Inhibition of Activated Factor XII by Antithrombin-Heparin Cofactor*

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The activation of Factor XII occurs via fragmentation of this zymogen into a diverse spectrum of enzymatically potent molecular species. To study the interaction of antithrombin-heparin cofactor and heparin with activated Factor XII, we have employed two forms of this enzyme with widely differing physical characteristics and biologic potencies. Antithrombin-heparin cofactor was found to be a progressive, time-dependent inhibitor of both forms. The addition of heparin dramatically accelerated the rates of these interactions. Furthermore, sodium dodecyl sulfate gel electrophoresis of reduced proteins has indicated that antithrombin-heparin cofactor functions by forming an undissociable complex with either species of the enzyme. This complex represents a 1:1 stoichiometric combination of activated Factor XII and inhibitor. In the presence of heparin, both species undergo virtually instantaneous complex formation with antithrombin heparin cofactor without exhibiting alterations in dissociability or stoichiometry.

The hemostatic mechanism is composed of two independent but, interrelated, series of linked proteolytic reactions which result in the evolution of thrombin and plasmin. At each stage of the coagulation or fibrinolytic system, a parent zymogen is converted to a corresponding serine protease which catalyzes a subsequent precursor-enzyme product transition (2). Once generated, thrombin and plasmin are responsible for the formation of the fibrin clot and its ultimate resolution.

Several plasma proteins localize and restrict the activity of the hemostatic process. Each of these inhibitory components appears to have a well defined, but narrowly limited, ability to oppose the action of certain serine proteases of this physiologic mechanism. Initially, it was thought that antithrombin-heparin cofactor shared this restricted pattern of inhibitor specificity and could inactivate only thrombin and Factor Xa (3–6).

Our laboratory has demonstrated that antithrombin-heparin cofactor neutralizes human thrombin by formation of a remarkably stable enzyme-inhibitor complex via an active center serine-reactive site arginine interaction, and that heparin dramatically accelerates the rate of this reaction (7). On the basis of this biochemical mechanism, we predicted that most, if not all, serine proteases of the hemostatic mechanism would be inactivated by this inhibitor and that heparin would accelerate each of these interactions (8).

To prove this thesis, we have previously shown that Factor IXa, Factor Xa, and plasmin are inhibited in this heretofore unsuspected fashion (8–10). In this communication, we examine the interaction of antithrombin-heparin cofactor with activated Factor XII (Hageman Factor). It is widely appreciated that a broad spectrum of enzymatic species can be generated from the latter zymogen. Several laboratories have isolated unique species with molecular weights ranging from 98,000 to 80,000 (11–14). For this reason, we have employed two forms of activated Factor XII which differ greatly in their physical characteristics and enzymatic potencies. Both of these species are progressively neutralized by antithrombin-heparin cofactor, and heparin dramatically accelerates the rates of both of these interactions. It is reasonable to assume that other serine proteases derived from Factor XII will be inactivated in a similar fashion. Thus, antithrombin joins C1 esterase inactivator as an inhibitor of Factor XIIa activity. C1 esterase inactivator was previously shown to bind and neutralize various forms of this enzyme (15, 16). We, therefore, suggest that, with the possible exception of tissue factor-Factor VII interaction,1 the antithrombin inhibitory mechanism may represent a critical control point in modulating each stage of the hemostatic system.

1 A preliminary report on the interaction of the tissue factor-Factor VII complex with antithrombin-heparin cofactor would suggest that neutralization of procoagulant activity is minimal. Furthermore, the addition of heparin resulted in a modest direct inhibition of procoagulant activity rather than an acceleration of enzyme-inhibitor complex formation (17). The studies require confirmation.
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**Materials and Methods**

Proteins and Polypeptides—Bovine serum albumin, β-galactosidase, phosphomonoesterase, chymotrypsinogen, ovalbumin, and cytochrome c were purchased from Sigma Corp. Human fibrinogen was obtained from AB Kabé, Stockholm, Sweden. Bradykinin triacetate was from Sandoz Pharmaceuticals Ltd, Basel, Switzerland.

**Factor XII activity was eluted, the CM-Sephadex column effluent was M sodium phosphate/sodium citrate buffer (pH 5.85), subsequent rather than pH 8.0. Third, the effluent from the second DEAE-Sephadex chromatography was performed at pH 9.1 and the effluent from the first DEAE-Sephadex chromatography contained Factor XIIa activity.**

**Preparative DEAE-Sephadex Chromatography**—Factor XII activity was measured by a minor modification of the standard method (10) and utilized human plasma deficient in this protein as substrate. A titration curve constructed from 10 normal plasmas was employed and the average value was arbitrarily assumed to be 100 units/ml. Factor XIIa activity was quantitated in an identical fashion except that the activator (kaolin) was omitted and the glass assay tubes were silicon-treated. Antithrombin-Heparin Cofactor activity was determined by the procedure of Abildgaard et al. (20). Factor X activity was assayed according to the method of Bachmann et al. (21). The activities of Factors V, VII, VIII, IX, XI, prothrombin, and thrombin were obtained by customary assay techniques (19, 22–24).

**Measurement of Component Activities of Kinin-generating System**—The concentration of bradykinin present in a given sample was measured by its ability to contract the isolated guinea pig ileum (25). The relationship between the height of ileal contraction and the concentration of added bradykinin was linear between 2 and 25 ng/ml. All samples were diluted to fall within this range. Kallikrein activity was quantitated by incubating the enzyme (10 to 100 μl) for 2 min at 37° with heat-inactivated human plasma (0.2 ml) and then determining the amount of bradykinin generated (26). The relationship between the concentration of added kallikrein and the level of bradykinin formed was linear with r = 0.98. The activity of Factor XIIa,, was measured by incubating the enzyme (5 to 190 μl) for 2 min at 37° with heat-inactivated human plasma (0.2 ml) treated with 2.4 × 10⁻³ M EDTA. The amount of bradykinin generated was quantitated by bioassay (27). The relationship between the concentration of added Factor XIIa,, and the quantity of bradykinin formed was linear with r = 0.98.

**Preparative DEAE-Sephadex Chromatography of Factor XII (Factor XIIa,,)**—Factor XIIa was purified from human plasma by a modification of the method of Cochrane and Wuepper (28). This technique utilized ammonium sulfate fractionation, separation on DEAE-Sephadex, rechromatography on DEAE-Sephadex, filtration through Sephadex G-200, and elution from CM-Sephadex. To obtain a physically homogeneous product, the following modifications were made in this preparative method. First, heparin (10 units/ml) was added to the plasma prior to fractionation in order to accelerate the removal of trace amounts of activated coagulation factors. Second, the initial DEAE-Sephadex chromatography was performed at pH 9.1 rather than pH 8.0. Third, the effluent from the second DEAE-Sephadex column (~340 ml) was concentrated by dialysis against 0.15 M sodium phosphate/sodium citrate buffer (pH 5.85), subsequent adsorption to a small column of CM-Sephadex (1.2 × 7 cm), and elution with 0.25 M NaCl in 0.017 M sodium phosphate/sodium citrate buffer (pH 7.0) of Factor XIIa,, activity. Preparations were concentrated approximately 20-fold by this procedure with minimal loss of Factor XII activity. Techniques utilized by other investigators, such as ammonium sulfate precipitation, or ultrafiltration, were considerably less effective. Fourth, as Factor XII activity was eluted, the CM-Sephadex column effluent was continuously concentrated via a "collector column" approach (29). This process entails continuous mixing of the column effluent with equal volumes of 0.025 M citric acid at pH 5.2 and filtration of the resultant solution through a small column of CM-Sephadex (1.2 × 7 cm). Factor XII activity was harvested from the "collector column" as described above. This continuous concentration procedure was necessary because samples otherwise obtained from the final CM-Sephadex chromatography lost activity during collection. We suspect that this instability was due to adsorption of the extremely dilute final product to the plastic fraction collector tubes. The average yield for the entire preparative technique was 11%, and the final product was purified approximately 3000-fold over the starting plasma.

**Purification of Low Molecular Weight Activated Form of Factor XII (Factor XIIa,,)**—Crude Factor XIIa,, was obtained from 500 ml of normal human plasma by QAE-Sephadex chromatography. These preparations were concentrated by ultrafiltration to ~20 mg/ml and stirred overnight in a glass vessel at 4° in order to permit extensive activation of Factor XII to smaller fragments (11). The activated species were rechromatographed on QAE-Sephadex, filtered through Sephadex G-100, and concentrated on SP-Sephadex (11). Purification of products was completed by electrophoresis on acrylamide gels (2 × 15 cm). The biologic activity was recovered by slicing appropriate portions of these gels into 5-mm sections and incubating each section with 0.5 ml of 0.15 M NaCl in 0.01 M sodium phosphate (pH 7.4) for 24 h at 37°. The eluates which contained Factor XIIa,, activity were subsequently centrifuged in order to remove particulate material, dialyzed extensively against the buffer described above, and finally concentrated by ultrafiltration. Factor XIIa,, was identical to the species termed Hageman Factor prealbumin fragments, or prekalikrein activator (27, 28).

**Purification of Prekallikrein**—Prekallikrein was prepared from 500 ml of normal human plasma by QAE-Sephadex chromatography, SP-Sephadex chromatography, Sephadex G-100 gel filtration, and passage over an anti-IgG immunoabsorbent as described by Kaplan and Aschot (30). The final yield of this procedure averaged 10%. The final product was homogeneous on disc gel electrophoresis and sodium dodecyl sulfate gel electrophoresis. Upon isofocusing gel electrophoresis, the stained protein band (pH 8.5 to 8.9) corresponded to the position of eluted biologic activity (11).

**Gel Electrophoresis**—The analytic disc gel electrophoretic procedure of Davis (31) as modified by Rosenthal and Waugh (29) was employed. The preparative disc gel electrophoretic technique, used to obtain Factor XIIa,, employed identical conditions except that larger acrylamide rods were utilized. Furthermore, these latter gels were cooled during electrophoresis by contact with continuously circulating water maintained at 4°C.

**Radioactivity Labeling of Factor XIIa,,** Fifty micrograms of purified Factor XIIa,, was radioiodinated with 0.5 mCi of Na¹¹¹¹ by the method of Greenwood and Hunter (34). The radiolabeled protein was completely separated from free iodoide by Sephadex G-50 gel filtration with the chromatographic matrix equilibrated with 0.15 M NaCl in 0.01 M sodium phosphate (pH 7.4). At equivalent concentrations, the kinin generating ability of the radiolabeled protein was 98 ± 3% of the unlabeled enzyme.

**Results and Discussion**

**Preparations of Factor XIIa,, and Factor XIIa,,**

Purified preparations with Factor XII activity were analyzed by appropriate assays and were found to be free of Factors V, VII, VIII, IX, X, XI, prothrombin, thrombin, fibrinogen, prekallikrein, and kallikrein. In addition, disc gel electrophore-
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sis of these products exhibited a single molecular species with 85% of the applied procoagulant activity located precisely at the band position (Fig. 1A). In some preparations, several very faint bands (~5% of stained protein) were present which had migrated approximately 5 mm into the gel. Furthermore, on sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents, most preparations exhibited a single component with an apparent molecular weight of 75,000 ± 1200 (Fig. 1B). On occasion, a second component of lower molecular weight, that accounted for approximately 3 to 5% of the applied protein, was noted (Fig. 3). Initially, these preparations were believed to be unactivated zymogen since their procoagulant potency could be increased 100- to 200-fold by the addition of kaolin, and their molecular weights, obtained by sodium dodecyl sulfate gel electrophoresis, were similar to those previously reported for Factor XII (12, 13). However, most products were found to be inactivated by 500-fold molar excess of diisopropyl fluorophosphate as well as inhibited by antithrombin-heparin cofactor (Figs. 2 and 3). Thus, these preparations represent a high molecular weight activated species of this zymogen which we have termed Factor XIIaHMw. This component has been utilized, throughout these studies, to investigate the interaction of an activated but minimally proteolyzed form of Factor XII with antithrombin-heparin cofactor. Preparations of an activated but extensively proteolyzed form of Factor XII (Factor XIIaLMw) were also analyzed by appropriate assay and were found to be free of Factors V, VII, VIII, IX, X, XI, prothrombin, thrombin,
inhibitor, and kallikrein. Disc gel electrophoretic analysis of these products showed two closely spaced bands with 100% of the applied kinin generating activity distributed over this region of the gel (Fig. 1C). Previous investigators have reported that preparations of Factor XIIaHMW typically exhibit this degree of charge microheterogeneity (27). Furthermore, these products migrate as single components on sodium dodecyl sulfate gel electrophoresis in the presence of reducing agents with an apparent molecular weight of 28,000 ± 1500 (Fig. 1D). However, the protein stains quite poorly under these conditions.

Interaction of Activated Forms of Factor XII with Antithrombin-Heparin Cofactor and Heparin

Interaction of Inhibitor and Heparin with Factor XIIaHMW — The interaction of Factor XIIaHMW with antithrombin-heparin cofactor was examined in the presence and absence of heparin. The final concentrations of Factor XIIaHMW, antithrombin-heparin cofactor, and heparin (when utilized) were 44 µg/ml, 128 µg/ml, and 4.0 units/ml, respectively. All studies were conducted at pH 7.5, ionic strength 0.175, and 37°C. The procoagulant potency of Factor XIIaHMW was initially measured without adding kaolin to "activate" the enzyme.

To establish the relative stability of our enzyme preparation, Factor XIIaHMW was incubated for 30 min under standard conditions. As shown in Curve A of Fig. 2, no significant loss in procoagulant activity was detected. To determine whether Factor XIIaHMW was inactivated by antithrombin-heparin cofactor, the enzyme and inhibitor were mixed together and the resulting solution was sequentially assayed for residual procoagulant activity. As shown in Curve B of Fig. 2, a time-dependent decay in Factor XIIaHMW procoagulant activity occurred with only 41 ± 3.9% of its initial value remaining after 30 min of incubation with the inhibitor.

To determine whether heparin accelerated the Factor XIIaHMW-antithrombin interaction, the inhibitor was incubated with mucopolysaccharide prior to the addition of the enzyme and the resulting solution was immediately assayed. However, the presence of this mucopolysaccharide necessitated a significant alteration in our usual technique for quantitating the activity of Factor XIIaHMW. The measurement of this enzyme’s procoagulant activity is dependent upon the extent to which Factor XIIaHMW can trigger subsequent zymogen-to-enzyme transitions of the coagulation cascade. Heparin is known to instantaneously inhibit these activation events via its interaction with antithrombin-heparin cofactor and must be removed before Factor XIIaHMW procoagulant activity can be measured. Therefore, heparinase and DEAE-cellulose were sequentially added to the incubation mixtures. After immediate centrifugation, the heparin-free supernatants were utilized to determine the Factor XIIaHMW procoagulant activity. Heparinase cleaves mucopolysaccharide chains and thus rapidly reduces their anticoagulant activity. DEAE-cellulose binds residual heparin as well as oligosaccharides. Neither of these treatments affect the procoagulant activity of Factor XIIaHMW. However, to prevent the Factor XIIaHMW from interacting with DEAE-cellulose the ionic strength of all incubation mixtures was increased to 0.9 prior to the addition of the chromatographic matrix.

Several control experiments demonstrated the validity of this approach. First, we have shown that our modified assay procedure has no direct effect upon Factor XIIaHMW procoagulant activity. For example, this serine protease was incubated under standard solvent conditions and at a final concentration of 44 µg/ml for 30 s to 5 min. Subsequently, 63 µg of heparinase was added for 60 s. Then the ionic strength of the incubation mixtures was increased to 0.2 prior to the addition of 2 mg of dried DEAE-cellulose. After centrifugation at 15,000 × g for 30 s, the supernatants were removed, and assayed for procoagulant activity. The potency of Factor XIIaHMW was determined to be 108 ± 2.5% of the added procoagulant activity. Second, we have demonstrated that the modified assay procedure can effectively remove heparin from incubation mixtures and permit the measurement of Factor XIIaHMW activity. For example, we have incubated this enzyme and heparin for times ranging from 30 s to 5 min at final concentrations of 44 µg/ml and 4.8 units/ml, respectively, before these solutions were processed as outlined above. The potency of Factor XIIaHMW was found to be 106 ± 5.4% of the added procoagulant activity.

Third, we have established that this modified assay procedure can eliminate mucopolysaccharide bound to antithrombin-heparin cofactor from incubation mixtures and allow the determination of Factor XIIaHMW activity. For example, we have incubated antithrombin-heparin cofactor and heparin at final concentrations of 112 µg/ml and 4.8 units/ml, respectively. To obtain supernatants, these solutions were processed in the manner outlined above. Then, Factor XIIaHMW was added and the mixture was incubated for 30 s to 5 min. When these solutions were assayed for Factor XIIaHMW potency, they were found to contain 97 ± 3.9% of the initial procoagulant activity added. Thus, our modified assay procedure represents a valid technique for determining Factor XIIaHMW potency after this enzyme has been in contact with heparin and antithrombin-heparin cofactor.

To analyze the interaction of Factor XIIaHMW with heparin and antithrombin-heparin cofactor, the inhibitor was preincubated with the mucopolysaccharide for 30 s at 37°C prior to the addition of enzyme. After an additional 30 s of incubation, the reaction mixtures were processed as described above and assayed for Factor XIIaHMW activity. As shown in Curve C of Fig. 2, the neutralization of this enzyme by the inhibitor in the presence of heparin is virtually instantaneous.

The interactions of enzyme, inhibitor, and mucopolysaccharide have also been analyzed by determining the residual Factor XIIaHMW activity after kaolin addition. This alteration in our customary assay procedure is employed in order to "fully activate" Factor XIIaHMW. These experiments utilized solvent conditions, reactant concentrations, and techniques for removal of heparin which were similar to those previously employed. The results obtained were identical to those described above.

In previous communications, we have demonstrated that the thrombin-antithrombin complex and the plasmin-antithrombin complex, as well as the Factor IXa-antithrombin complex remain intact after exposure to varying combinations of denaturing and reducing agents. This property permitted us to monitor these enzyme-inhibitor interactions with sodium dodecyl sulfate gel electrophoresis (7, 9, 10). Initial experiments with the Factor XIIaHMW suggested that we could employ the same technique to analyze this enzyme-inhibitor interaction.

The inhibitor and enzyme were mixed at final concentrations of 95 and 60 µg/ml, respectively. In order to avoid secondary proteolysis of the complex (7), the concentration ratio was chosen so that a significant excess of inhibitor would be present on completion of this reaction. The solvent conditions utilized were pH 7.6, ionic strength 0.16, and 37°C. Aliquots were...
separately removed, immediately denatured, and treated as
described in Fig. 3, prior to their analysis by sodium dodecyl
gel electrophoresis in the presence of reducing agents.

In this electrophoretic system, antithrombin-heparin cofac-
tor and Factor XIIa,LMW migrated as single components with
apparent molecular weights of 61,500 ± 1000 and 75,000 ±
d respectively. When these proteins were incubated to-
gether over 40 min, the enzyme band virtually disappeared
while the inhibitor band decreased in intensity. Simultane-
ously, a new species emerged with an apparent molecular
weight of 117,000 ± 1500 (Fig. 3).

The interaction of antithrombin-heparin cofactor and Factor
XIIa,LMW was also studied in the presence of heparin (10
units/ml). The protein concentrations, conditions of incuba-
tion and analytic methods were identical to those described
above. Gel 7 of Fig. 3 reveals that formation of the new
molecular species was complete within 30 s. The apparent
molecular weight of this complex was identical to that
obtained in the absence of heparin.

Interaction of Inhibitor and Heparin with Factor XIIa,LMW

The interaction of Factor XIIa,LMW with antithrombin-hepa-
arin cofactor was also analyzed in the presence and absence
of heparin. The final concentrations of Factor XIIa,LMW, inhibitor
and heparin (when utilized) were 15 μg/ml, 100 μg/ml, and 10
units/ml, respectively. All investigations were carried out at
pH 7.4, ionic strength 0.16, and 37°. The potency of Factor
XIIa,LMW was quantitated by incubating an aliquot of the
solution with 0.2 ml of fresh human plasma for 2 min and then
determining the amount of bradykinin generated via a bioas-
say procedure (see "Materials and Methods" for detailed
description).

To illustrate the relative stability of our enzyme prepara-
tions, Factor XIIa,LMW was incubated for 20 min under standard
solvent conditions. As shown in Curve A of Fig. 4, minimal loss
in bradykinin-generating activity was noted. To determine
whether Factor XIIa,LMW was inactivated by antithrombin-
heparin cofactor, the enzyme and inhibitor were mixed to-
gether and the resultant solution was sequentially assayed for
residual bradykinin-generating activity. As depicted in Curve
B of Fig. 4, a time-dependent decay in Factor XIIa,LMW potency
was apparent with only 31 ± 6% of its initial value remaining
after 20 min of incubation with the inhibitor. To demon-
strate the effect of heparin upon the Factor XIIa,LMW-antithrombin
interaction, the inhibitor was admixed with the mucopolysac-
charide prior to the addition of enzyme, and the resulting
solution was assayed after varying times of incubation. As
shown in Curve C of Fig. 4, the neutralization of this enzyme
by the inhibitor is dramatically accelerated by the presence
of heparin.

Previous investigators have demonstrated that addition of
Factor XIIa,LMW to plasma converts prekallikrein to kallikrein
(27, 28, 36). This latter enzyme cleaves kininogen and thus
releases bradykinin. In the absence of heparin, we have
established that a progressive, time-dependent decay in
potency occurs after exposure of this serine protease to anti-
thrombin-heparin cofactor (Fig. 4). Since the samples for each
time point are incubated with substrate for an equivalent pe-
tiod, the data indicates that antithrombin-heparin cofactor
directly neutralizes Factor XIIa,LMW rather than indirectly in-
hibiting kallikrein or other molecular species generated within
plasma during the assay procedure. In the presence of hepa-
rin, we have shown, however, that this process is virtually
instantaneous (Fig. 4). Thus, one might argue that heparin-
activated inhibitor neutralizes kallikrein or other serine pro-
tases formed by Factor XIIa,LMW and that these events are
responsible for the accelerated inactivation of bradykinin-
generating activity. Indeed it is known that kallikrein is inhib-
ited slowly by antithrombin-heparin cofactor and that heparin
modestly accelerates this reaction (37, 38).

Therefore, we directly examined the effects of the inhibitor
and the mucopolysaccharide upon the Factor XIIa,LMW-dependent
conversion of prekallikrein to kallikrein. This was accom-
plished by incubating heparin or antithrombin-heparin cofac-
tor, or both, with Factor XIIa,LMW prior to, or immediately after,
this enzyme had generated kallikrein from its precursor zymo-
genin. The quantity of kallikrein formed was estimated with the
standard bioassay technique using heated plasma as substrate.
This treatment of the plasma substrate is employed since heat
denatures Factor XII and prekallikrein but only minimally
alters kininogen (26, 27). The solvent conditions utilized
throughout these studies were pH 7.4, ionic strength 0.16, and
37°.

In the first set of experiments, we have demonstrated that
this bioassay can quantitate prekallikrein to kallikrein conver-
sion. In addition, we have shown that antithrombin-heparin
cofactor and heparin have minimal effect upon the bradykinin-
producing ability of the Factor XIIa,LMW-prekallikrein system
when the inhibitor and mucopolysaccharide are added after
kallikrein has been generated. In control experiments (not
shown) either Factor XIIa,LMW or prekallikrein were added to
buffer at concentrations of 75 and 50 μg/ml, respectively.
The resultant solutions were incubated for 2 min and then assayed
for their ability to generate bradykinin. No bradykinin was
detected under these conditions. Thus the bioassay is insensi-
tive to either Factor XIIa,LMW or prekallikrein if these compo-
nents are added alone. Subsequently, Factor XIIa,LMW was
mixed with purified prekallikrein, in order to allow maximal
generation of kallikrein (Table I, First Reaction Sequence,
Solution I). After 4-min of incubation, an equal volume of
buffer (Solution IIa) was admixed with Solution I. After an
additional incubation of 2 min, aliquots were removed and

![Fig. 4. Per cent of initial kinin-generating activity at different times
following the incubation of Factor XIIa,LMW with buffer (Curve A),
antithrombin-heparin cofactor (Curve B), or antithrombin-heparin
cofactor and heparin (Curve C). Each point represents the mean ±
S.E. of at least three separate incubation mixtures.](http://www.jbc.org/
assayed. As shown in Table I, significant quantities of bradykinin had been generated. If the level of prekallikrein utilized in Solution I is gradually reduced, a proportional linear decline in bradykinin formation is noted (not shown). Therefore, the presence of molecular species in Solution II which inhibit kallikrein ought to be detectable via suppression of bradykinin generation. However, when antithrombin-heparin cofactor together with heparin (Solution IIb) were employed in place of buffer, no reduction in bradykinin formation was noted. Thus the inhibitor and its mucopolysaccharide cofactor have virtually no effect upon kallikrein if incubated for short periods of time with this enzyme.

In the second set of experiments (Table I, Second Reaction Sequence) we have shown that neither heparin nor antithrombin-heparin cofactor, alone, can reduce the Factor XIIaMw dependent generation of kallikrein from its zymogen precursor, if the inhibitor or mucopolysaccharide are preincubated with Factor XIIaMw for a short period of time. If, however, heparin and antithrombin-heparin cofactor, together, are preincubated with Factor XIIaMw for the same brief time period, subsequent conversion of prekallikrein to kallikrein is greatly reduced. Initially, Factor XIIaMw (Solution Ia) was incubated for 2 min with either buffer (Solution Ib,) or antithrombin-heparin cofactor (Solution Ib), or heparin (Solution Ib,). Then prekallikrein (Solution II) was added and the resultant reaction mixture was incubated for an additional 4 min prior to bioassay. As shown in Table I, neither of these components, when added separately, can influence the generation of kallikrein under these conditions. If, however, both the inhibitor and heparin are present (Solution Ib,), then addition of prekallikrein (Solution II) does not result in significant bradykinin formation as judged by the standard bioassay technique. These data demonstrate that heparin dramatically accelerates the inactivation of Factor XIIaMw by antithrombin-heparin cofactor. Thus, these experiments substantiate our previous conclusions based upon bradykinin generation in whole plasma (Fig. 4).

We have already shown that Factor XIIaMw forms a stable complex with antithrombin-heparin cofactor and have utilized sodium dodecyl sulfate gel electrophoresis to study the enzyme-inhibitor interactions. Initial experiments with Factor XIIaMw and antithrombin-heparin cofactor revealed a complex of similar stability. Thus, we attempted to employ sodium dodecyl sulfate gel electrophoresis to monitor complex formation between Factor XIIaMw and inhibitor in the presence and absence of heparin. Since the enzyme stains poorly in this electrophoretic system, we utilized 125I-labeled Factor XIIaMw to facilitate the analysis.

Antithrombin-heparin cofactor and 125I-labeled Factor XIIaMw were mixed at final concentrations of 100 and 15 μg/ml, respectively. This concentration ratio was chosen so that an excess of inhibitor would be present at the completion of the reaction in order to avoid secondary proteolysis of the complex. The solvent conditions utilized were pH 7.4, ionic strength 0.16, and 37°. Aliquots were sequentially removed, immediately denatured, and treated as described in Fig. 5 prior to

### Table I

**Conversion of prekallikrein to kallikrein**

<table>
<thead>
<tr>
<th>Solution I</th>
<th>Solution II</th>
<th>Bradykinin generation (ng)</th>
</tr>
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<tbody>
<tr>
<td>First Reaction Sequence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Factor XIIaMw, 30 μg/ml (Ia)</td>
<td>Buffer (IIa)</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>+ prekallikrein, 200 μg/ml (Ib)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Factor XIIaMw, 20 μg/ml (Ia)</td>
<td>Antithrombin-heparin cofactor, 200 μg/ml + heparin, 20 units/ml (Ib)</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>+ prekallikrein, 200 μg/ml (Ib)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second Reaction Sequence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Factor XIIaMw, 30 μg/ml (Ia)</td>
<td>Prekallikrein, 100 μg/ml</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>+ Buffer (Ib,)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Factor XIIaMw, 30 μg/ml (Ia)</td>
<td>Prekallikrein, 100 μg/ml</td>
<td>88 ± 6</td>
</tr>
<tr>
<td>+ antithrombin-heparin cofactor, 200 μg/ml (Ib)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Factor XIIaMw, 30 μg/ml (Ia)</td>
<td>Prekallikrein, 100 μg/ml</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>+ heparin, 20 units/ml (Ib)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Factor XIIaMw, 30 μg/ml (Ia)</td>
<td>Prekallikrein, 100 μg/ml</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>+ antithrombin-heparin cofactor, 200 μg/ml + heparin, 20 units/ml (Ib)</td>
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**Fig. 5.** Reduced sodium dodecyl sulfate gel electrophoretic analysis of Factor XIIaMw-antithrombin-heparin cofactor (AT-HCF) interaction in the presence and absence of heparin. Factor XIIaMw radiolabeled with 125I was mixed with buffer, antithrombin-heparin cofactor, or heparin and antithrombin-heparin cofactor for varying periods of time prior to electrophoretic analysis. After completion of the separation, gels were sliced into 1.2-mm segments and counted for 125I. The direction of protein migration is from right (cathode) to left (anode). See text, "Materials and Methods," and Fig. 3 for additional experimental details.
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electrophoretic analysis in the presence of reducing agents. In this electrophoretic system, the inhibitor and enzyme migrate as single components with apparent molecular weights of 58,000 ± 2000 (stained gel) and 28,000 ± 1500, respectively. When these proteins are incubated together, the isotopically labeled bands gradually waned, exhibiting a 50% reduction after 15 min. Simultaneously, a new component is evident with an apparent molecular weight of 85,000 ± 3,000 (Fig. 5, upper panel). The interaction of antithrombin-heparin cofactor and Factor XIaLMW has also been studied in the presence of heparin (10 units/ml). The protein concentrations, conditions of incubation, and analytic methods were identical to those described above. The lower panel of Fig. 5 reveals that complex formation was complete within 30 s. The apparent molecular weight of this complex was identical to that obtained in the absence of heparin.

The major difficulty in examining the interaction of activated Factor XII with antithrombin-heparin cofactor and Factor XIIaLMW has been the selection of appropriate form(s) of this serine protease to employ in our studies. Indeed, the complicated nature of this zymogen's activation mechanism as well as the resultant molecular heterogeneity of the enzymatically active end products are widely appreciated (11, 12, 27, 39-42). Several laboratories have demonstrated that Factor XII can be converted to an active form via adsorption to specific surfaces or by limited proteolysis with kallikrein, plasmin, or trypsin (11-14, 27, 43). Furthermore, these enzymatically active species range in size from products with molecular weight of ~80,000 to those with molecular weights of ~30,000 (11,14). All of these diverse molecular forms have the capacity to activate the coagulation mechanism via conversion of Factor XI to Factor XIa, the bradykinin system via transformation of prekallikrein to kallikrein (14), or the fibrinolytic pathway by generation of plasminogen activator from plasminogen proactivator (30). However, their relative ability to initiate blood clotting, bradykinin generation, or fibrinolysis may differ considerably from one molecular species to another (11, 12, 27, 28). It is, as yet, unclear whether one or more of these products represents the physiologically relevant species.

Therefore, we have chosen to utilize two activated forms of Factor XII which greatly differ in their physical characteristics and enzymatic potencies. First, we have employed a high molecular weight activated form of this zymogen which we have termed Factor XIIaLMW. This molecular species is similar in size to its parent proenzyme and retains the capacity to dramatically increase its procoagulant activity after contact with kaolin. Thus this component represents a minimally altered but enzymatically potent end product of Factor XII activation. Second, we have utilized a low molecular weight active form of this zymogen, Factor XIaLMW, which is generated after extensive proteolysis of Factor XII.

Both molecular species are slowly neutralized by antithrombin-heparin cofactor as judged by coagulation and bradykinin bioassay techniques. Furthermore, the addition of heparin dramatically accelerates neutralization of either enzymatic form by this protease inhibitor. The occurrence of stable enzyme-inhibitor complexes has permitted us to further demonstrate that either form of activated Factor XII can interact with antithrombin-heparin cofactor and that heparin can accelerate this process. The apparent molecular weight of both types of enzyme-inhibitor complexes are in reasonable accord with the sum of the apparent molecular weights of the individual constituents (Factor XIaLMW, antithrombin complex ~117,000 versus reactants ~136,000; Factor XIIaLMW, antithrombin complex ~85,000 versus reactants ~90,000). Thus the molar stoichiometry of these interactions appears to be 1:1.

Given the two widely differing species of activated Factor XII used in these studies, we would suggest that other enzymatically potent forms of this zymogen must be inhibited in a fashion similar to that described above. Thus Factor XIa, like Factor IXa, Factor Xa, Factor Xla, thrombin, and plasmin, is slowly, but progressively inactivated by antithrombin-heparin cofactor in the absence of heparin and virtually instantaneously neutralized by the inhibitor in the presence of this mucopolysaccharide.

The interaction of heparin and antithrombin-heparin cofactor with virtually all of the serine proteases of the coagulation-fibrinolytic systems represents a highly specific process for inactivating enzymes of the hemostatic mechanism. Serine proteases which are generated within physiologic systems are linked to, but separate from, the coagulation-fibrinolytic mechanism do not appear to be handled in the above fashion (44).

The speed and selectivity of this inhibitory mechanism suggests that it may be of great importance in modulating the activity of the hemostatic system. Heparan sulfate, a heparin-like mucopolysaccharide which possesses some anticoagulant activity, has recently been found on the surface of endothelial cells and of platelets (45, 46). This component would permit antithrombin-heparin cofactor to be selectively activated in vivo at blood-surface interfaces where enzymes of the coagulation-fibrinolytic pathways, such as Factor XIIa, are assumed to be generated. In this manner, the plasma protease inhibitor would be critically placed to neutralize these enzymes and thereby protect natural surfaces against thrombus formation or proteolytic attack.

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