1	0.02
sphingomyelin	10	0.03
	1	0.03
sphingosine sulfate	10	0.01
	1	0.02
control		0.04

[a] Reaction mixtures contained 3 μg of factor XII, 0.03 μg of kallikrein, 10 μmol of Tris-HCl buffer, pH 7.6, and two different amounts (10 and 1 μg) of lipid in a final volume of 50 μL. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 10 μL of trasylol (2.5 μg) solution, and the TAME esterase activity was measured as described under Methods.

The finding that dextran sulfate is an excellent surface for the contact activation of factor XII led us to examine other acidic lipids and conjugated carbohydrate compounds. Compounds of this type were important to examine since they could play a role in vivo as a contact surface for initiating blood coagulation through the activation of factor XII. Among the compounds tested, sulfatide, a sulfonated glycolipid from bovine brain, was found to be very effective in the activation of factor XII by kallikrein (Table I). Various compounds, which are structurally related to sulfatides, were also tested, including sphingomyelin and galactoceramide from bovine brain. In these experiments, two different concentrations of lipid (1 and 10 μg) were studied for the activation of 3 μg of factor XII by 0.03 μg of kallikrein. Of the various compounds tested, ganglioside and stearic acid showed significant activity (Table I). The two most abundant lipid compounds in brain tissue, phosphatidylcholine and sphingomyelin, were not active in the activation of factor XII. Also, galactoceramide, which has the same structure as sulfatides except the 3-O position of the sugar moiety lacks a sulfate group, was totally inactive. Synthetic sphingosine sulfate and glucose 6-*O*-sulfate also failed to activate factor XII. Bovine and rabbit brain cephalin preparations (Bell & Alton, 1954), which are rich in phosphatidylcholine and phosphatidylserine, were also inactive.

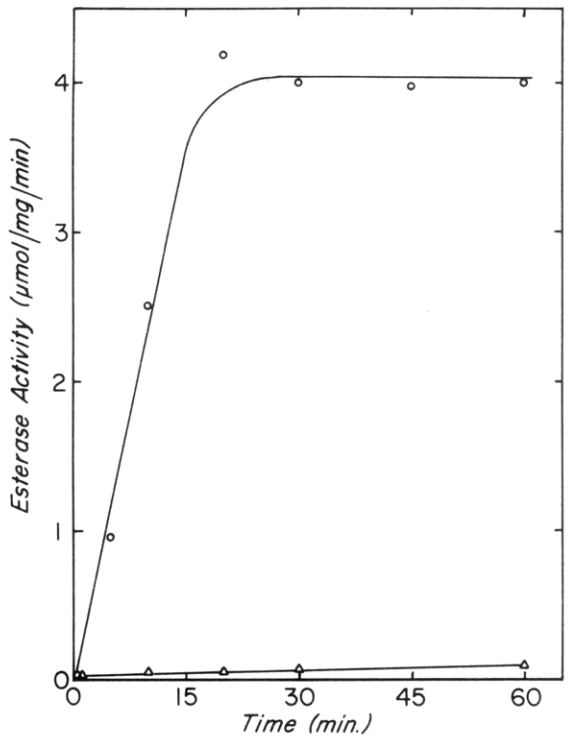

FIGURE 4: Time curve for the activation of factor XII by kallikrein in the presence of sulfatide and galactoceramide. The reaction mixture contained 50 μg of factor XII, 1 μg of kallikrein, 240 μmol of Tris-HCl buffer, pH 7.6, and 25 μg of sulfatide or 50 μg of galactoceramide in a final volume of 1.2 mL. The reactions were started by the addition of kallikrein and incubated at 37 °C. Aliquots (150 μL) were removed at various times and added to 10 μL of a trasylol solution. An aliquot (40 μL) of this sample was used for the determination of factor XII_a by its TAME esterase activity, and the remaining portion was employed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (○) Factor XII and kallikrein plus sulfatide; (△) factor XII and kallikrein plus galactoceramide.

Various polysaccharides and skin collagen were then tested under the same conditions by using 0.5–10 μg of compounds in 50 μL of the reaction mixture. None of these compounds, however, was found to be active in the activation of factor XII by kallikrein. These materials included bovine aorta proteoglycan, bovine cornea keratan sulfate, whale cartilage keratan sulfate, shark cartilage keratan sulfate, porcine dermatan sulfate, crude or purified heparin sulfate, shark cartilage chondroitin sulfate, bovine vitreous humor hyaluronic acid, bovine submaxillary mucin, soluble calf skin collagen, and purified soluble rat skin collagen (types I and III). In the control sample without lipid or other compounds added, 0.036 μmol of TAME was hydrolyzed per mg per min. This was due to a very small contamination (1.8%) of factor XII by factor XII_a.

The effects of sulfatide on the activation reaction were then studied in greater detail. A time curve for the activation of factor XII in the presence of 25 μg of sulfatide is shown in Figure 4. In these experiments, 50 μg of factor XII was completely activated by kallikrein in about 25 min in the presence of 25 μg of sulfatide (open circles). The enzyme to substrate weight ratio was 1:50. The rate of the activation reaction was slower with sulfatide than that observed with dextran sulfate. In these experiments, the sulfatide suspensions were prepared by the aid of a vortex mixer. Sulfatide suspensions prepared by sonication were only slightly more effective (1.2–1.5 times) than those prepared by the vortex mixer. The maximum esterase activity generated during this activation reaction was 4 μmol of TAME hydrolyzed per mg per min. This value is twice as high as that expected for factor XII_a. The higher specific activity was shown to be due to the direct effect of sulfatide on the esterase activity of factor XII_a, as described below.

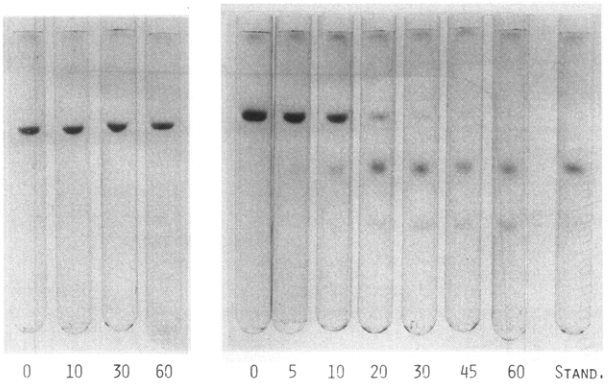

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of samples removed during the activation of factor XII by kallikrein in the presence of sulfatide. Samples were prepared as described in the legend to Figure 4. The left panel is for the gels of the nonreduced samples (1.7 μg of protein), and the right panel is for the gels of the reduced samples (3.4 μg of protein). Electrophoresis was carried out as described in the legend to Figure 3. The anode was at the bottom of the gels.

The sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of aliquots removed from the activation reaction of factor XII by kallikrein in the presence of sulfatide is shown in Figure 5. The gel pattern for the nonreduced and reduced samples shows that the single polypeptide chain of factor XII was completely converted to factor XII_a within 25–30 min. This activation mechanism appeared to be essentially identical with that observed in the presence of dextran sulfate.

An amino-terminal analysis of factor XII_a was then carried out to clearly establish that the factor XII_a generated in these reactions was identical with that previously characterized (Fujikawa et al., 1977a). In these studies, factor XII_a generated in the presence of kallikrein and sulfatide was examined in a Beckman sequenator. The following residues from each turn were observed: (1) Thr, Val, and Lys; (2) Pro, Val, and Trp; (3) Pro, Gly, and Leu; (4) Trp, Gly, and Leu; (5) Lys and Leu. These residues correspond to the amino-terminal sequence of Thr-Pro-Pro-Trp-Lys from the heavy chain of factor XII_a and Val-Val-Gly-Gly-Leu from the light chain of factor XII_a (Fujikawa et al., 1977a). The third sequence (Lys-Trp-Leu-Leu) probably resulted from some minor degradation products that were formed during the 2-h incubation. These data provide strong evidence to indicate that the activation of factor XII by kallikrein is due to the cleavage of a specific internal arginyl–valine peptide bond in the precursor molecule (Fujikawa et al., 1977a).

When factor XII was activated by kallikrein on the surface of kaolin, HMW kininogen stimulated this reaction several-fold (Figure 1). Thus, HMW kininogen appeared to be an important component of the activation of factor XII when the reaction occurred on a kaolin surface. Accordingly, it was of interest to test whether HMW kininogen influenced the activation reaction in the presence of dextran sulfate or sulfatide. In these studies, it was found that the addition of HMW kininogen to the activation reaction in the presence of sulfatide or dextran sulfate inhibited the reaction 70–80% (Table II). Furthermore, the addition of kaolin also caused a 50% inhibition of the activation reaction when assayed in the presence of sulfatide or dextran sulfate. In contrast, HMW kininogen has a substantial accelerating effect on the kaolin system in

Table II: Effect of Various Components on the Activation of Factor XII by Kallikrein[a]

components added	factor XII_a esterase act. (μmol of TAME hydrolyzed per mg per min)
dextran sulfate	2.4 (100)
dextran sulfate plus HMW kininogen	0.7 (29)
dextran sulfate plus kaolin	1.3 (54)
sulfatide	1.5 (100)
sulfatide plus HMW kininogen	0.3 (20)
sulfatide plus kaolin	0.8 (53)
kaolin plus HMW kininogen	1.5 (100)
kaolin	0.6 (40)
HMW kininogen	0.0 (0)

[a] Reaction mixtures for the dextran sulfate system contained 3 μg of factor XII, 10 μmol of Tris-HCl buffer, pH 7.6, 0.03 μg of kallikrein, and 0.5 μg of dextran sulfate in a final volume of 50 μL. Kaolin (250 μg) or HMW kininogen (9 μg) was added as shown. Reaction mixtures for the sulfatide system contained 1.5 μg of sulfatide in place of dextran sulfate. Reaction mixtures for the kaolin system contained 250 μg of kaolin and/or 9 μg of HMW kininogen in addition to enzyme and substrate. All reactions were initiated by the addition of kallikrein (0.03 μg). After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 10 μL of trasylol (2.5 μg) solution, and the TAME esterase activity was measured as described under Methods. The values in parentheses are percentages.

Table III: Effect of Sulfatide, Dextran Sulfate, and HMW Kininogen on the TAME Esterase Activity of Factor XII_a[a]

components added	amt (μg)	factor XII_a esterase act. (μmol of TAME hydrolyzed per mg per min)
control		2.0
sulfatide	0.25	2.7
	0.50	2.8
	1.0	3.8
	2.0	4.0
dextran sulfate	0.25	2.2
	0.50	2.4
	1.0	2.7
	2.0	2.7
HMW kininogen	0.25	2.2
	0.50	2.4
	1.0	2.5
	2.0	2.5

[a] Reaction mixtures contained 1.3 μg of factor XII_a, 10 μmol of Tris-HCl buffer, pH 7.6, 1 μmol of [^{3}H]TAME, and increasing concentrations of sulfatide, dextran sulfate, or HMW kininogen plus 2.5 μg of trasylol. The final volume was 60 μL. An aliquot (55 μL) of each sample was transferred to the toluene scintillator, and the radioactivity was determined as described under Methods.

Table IV: Effect of Sulfatide and Kaolin on Various Deficient Plasmas[a]

plasma	clotting time (s) sulfatide (10 μg)	kaolin (250 μg)	control
human, normal pooled	112	113	580
human factor XII deficient	564	>600	>600
human prekallikrein deficient	150	223	482
human HMW kininogen deficient	228	280	540
bovine factor XI deficient	>600	>600	>600

[a] Clotting assays were carried out as described under Methods. Plasma and surface materials were preincubated for 5 min at room temperature before the $CaCl_2$–phospholipid solution was added. In the control experiments, no contact surface materials were added to the assay mixture.

the absence of sulfatide or dextran sulfate (Table II and Figure 1). It should also be noted that dextran sulfate or sulfatide is more effective than kaolin when studied under the optimal conditions. In addition, these results show that HMW kininogen is a strong inhibitor of the contact activation of factor XII when a more active contact surface, such as dextran sulfate or sulfatide, is employed. The inhibitory effect of HMW kininogen may be due to several different reasons. For instance, HMW kininogen and factor XII are both substrates of kallikrein and, therefore, the two substrates compete for the same enzyme. Indeed, in preliminary experiments, it has been observed that kallikrein hydrolyzed HMW kininogen at a much faster rate than factor XII when both substrates were present at concentrations employed in the present experiments. Another possible explanation is that one of the fragments (fragment 1-2) of HMW kininogen formed in the presence of kallikrein is inhibiting the contact activation reaction, as previously noted by Oh-ishi et al. (1977). Accordingly, the effect of HMW kininogen on the activation of factor XII is strongly dependent on the type of surface material employed in the activation reaction.

As previously noted, factor XII_a formed in the presence of sulfatide had twice the specific esterase activity of factor XII_a formed in the absence of sulfatide. This was shown to be due to a direct stimulation of the esterase activity by sulfatide (Table III). In these experiments, the esterase activity of factor XII_a was increased from 2 to 3.8 μmol/(mg min) at 1.0 μg of sulfatide. Increases of 25 and 30% were also observed with dextran sulfate and HMW kininogen, respecitvely. However, the addition of kaolin did not influence the esterase activity of factor XII_a in these experiments. An increase of esterase activity of the human factor XII fragment by HMW kininogen with Ac-Gly-Lys-EtOH as the substrate was reported earlier by Liu et al. (1977). These results indicate that sulfatide and dextran sulfate provide a contact surface for the activation of factor XII as well as stimulate the esterase activity of the resulting factor XII_a.

It has been reported that the proteolytic activation of human factor XII occurs on a kaolin surface or in the fluid phase by plasma enzymes, such as factor XI_a and plasmin. These enzymes, however, were 10–100 times less effective than kallikrein (Kaplan & Austen, 1971; Cochrane et al., 1973; Revak et al., 1974; Griffin, 1978). Similar studies were carried out with bovine factor XII in the presence of sulfatide using an enzyme to substrate weight ratio of 1:50 (Figure 6). The initial rate of activation by factor XI_a (solid circles) was about 4 times slower than that by kallikrein (open circles), and the activation by plasmin (open triangles) was about 6 times slower. The activation of factor XII by factor XI_a was not influenced by HMW kininogen, while the activation by plasmin was inhibited by HMW kininogen. In the absence of sulfatide, factor XI_a or plasmin had no effect on factor XII. The activation of factor XII by factor X_a, factor IX_a, and thrombin was also studied in the presence of kaolin or sulfatide with an enzyme to substrate weight ratio of 1:25. In these experiments, no fragmentation of the factor XII molecule was observed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. These data are consistent with the concept that kallikrein is the initial activator of factor XII in plasma, and factor XI_a and plasmin have only a secondary role in the activation of this protein.

The effect of sulfatide on the clotting time of various deficient plasmas was also tested and compared with the kaolin clotting time of normal plasma (Table IV). In these experiments, plasma and either kaolin or sulfatide were preincubated

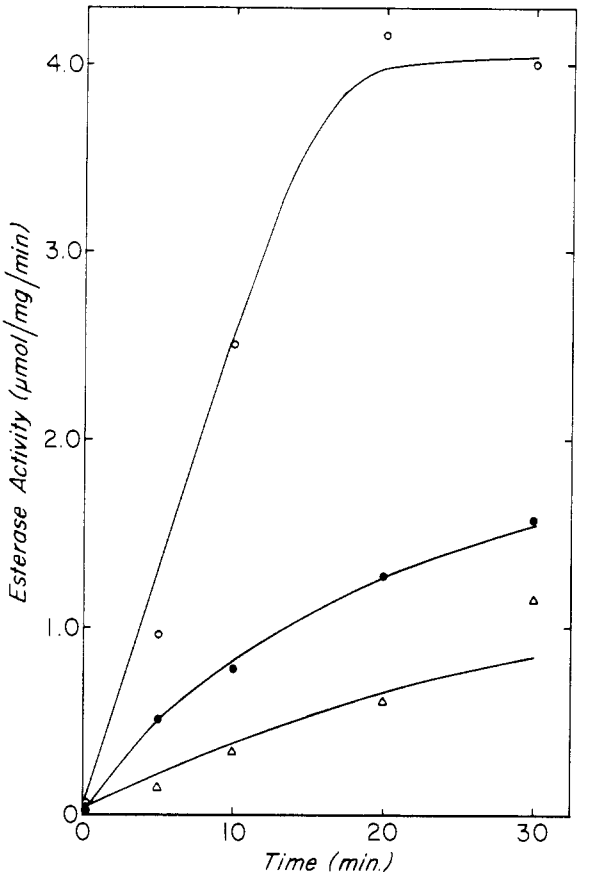

FIGURE 6: Time curve for the activation of factor XII by kallikrein, factor XI_a, or plasmin in the presence of sulfatide. The reaction mixture contained 18 μg of factor XII, 60 μmol of Tris-HCl buffer, pH 7.6, 9 μg of sulfatide, and 0.36 μg of kallikrein, factor XI_a, or plasmin in a final volume of 0.3 mL. The incubation temperature was 37 °C. Aliquots (50 μL) were removed at various times and the reaction was stopped by the addition of 10 μL of trasylol (10 μg) or Russell's viper venom inhibitor. TAME esterase activity was determined as described under Methods. The curve for the activation of kallikrein was taken from Figure 4 for comparison, where the same concentrations of factor XII, kallikrein, sulfatide, and buffer solution were employed. (○) Activation by kallikrein; (●) activation by factor XI_a; (△) activation by plasmin.

for 5 min, and the clotting time was then measured following the addition of a phospholipid–$CaCl_2$ solution. In the control experiments, neither kaolin nor sulfatide was added. With normal plasma, 10 μg of sulfatide gave the same clotting time as that with 250 μg of kaolin. This indicates that sulfatide is far more effective than kaolin in the surface activation of factor XII. It should be noted that the clotting time of both prekallikrein-deficient and HMW kininogen deficient plasma was significantly shorter with sulfatide than with kaolin. This result suggests that prekallikrein and HMW kininogen may have a less important role in the initiation of the coagulation system than that expected from clotting data obtained in the presence of kaolin. Although dextran sulfate is more active than sulfatide in the surface activation of factor XII, it does not shorten the clotting time of whole plasma. This is probably due to the fact that dextran sulfate has a heparin-like activity (Ricketts, 1952) which inhibits clotting via antithrombin III and nullifies its effect on the contact phase of coagulation.

Discussion

Bovine factor XII contains a single polypeptide chain (M_r 74000) with an amino-terminal sequence of Thr-Pro-Pro-Trp-. Factor XII_a, which has also been isolated from bovine plasma, is composed of a heavy and a light chain, and these two chains are linked together by a disulfide bond(s). The heavy chain of factor XII_a (M_r 46000) has the same amino-terminal sequence as that of the zymogen. It contains arginine as its carboxyl-terminal residue. The light chain (M_r 28000) has an amino-terminal sequence of Val-Val-Gly-Gly- and contains the active-site serine residue. Thus, it has been suggested that factor XII is converted to factor XII_a by a plasma enzyme, such as kallikrein, by the cleavage of a single internal peptide bond between arginine and valine (Fujikawa et al., 1977a,b). The present studies demonstrate that plasma kallikrein will indeed cleave this arginyl–valine peptide bond in factor XII, yielding factor XII_a.

The formation of factor XII_a by kallikrein requires the presence of a negatively charged surface, such as dextran sulfate. Accordingly, it seemed likely that other acidic mucopolysaccharides, such as sulfonated mucopolysaccharides, would be effective in this reaction. None of the mucopolysaccharides from bovine or other sources that have been tested thus far, however, was capable of initiating the activation of factor XII. These compounds included bovine cornea keratan sulfate, shark cartilage keratan sulfate, whale cartilage keratan sulfate, crude or purified porcine intestine heparin sulfate, shark cartilage chondroitin sulfate, bovine aorta proteoglycan, porcine dermatan sulfate, and bovine vitreous humor hyaluronic acid. These results are in contrast to the findings in whole plasma that chondroitin sulfate, heparin sulfate, or chitin will initiate a factor XII dependent kinin-like activity (Moskowitz et al., 1970).

Calf skin collagen (Cochrane et al., 1972) and stearic acid (Nossel, 1964) have been reported previously as potentially important surfaces for the contact activation of factor XII under physiological conditions. Detailed studies with these compounds with purified reagents, however, were not made. In the present experiments, soluble calf skin collagen and purified soluble rat skin collagen (types I and III) were found to be inactive in the surface activation of bovine factor XII. Stearic acid, however, was found to have some activity, suggesting that this compound, as well as sulfatide and ganglioside, may be involved in the contact activation of blood coagulation under physiological conditions. These reactions probably occur on the surface of micelles or vesicles formed by these lipids. Sulfatides, such as galactoceramide 3-*O*-sulfate, are classified either as glycolipids or sphingolipids since they contain both a carbohydrate and a sphingosine moiety. They are fairly abundant in brain tissue and are also found in other organs as well as whole blood (Soper, 1963) and red cells (Hansson et al., 1978). The contact activation of factor XII by lipids may be similar to that initiated by bacterial lipoprotein (endotoxin) (Morrison & Cochrane, 1974). The present data, as well as those of Nossel (1964), suggest that the initiation of the intrinsic pathway of blood coagulation and the middle-phase reactions including (1) factor IX_a, factor $VIII_a$, and factor X and (2) factor X_a, factor V_a, and prothrombin occur on lipid vesicles. It should be emphasized, however, that sulfatides will not replace phospholipids in these latter two reactions (unpublished data), nor will phospholipids replace sulfatides in the contact phase.

The effects of HMW kininogen on the coagulation process initiated by kaolin are well established (Wuepper et al., 1975; Saito et al., 1975; Colman et al., 1975). This is due in part to the effect of HMW kininogen on the activation of factor XII by kallikrein in the presence of kaolin (Griffin & Cochrane, 1976; Meier et al., 1977; Griffin, 1978). Recently, "active kininogen", which is formed by the brief treatment of HMW kininogen with kallikrein, was found to have a re-

markable effect on the contact activation reaction in the presence of kaolin (Kato et al., 1979). In the present studies, it was found that HMW kininogen does not stimulate the activation of factor XII when the reaction is examined in the presence of sulfatide or dextran sulfate. With plasma, the addition of sulfatide shortened the clotting time of normal plasma, prekallikrein-deficient plasma, and HMW kininogen deficient plasma. However, the clotting time of HMW kininogen deficient plasma was not as short as that of normal plasma, whereas the clotting time of prekallikrein-deficient plasma was rather close to that of normal plasma. These results are consistent with the fact that HMW kininogen also stimulates the activation of factor XI by factor XII_a (Griffin & Cochrane, 1976; Saito, 1977; Kurachi et al., 1980). Indeed, it is possible that HMW kininogen may play a role as an inhibitor when contact activation is initiated by sulfatide, since this protein was a reasonably strong inhibitor for the activation of factor XII under these conditions.

Recent findings in our laboratory have demonstrated that factor XII can activate factor XI at the same rate as factor XII_a in the presence of dextran sulfate or sulfatide (Kurachi et al., 1980). Also, no fragmentation of human factor XII was found when human factor XI was activated with factor XII and HMW kininogen in the presence of ellagic acid (Saito, 1977). This raises the possibility that the proteolytic activation of factor XII by kallikrein may not be the first event for the initiation of blood coagulation. This possibility is supported by the present data indicating a rapid clotting time for prekallikrein-deficient plasma in the presence of sulfatide (Table III). Radiolabeled human factor XII is cleaved in whole plasma shortly after clotting occurs (Revak et al., 1977), but it is not clear whether this fragmentation is necessary for the initiation of coagulation or whether it occurred primarily after the initiation of coagulation.

The proteolytic activation of factor XII may be necessary for the initiation of the fibrinolytic system. An inhibitor of plasminogen activation, called PA inhibitor, has been described (Hedner et al., 1970; Hedner, 1973) and shown to inhibit factor XII_a but not factor XII, factor XI_a, or factor X_a (Hedner & Martinsson, 1978). Furthermore, patients with high levels of this inhibitor have a high incidence of deep venous thrombosis (Nilsson et al., 1961; Pandolfi et al., 1970), suggesting that factor XII_a formation may be required for the initiation of normal fibrinolysis (Hedner & Martinsson, 1978).

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Mechanism of Activation of Bovine Factor XI by Factor XII and Factor XII_a†

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ABSTRACT: Factor XI (plasma thromboplastin antecedent) is one of several plasma proteins that participate in the contact phase of blood coagulation. Bovine factor XI (M_r 124 000) is a glycoprotein composed of two nearly identical polypeptide chains held together by a disulfide bond(s). The amino-terminal sequence of each of the two chains is Glu-Cys-Val-Thr-Thr-Leu-Phe-Gln-Asp-Ala-Cys-Phe-Lys-Gly-Gly-Asp-, a sequence which shows considerable homology with the amino-terminal sequence of bovine plasma prekallikrein. During the coagulation cascade, factor XI is converted by limited proteolysis to factor XI_a by either factor XII (Hageman factor) or factor XII_a. The activation of factor XI by factor XII requires the presence of sulfatide, dextran sulfate, or kaolin plus high molecular weight kininogen. In these reactions, factor XII, a zymogen, participates as an enzyme in the conversion of factor XI to factor XI_a. Accordingly, this reaction appears to be a very specific substrate-induced catalysis by a zymogen protein. Factor XII_a, a well-characterized serine protease, also activates factor XI in a reaction stimulated by sulfatide, dextran sulfate, or kaolin plus high molecular weight kininogen. During the activation of factor XI, each of the two chains of the zymogen is cleaved. This gives rise to factor XI_a composed of two heavy chains (M_r ~35 000 each) and two nearly identical light chains (M_r ~24 000 and ~26 000), and these four chains are held together by disulfide bonds. The heavy chains originate from the amino-terminal portion of the precursor molecule, while the light chains originate from the carboxyl-terminal portion of the precursor molecule. The amino-terminal sequence of each of the light chains is Ile-Val-Gly-Gly-Thr-Gln-Ala-Val-?-Gly-Glu-Trp-Pro-Tyr-Gln-Ile-. The light chains also contain an active-site serine residue. These data indicate that factor XI is converted to a serine protease by limited proteolysis, and this reaction, catalyzed by either factor XII or factor XII_a, readily occurs in the presence of sulfatide, dextran sulfate, or kaolin plus high molecular weight kininogen.

Factor XI (plasma thromboplastin antecedent),[1] factor XII (Hageman factor), prekallikrein, and HMW kininogen[2] participate in the early or contact phase of blood coagulation (Kaplan, 1978). When the coagulation process is initiated in the presence of a surface, a series of reactions are triggered, leading to an enzyme cascade and fibrin formation (Davie et al., 1979). The various clotting factors participating in this enzyme cascade are present in plasma in a precursor form, and most are converted to serine proteases during the coagulation process. This includes factor XI which has been extensively purified and characterized from human and bovine plasma (Koide et al., 1977a; Bouma & Griffin, 1977; Kurachi & Davie, 1977). Factor XI is composed of two similar or identical polypeptide chains, and these two chains are held together by a disulfide bond(s). The conversion of human factor XI to factor XI_a by factor XII_a has been studied in detail (Kurachi & Davie, 1977; Bouma & Griffin, 1977). This reaction involves the cleavage of an internal peptide bond in each of the two chains of the precursor molecule, giving rise to a serine protease called factor XI_a. Accordingly, human factor XI_a is composed of two identical heavy chains (M_r 35 000 each) and two identical light chains (M_r 25 000 each), and these four chains are held together by disulfide bonds. The activation of human factor XI, as well as the activation of factor XII and prekallikrein, is stimulated by the presence of kaolin and HMW kininogen (Griffin & Cochrane, 1976; Webster et al., 1976; Schiffman et al., 1977; Saito, 1977; Bouma & Griffin, 1977; Liu et al., 1977; Fujikawa et al., 1980).

In the present study, the mechanism of activation of bovine factor XI by factor XII as well as factor XII_a has been studied in detail. The data provide clear evidence that factor XII, a

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959). In the present studies, factor XII refers to the single-chain molecule (M_r 74 000) and factor XII_a refers to the two-chain molecule (M_r 74 000) held together by a disulfide bond(s).
[2] Abbreviation used: HMW kininogen, high molecular weight kininogen.

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