Identity of Plasma-activated Factor X Inhibitor with Antithrombin III and Heparin Cofactor*

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SUMMARY

Antithrombin III activity cochromatographed with the activity of the inhibitor to activated Factor X during the purification of the inhibitor on Sephadex G-200, DEAE-Sephadex, and DEAE-cellulose columns. Both activities also cochromatographed on preparative polyacrylamide gel disc electrophoresis. When a fraction of activated Factor X inhibitor was incubated at 56°, at pH 7.5, a simultaneous loss of antithrombin III and activated Factor X inhibitor activities resulted. A complete neutralization of the antithrombin III activity of the activated Factor X inhibitor fraction with thrombin rendered the inhibitor incapable of subsequently inhibiting activated Factor X and vice versa.

When the inhibitor was chromatographed on a Sephadex G-200 column, antithrombin III, heparin cofactor, and activated Factor X inhibitor activities cochromatographed with the 280 mμ absorbing material in the activated Factor X inhibitor sample applied.

To demonstrate further the identity of these three anticoagulant activities, a series of gel filtration experiments on a single column of Sephadex G-200 was performed in which fractions of activated Factor X inhibitor alone, heparin alone, and inhibitor mixed with the inhibitor were separately chromatographed in the presence of 0.01 M NaCl in 0.02 M Tris-maleate, pH 7.2, at 26°. The relative elution volume, Vr/Vo of activated Factor X inhibitor alone in 0.01 M NaCl was 1.83, and in 0.10 M to 0.30 M NaCl was 1.77. Heparin behaved as a heterogenous substance with molecular weights ranging from 40,000 to greater than 200,000, at the salt concentrations studied. When a mixture of activated Factor X inhibitor and heparin was chromatographed, the two components eluted as a complex. A single broad heparin cofactor peak cochromatographed with the activated Factor X inhibitor protein and the heparin when high ionic strength eluent was employed. The fractions demonstrated antithrombin III activity when the heparin in the heparin cofactor peak fractions was neutralized with protamine sulfate.

The plasma-activated Factor X inhibitor was shown to be unrelated to a hepatic anticoagulant that had been claimed to be a specific inhibitor of activated Factor X.

It is concluded, therefore, that the biological activities variously termed activated Factor X inhibitor, antithrombin III, and heparin cofactor activities all belong to a single blood proteinase inhibitor with broad specificity. Together with other data presented, it is suggested that the key function of activated Factor X inhibitor as a natural anticoagulant may be primarily concerned with regulating hemostatic balance through neutralization of activated Factor X, a reaction profoundly enhanced by traces of heparin. It is for these reasons we propose that this low molecular weight α2-globulin inhibitor be termed activated Factor X inhibitor.

In the previous report (1), we suggested that the primary physiological target of the inhibitor to activated Factor X was activated Factor X itself rather than thrombin. Although the inhibitor was also capable of slowly neutralizing the coagulant and esterase activities of thrombin, this inhibitor, in the presence of heparin, exerted an instantaneous, but operationally reversible blockade of the clotting activity of thrombin, without any apparent effect on its esterolytic function. These findings raised questions as to the identity of the plasma inhibitor with primary physiological significance for thrombin.

For many years, antithrombin III (progressive antithrombin) has been considered by numerous investigators to be the principal circulating inhibitor to thrombin. However, some of the biological properties of the activated Factor X inhibitor observed by us resemble those reported in the literature and attributed by others to plasma antithrombin III. In fact, in a short communication (3), it was reported that a heterogeneous fraction of antithrombin III from bovine plasma was capable of neutralizing autoprotrombin C (equated with activated Factor X). The authors postulated that the capacity of this

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fraction to neutralize both activated Factor X and thrombin belonged to a single proteinase inhibitor termed antithrombin III.

Many observations have been made regarding antithrombin III in the past 50 years, so that it is almost impossible to refer to all of them; we have selected for reference, therefore, those investigations most pertinent to this communication. It has also been suggested that antithrombin III is identical with heparin cofactor (antithrombin II), a plasma protein that, in conjunction with heparin, exerts an instant antithrombin effect (4-13). A number of other investigators, however, have claimed a lack of identity for these two activities (14-22).

Finally, it has also been claimed that liver contains a specific inhibitor to activated Factor X (23). This observation prompted us to investigate and compare its activity with that of the plasma inhibitor of activated Factor X.

The present report presents data to show that the activities of activated Factor X inhibitor, antithrombin III, and heparin cofactor are inseparable from each other by presently available physical methods including gel filtration, ion exchange chromatography, preparative disc electrophoresis on polyacrylamide gel, differential titration, and heat stability studies. These results are consistent with the view that these variously termed activities belong to a single protein.

Finally, we have been unable to substantiate the claim that liver homogenates contain an inhibitor specific for activated Factor X and have also determined that the inhibitor obtained from liver homogenates is entirely distinguishable from the inhibitor we have isolated from plasma.

**EXPERIMENTAL PROCEDURES**

**Materials**

The reagents used in this report have already been described in the preceding two communications (1, 24).

**Methods**

The activated Factor X inhibitor activity in the chromatographic effluents, and other investigative techniques, unless otherwise stated, are described in the preceding two reports (1, 24). The antithrombin III activity from column effluents were routinely assayed as follows. A 0.2-ml fraction (1:2 to 1:5 dilution in 0.145 M NaCl), 0.2 ml of NaCl (0.145 M), and 0.1 ml of thrombin (2 units) were added in the indicated order to a siliconized tube at 37°, and at 15 s and 5 min after the addition of the thrombin, 0.1-ml aliquots were removed and added to 0.2 ml of fibrinogen solution. The difference in clotting times between the 15-s and 5-min samples represented the potency of the antithrombin III activity. All experiments involving thrombin were performed with siliconized glassware. The heparin cofactor and heparin assays were performed as described in the appropriate sections.

**RESULTS**

Identity of Activated Factor X Inhibitor with Antithrombin III

It has been observed (3, 25) that an heterogeneous fraction of plasma antithrombin III was also capable of inhibiting activated Factor X (autoprotrombin C). When the antithrombin III fraction was saturated with thrombin, it was found that the resulting mixture subsequently became incapable of neutralizing activated Factor X. From this it was concluded that the two activities responsible for the neutralization of thrombin and activated Factor X belonged to a single molecule. As we have previously demonstrated, highly purified fractions of activated Factor X inhibitor were capable of inhibiting thrombin (1). The following experiments were performed to establish the relationship between the inhibitor and antithrombin III.

Titration of Activated Factor X Inhibitor with Thrombin and Activated Factor X—When 1 ml of inhibitor (100 μg) was incubated with 1 ml of thrombin (200 NIH units) in a siliconized tube at 37° for 180 min, less than 0.05 unit of thrombin remained in the reaction mixture. From this primary reaction mixture, 0.3 ml was removed and incubated with 0.1 ml (2 units) of thrombin for 10 min at 37°. Likewise, another 0.3-ml sample from the primary incubation mixture was removed and allowed to react with 0.1 ml (2 units) of activated Factor X. Approximately 98% of the thrombin and 100% of the activated Factor X remained in the secondary incubation mixtures.

Heat Stability of Two Anticoagulant Activities in Purified Fraction of Activated Factor X Inhibitor—A purified fraction of activated Factor X inhibitor was heated in a stopped tube at 50° and pH 7.5 for 6 hours. Samples of the heated fraction were removed hourly and assayed for antithrombin III and activated Factor X inhibitor activities. Prior to heat treatment, the fraction was serially diluted to establish a calibration curve for each activity. The concentration of the fraction to be heated was adjusted such that 2 units of activated Factor X and 18 units of thrombin were inactivated separately in 10 min under the described conditions (1). The loss of both the antithrombin III and activated Factor X inhibitor activities closely followed each other (Fig. 1).

Chromatographic and Electrophoretic Behavior of Antithrombin III during Purification of Activated Factor X Inhibitor—As shown in Fig. 2, in the initial isolation of the activated Factor X inhibitor on Sephadex G-200, the antithrombin III activity cochromatographed with it. When this peak was pooled and further chromatographed on a DEAE-Sephadex column, Fig. 3, the same pattern as in Fig. 2 was obtained. During the subsequent steps in the purification procedure (24), antithrombin III activity consistently cochromatographed with the activated Factor X inhibitor activity on the DEAE-cellulose column. When the DEAE-cellulose chromatographed, pooled fraction containing high activated Factor X inhibitor activity was filtered either on a Sephadex G-100 or Sephadex G-200 column, the antithrombin III also cochromatographed with the activated Factor X inhibitor (not shown). These two activities were also eluted together at pH 9 on the ion exchange cellulose columns.

A pooled fraction of activated Factor X inhibitor from three different DEAE-cellulose runs was fractionated by preparative polyacrylamide gel disc electrophoresis at pH 8.1 (24). The antithrombin III and activated Factor X inhibitor activities were assayed immediately upon elution, because, as previously shown, the purified inhibitor rapidly lost its biological activity when subjected to electrophoresis on the polyacrylamide gel.
Heat stability of antithrombin III and activated Factor X inhibitor activities in a purified fraction of activated Factor X inhibitor. A primary reaction mixture containing 1 ml of inhibitor (500 μg) and 9 ml of Tris-maleate, 0.02 M, pH 7.5, was heated at 56° in a stoppered tube. At the indicated time intervals aliquot samples were removed and tested for residual activated Factor X inhibitor and antithrombin III activities. The activated Factor X inhibitor activity was measured by incubating, in the following order, 0.1 ml of a 1:2 dilution of the heated sample, 0.3 ml of Tris-maleate, 0.02 M, pH 7.5, and 0.1 ml of activated Factor X (3 units) at 37°. At 15 s and 10 min after the addition of the activated Factor X, 0.1 ml aliquots were removed and assayed for residual activated Factor X activity. The difference between the 15 s and 10 min values represented the amount of activated Factor X inhibited, and from the calibration curve of the inhibitor fraction prior to heating, the amount of inhibitor required to inactivate this amount of activated Factor X under similar conditions was calculated. The residual antithrombin III activity of the heated inhibitor fraction was measured by incubating, in the following order in a siliconized tube, 0.1 ml of a 1:5 dilution of the heated sample, 0.2 ml of Tris-maleate, 0.02 M, pH 7.5, and 0.2 ml of thrombin (20 units) at 37°. At 15 s and 10 min after addition of the thrombin, 0.1 ml samples were removed and tested for residual thrombin activity. Thrombin was assayed by adding 0.1 ml of the test material, appropriately diluted in 0.145 M NaCl, to 0.2 ml fibrinogen solution in a siliconized tube. The difference between the 15 s and 10 min values represented the amount of thrombin inhibited. The amount of antithrombin III activity required to inhibit this quantity of thrombin was then calculated from the antithrombin III calibration curve performed on the inhibitor fraction before heat treatment. The calibration curves for each activity were established by serially diluting the inhibitor fraction in Tris-maleate 0.02 M, pH 7.5, and assaying as described above.

As can be seen in Fig. 4, again the antithrombin III cochromatographed with the activated Factor X inhibitor.

Despite the present lack of more accurate methods for the quantitative assessment of either activity, there appears to be a fairly constant ratio of these two activities (activated Factor X inhibitor and antithrombin III) in the chromatographic and electrophoretic experiments. These data, together with the titration and heat stability studies, are consistent with the view that antithrombin III and activated Factor X inhibitor activities belong to a single substance.

Identity of Antithrombin III and Heparin Cofactor

In view of the data presented above, supporting the identity of antithrombin III with activated Factor X inhibitor, an examination of the relationship of antithrombin III with heparin cofactor (defined as the substance present in both plasma and serum which is required for the anticoagulant action of heparin) was undertaken.
Factor X inhibitor activities. All three activities cochromatographed with the 280 mg absorbing material in the inhibitor sample applied, Fig. 5. Identical elution patterns were also obtained when the inhibitor fraction was chromatographed on Sephadex G 75 and G 100 under the same conditions. Replacing 0.145 M NaCl with the Tris-maleate buffer did not alter the chromatographic profile of the different activities in the inhibitor fractions on the Sephadex columns.

**Gel Filtration Behavior of Heparin Cofactor and Activated Factor X Inhibitor**—The fact that the heparin cofactor, the activated Factor X inhibitor, and the antithrombin III activities cochromatographed with the protein in a purified fraction of activated Factor X inhibitor further suggested that all three activities might belong to a single protein molecule. The following experiments were performed to demonstrate further the relation of heparin cofactor to activated Factor X inhibitor and, therefore, to antithrombin III.

Sephadex G-200 packed in a single Lucite column, 0.95 × 40 cm, was used for all the filtration experiments carried out at 20°C with a flow rate of 3 ml per hour controlled with a hydrostatic head. Fractions of 1 ml each were collected in a LKB fraction collector. For each set of filtrations and using the same salt concentration, 50 ml of the buffer were allowed to pass through the column between each run, and when a buffer of a different ionic strength was used, the column was re-equilibrated with 200 ml of the desired buffer before use. The experiments presented in Fig. 6 were chromatographed in the following order: First, System A; second, System D; third, System B; and last, System C. Dextran blue was used as a void volume (V₀) marker for each set of filtrations using different buffers. The total volume of the samples loaded was always 1 ml for each experiment and the materials to be chromatographed were contained in the same buffer as that for elution. The various concentrations of NaCl were dissolved in 0.02 M Tris-HCl, pH 7.2.

When 1.25 mg of the inhibitor alone was chromatographed on the Sephadex G-200 column, it emerged as a symmetrical peak irrespective of the ionic strength of the buffers used. The relative elution volume, Vₑ/V₀ (where Vₑ is the elution volume of the inhibitor and V₀ is the void volume), of the inhibitor with 0.01 M NaCl was 1.83 and that with 0.10 M, 0.20 M, and 0.30 M NaCl was 1.77. When 500 USP units of heparin alone were chromatographed, the elution profiles were typical of those for macromolecular substances. At a low salt concentration, such as 0.01 M, part of the heparin fraction behaved like a compound with a molecular weight approaching 200,000 (the exclusion limit of Sephadex G-200) with Vₑ/V₀ equal to 1. The remainder of the fraction was eluted over a broad peak with a Vₑ/V₀ equal to 1.83, the same as that for the inhibitor alone. However, when the heparin was eluted with 0.10 M NaCl, the Vₑ/V₀ was 1.38. The activity spread over the entire bed with slight shoulderings on the descending slope of the peak. At 0.20 M NaCl, the heparin was eluted in a slightly narrower peak with a Vₑ/V₀ equal to 1.53. Finally, when the ionic strength was increased to 0.30, there was less activity near the V₀ region and the Vₑ/V₀ of the heparin was 1.77, the same as that of the inhibitor alone.

In all instances, the heparin behaved like a heterogeneous compound with a molecular weight ranging from approximately 40,000 to greater than 200,000. When a fraction of activated Factor X inhibitor was chromatographed together with 500 units of heparin, the elution profile for each substance was altered for each salt concentration used. The inhibitor protein (measured by 280 mg absorbance), when eluted at an ionic strength of less than 0.3, behaved as a heterogeneous substance with a wide range of molecular weights. It cochromatographed with the heparin. At 0.30 M NaCl, the inhibitor was eluted near symmetry. In all four experiments, both the heparin activity and the inhibitor peaked together, and had the same Vₑ/V₀ as that of the inhibitor when chromatographed alone under the same conditions. Therefore, the relative elution volume of the heparin, when chromatographed together with the inhibitor, was shifted to the right, indicative of complex formation with the inhibitor protein.

The fractions were furthermore tested for heparin cofactor activity, that is, the "immediate" antithrombin effect. A major heparin cofactor activity peak was found in the void volume region of the Sephadex column using 0.01 M NaCl as eluent. The rest of the activity spread over the entire column. The activity peak did not cochromatograph with either the inhibitor protein or the heparin activity. When the heparin plus inhibitor mixture was chromatographed and eluted with either 0.10 M or 0.20 M NaCl, two distinct heparin cofactor activity peaks separated from both the inhibitor protein and heparin peaks were seen for each run. The inhibitor protein and heparin peaks showed less skewing with increasing ionic strength. At an ionic strength of 0.3, the inhibitor protein peak was nearly symmetrical. There was a single high plateau of heparin cofactor activity that cochromatographed with the inhibitor protein. When the heparin in the heparin cofactor activity peaks (Figs. 14 and 20) from System B was neutralized with protamine sulfate, and then tested for antithrombin III activity, it was found that antithrombin III activity was present in proportion to the amount of inhibitor protein. If the peak fraction of the heparin cofactor activity peak (Fraction 13) from System A was...
Fig. 6. Gel filtration of heparin, activated Factor X inhibitor, and heparin plus inhibitor mixture on Sephadex G-200 packed in a Lucite column, 0.95 X 40 cm, at 26°. Heparin was measured by incubating, in a siliconized tube, 0.1 ml of inhibitor (20 μg), 0.1 ml of the fraction (diluted with 0.145 M NaCl at 1:10 to 1:100), and 0.2 ml of fibrinogen (Fraction I-4) at 37° for 10 s. The mixture was then clotted with 0.1 ml of thrombin (15 units). The amount of thrombin inhibited was noted and the level of heparin causing any fraction with an activity that could neutralize activated Factor X, Dr. Deykin generously provided us with all the remaining fractions employed in his original study. These fractions were tubes 35, 36, and 37 from Fig. 1 of Reference 23 obtained by Deykin, Cochios, and Mosher from their gel filtration on a Sephadex G-200 column. Since tube 36 was exclusively used for their experiments as reported in their Figs. 2 and 3, we examined its effect in our system on activated Factor X inhibitor.

All three fractions, transported in Dry Ice, contained, upon thawing, some insoluble material and after removal of the precipitate the supernatants appeared opaque and mucoid. Tube 36 had a total protein content of 3.5 mg per ml. On polyacrylamide gel disc electrophoresis at pH 9.5 the material revealed 10 globulin bands without any detectable protein migrating in the albumin region. At low protein content the material had a high absorbance at 260 μm. Confirming the published observations of Deykin et al., all three fractions greatly prolonged the prothrombin and partial thromboplastin times, and only slightly prolonged the thrombin time. Upon heating at 56° for 10 min, all fractions completely lost their anticoagulant effect.

The action of the hepatic inhibitor on activated Factor X was tested in our specific assay system for activated Factor X inhibitor activity. When 0.2 ml of a 1:2 dilution of tube 36 (containing 350 μg of protein) was incubated with 0.1 ml of activated Factor X (10 units) and 0.7 ml of 1% bovine serum albumin in 0.145 M NaCl, at the end of 60 min, 98% of the any fraction with an activity that could neutralize activated Factor X, Dr. Deykin generously provided us with all the remaining fractions employed in his original study. These fractions were tubes 35, 36, and 37 from Fig. 1 of Reference 23 obtained by Deykin, Cochios, and Mosher from their gel filtration on a Sephadex G-200 column. Since tube 36 was exclusively used for their experiments as reported in their Figs. 2 and 3, we examined its effect in our system on activated Factor X inhibitor.

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Dependence

0.1 ml of heparin, which replaced the heparin, indicated an acceleration effect on the activated Factor X instead of 60 min when the hepatic fraction was omitted. This anticoagulant effect was partially corrected by protamine sulfate. When 350 µg of the material (antithrombin III and activated Factor X inhibitor) belong to the same protein molecule. Similarly, Figs. 5, 6, and Table I provide support for our view, as well as that of others (4-13), that antithrombin III and heparin cofactor activities are associated with the same protein. These findings permit us to reinterpret the data of Porter et al. (21) by first considering briefly certain physical aspects of Sephadex gel filtration.

In general, gel filtration permits the fractionation of compounds with different molecular weights or sizes. In rare instances, difficulties are encountered because of the nature of the dextran gel or the molecular structure of the materials fractionated. Changes occur, moreover, with the varying ionic strength of the solvent employed. Often, the factor of molecular sieve behavior is observed. Dextran gel contains a small number of free hydroxyl groups and behaves as a weak polyanion. Unless these charges are suppressed, highly acidic compounds will be excluded from the gel matrix regardless of their molecular weights (26-36). Heparin is one of these compounds, as demonstrated by Skalka (32), whose findings were confirmed here when heparin alone was chromatographed on Sephadex G-200 and eluted with different ionic strength buffers (Fig. 6). In all instances heparin was eluted as a heterogeneous compound with apparent molecular weights ranging from approximately 40,000 to greater than 200,000. The elution profiles of heparin alone on the Sephadex column have served as controls for the behavior of this compound under the experimental conditions chosen, in this study, to demonstrate the identity of activated Factor X inhibitor with heparin cofactor activity. Porter et al. (20, 21) did not indicate that they had performed such controls, but had simply assumed that since heparin is a low molecular weight substance (less than 16,000) it should completely penetrate the gel matrix and thus be eluted much later than albumin. Such an assumption, however, is valid only for globular substances that do not interact with the gel.

The experiments in which heparin was chromatographed together with the inhibitor to activated Factor X (Fig. 6, bottom, horizontal column) suggest a complex formation between these two substances. The fractions containing the heparin + inhibitor complex blocked the thrombin-fibrinogen reaction instantaneously, in a manner similar to the well known antithrombin action of heparin plus the plasma cofactor. With 0.01 M NaCl a heparin cofactor activity peak was eluted in the void volume with much of the remaining activity spread over

| Inhibitor concentration | Activated Factor X inhibited | Thrombin inhibited |
|-------------------------|-----------------------------|------------------|
|                         | Without heparin | With heparin | Without heparin | With heparin |
| µg/reaction | units/reaction | units/reaction | units/reaction | units/reaction |
| 5          | 15            | 20           | 5              | 8             |
| 4          | 13            | 20           | 0.5            | 1.50          |
| 3          | 12            | 19.4         | 0              | 0.30          |

The mixture contained 0.2 ml of activated Factor X (20 units), 0.1 ml of 1% bovine serum albumin in 0.02 M Tris-maleate, pH 7.5, and 0.2 ml of inhibitor incubated at 37°C for 30 min.

In the control experiments, an equal volume of 0.145 M NaCl replaced the heparin, and 0.02 ml Tris-maleate pH 7.5 replaced the inhibitor.

**Table I**

Dependence of heparin cofactor and antithrombin III activities on activated Factor X inhibitor concentration

In the control experiments, an equal volume of 0.145 M NaCl replaced the heparin, and 0.02 ml Tris-maleate pH 7.5 replaced the inhibitor.

These data strongly suggest that the liver inhibitor is not identical with our plasma inhibitor and is not a specific inhibitor of activated Factor X.

**DISCUSSION**

As one reviews the literature on this subject, one becomes aware that the issue of the identity of antithrombin III with heparin cofactor has remained unresolved. Seegers et al. (9) in 1942, for example, claimed identity for antithrombin III and heparin cofactor. Yet, in 1954, Poll et al. (17) reported that these two activities belonged to different substances without referring to Seeger’s earlier findings.

More recently, Abildgaard (13) prepared a fraction of antithrombin III by disc electrophoresis and found it to possess heparin cofactor activity. From this single observation he concluded that both antithrombin III and heparin cofactor were identical with one another. Porter, Porter, and Shanberry (21), using Sephadex gel filtration and electrophoretic techniques on heparinized plasma, obtained certain elution patterns that they interpreted as proof that they had partially separated heparin cofactor from antithrombin III. Based on their observations they concluded that these two activities were different plasma components. Ganrot (22), on the basis of his work, came to a similar conclusion.

The data presented in Figs. 2 through 5 indicate that antithrombin III and activated Factor X inhibitor are inseparable from each other by essentially all of the physical techniques commonly employed in the separation of macromolecules. Combined with the results obtained from heat stability and titration studies, these data are consistent with the interpretation that these two naturally occurring anticoagulant activities (antithrombin III and activated Factor X inhibitor) belong to the same protein molecule. Similarly, Figs. 5, 6, and Table I provide support for our view, as well as that of others (4-13), but not that of Porter et al. (21), that antithrombin III and heparin cofactor activities are associated with the same protein. These findings permit us to reinterpret the data of Porter et al. (21) by first considering briefly certain physical aspects of Sephadex gel filtration.

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the entire column. Upon increasing the salt concentration to 0.3 M NaCl, the heparin cofactor activity cochromatographed with both the heparin and the inhibitor protein. The heparin cofactor peaks observed at lower ionic strength do not represent a separation of this activity from the activated Factor X inhibitor or antithrombin III. The displacement of the heparin cofactor peaks from the inhibitor peak cannot be explained on the basis of differences in molecular weight or size of the heparin-inhibitor complex, because no definite inhibitor protein peaks coinciding with those of heparin cofactor activity were obtained. The following explanations are offered for this phenomenon. In low ionic strength solvents there is much electrostatic interaction between the heparin molecule of the heparin-inhibitor complex and the dextran gel. This causes the electroheterogeneous population of heparin molecules to rearrange into groups carrying a similar number of electrical charges. The group capable of causing higher electrostatic repulsion will, therefore, be excluded faster from the gel. These highly charged groups possess greater anticoagulant activity and may reflect the presence of larger amounts of sulfate ester groups at all primary hydroxyl positions. It is the 6-O sulfate group that confers the anticoagulant activity to the heparin molecule rather than the actual molecular weight (37). Using heparinized whole plasma, Porter et al. (21) interpreted a similar, but less defined, phenomenon as a separation of the heparin cofactor from the antithrombin III. This separation occurred, in their view, by virtue of the heparin complexing with its plasma cofactor thereby resulting in the formation of a larger molecular weight complex that was eluted earlier than antithrombin III. In our study, when ionization of the free charged groups is suppressed by high salt concentration, e.g. in the experiment employing 0.3 M NaCl (Fig. 6), electrostatic interactions are minimized, and, therefore, the heparin cofactor cochromatographed with the activated Factor X inhibitor.

Porter et al. (21) attempted further to support their claim that heparin cofactor and antithrombin III are two different substances by their failure to detect the latter activity in a fraction containing heparin plus cofactor, after neutralization of the heparin. They reasoned that, if heparin cofactor and antithrombin III belonged to a single substance, then the neutralization of the anticoagulant in their isolated fraction containing heparin cofactor activity should demonstrate progressive antithrombin activity. Although this is a valid argument, they did not determine the amount of heparin cofactor protein in the fractions they examined. When the heparin in the fractions containing heparin cofactor activity (Fig. 6) is neutralized with protamine sulfate, these fractions demonstrate antithrombin III activity. The amount of antithrombin III activity detected was dependent upon the quantity of activated Factor X inhibitor protein present in each fraction. The data presented in Table I further substantiate the thesis that heparin cofactor, antithrombin III, and activated Factor X inhibitor activities are identical. The observed increase in the inhibition of either enzyme in the presence of heparin over those values in the absence of heparin is related to the fact that the control experiments (no added heparin) were not allowed to proceed to equilibrium with the inhibitor. It is evident that, in the presence of heparin, the inhibitory action of minute quantities of activated Factor X inhibitor on either activated Factor X or thrombin can be maximally achieved instantaneously, whereas, in its absence, the time period required to achieve the same degree of inhibition would be so great that it might even exceed the half-life of the inhibitor.

Great precautions should also be exercised in all these studies, to rule out any contamination of activated Factor X in the fibrinogen preparations employed. We have consistently obtained spuriously high degrees of thrombin inhibition with crude fibrinogen preparations contaminated by trace amounts of the activated Factor X inhibitor in the presence of heparin. Even the mere addition of small amounts of heparin to the crude fibrinogen preparations caused a great delay in clotting, when compared with the Blombäck Fraction I-1 (38) fibrinogen preparation, or even the Kabi product (human fibrinogen, grade L, AB Kabi, Stockholm, Sweden). These points have not been considered previously by investigators who have claimed that heparin cofactor and antithrombin III are separate entities. In fact, most experiments reported in the literature have employed crude fibrinogen preparations that in our laboratory have been shown to contain significant amounts of activated Factor X inhibitor (heparin cofactor).

Liver inhibitors of blood coagulation have been isolated by several investigators (25, 39-41). Recently Deykin et al. (23) reported that they had isolated a specific inhibitor of activated Factor X from bovine liver. This inhibitor prolonged both the intrinsic and extrinsic coagulation assay systems. No specific assay for activated Factor X inhibition was employed. The inhibitor was completely inactivated at 56°C within 10 min, and prolonged slightly the thrombin-fibrinogen reaction. The addition of the crude liver inhibitor fraction to a thrombin generation test system caused a reduction of the final amount of thrombin formed from 0.65 to 0.35 unit. From this experiment they inferred that the inhibitor acted specifically against activated Factor X, despite the fact that they were working with a multi-component reaction system, in which an adverse effect on any one of the reactants could influence the overall kinetics. We were interested in this hepatic inhibitor primarily to determine its relationship to our plasma inhibitor. Having repeatedly failed to detect any specific inhibition on purified activated Factor X with hepatic inhibitor fractions that prolonged the Quick prothrombin time and the partial thromboplastin time, prepared according to their published method (23), we obtained from Dr. Deykin for comparative studies the original fractions used in his reported experiments. No inhibition of activated Factor X was observed when it was incubated with the hepatic inhibitor fractions supplied by Dr. Deykin. This was true even though the concentration of this inhibitor fraction employed in our test system was increased up to 12-fold the amount used in their published report without any loss of activated Factor X activity. The behavior of this hepatic inhibitor was, in fact, rather reminiscent of the one observed by us previously in liver perfusates (42).

In conclusion, based on the data presented both in this and the preceding reports (1, 43), we propose that the key function of activated Factor X inhibitor as a natural anticoagulant may be primarily concerned with regulating hemostatic balance through neutralization of activated Factor X; a reaction profoundly enhanced by traces of heparin. Whether we are dealing with a polyanivalent inhibitor or one with a single substrate site for the enzymes studied is not conclusively demonstrated. However, the fact that both activated Factor X and thrombin (as well as plasmin and trypsin\(^2\)) are all inhibited by the activated Factor X

\(^2\) E. T. Yin and S. Wessler, unpublished data.
inhibitor, and that these activities belong to the serine group of enzymes, tends to favor the view that these anticoagulant activities are functions of a single site. We further propose that this α2-globulin inhibitor previously termed antithrombin III or heparin cofactor, be now appropriately designated activated Factor X inhibitor.

Finally, there are several implications of this investigation that are of clinical interest. One of these, perhaps, deserves mention here: namely, the possibility that an altered level of plasma-activated Factor X inhibitor may serve as an indicator that intravascular coagulation may have occurred. For, if systemic hypercoagulability is reflected in the level of circulating activated Factor X inhibitor activity, the methodology herein presented is capable of determining the validity of such an important hypothesis.

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