Preparation, Characterization, and Turnover Properties of Heparin-Antithrombin III Complexes Stabilized by Covalent Bonds*

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High affinity heparin obtained by chromatography of clinical grade heparin on antithrombin III-Sepharose was covalently coupled to antithrombin III using a three-step procedure: 1) introduction of free amino groups in the heparin molecule; 2) reaction of these amino groups with the bifunctional reagent tolylene-2,4-diisothiocyanate; and 3) reaction of the remaining isothiocyanate group with amino groups on antithrombin III. Amino groups were introduced in the heparin molecule by limited N-desulfation or by reaction of carboxyl groups with hexamethylenediamine with the use of a water-soluble carbodiimide. On average between 1 and 2 mol of NH₂ groups were introduced/15,000 g of heparin. The yield of coupling of the isothiocyanate-substituted heparin to antithrombin III was around 30% and coupling occurred preferentially with an apparent 1:1 stoichiometry for both substituted hep- arins. The complexes were separated from free heparin by chromatography on antithrombin III-Sepharose or on DEAE-Sephadex and from reacted antithrombin III by gel filtration on a high performance liquid chro- matography column or by gradient elution from hepa- rin-Ultrogel.

The covalent heparin-antithrombin III complexes inhibited factor Xa with a second order rate constant of 2.1 ± 0.1 × 10⁴ M⁻¹ s⁻¹, which is similar to that found for the noncovalent complex. Neither addition of heparin nor antithrombin III significantly influenced the inhibition rate of factor Xa by the covalent complex.

The half-life of the stabilized complexes in blood fol- lowing intravenous injection in rabbits was 0.68 ± 0.08 h for the N-desulfated heparin-antithrombin III complex and 0.99 ± 0.27 h for the hexamethylenediamine-substituted heparin-antithrombin III complex, which are 2.4 and 3.5 times longer than the half-lives of free or hexamethylenediamine-substituted heparin in the blood. This finding indicates that the main mechanism of disappearance of the anticoagulant activity following intravenous injection of heparin is by removal of free heparin and dissociation of the heparin-antithrombin III complex and not by cleaving of the intact com- plex.

Heparin, a mucopolysaccharide isolated from pig intestine or bovine lung, is heterogeneous with respect to molecular size, chemical structure, and anticoagulant activity. It contains material with molecular weights between 6,000 and 30,000 (1). It is a polymer of repeating uronic acid (iduronic and glucuronic acid) and glucosamine disaccharide units with a varying degree of derivatization (sulfation or acetylation) of amino and hydroxyl groups (2-6). Heparin can be fractionated by affinity chromatography on antithrombin III-Sepharose into high affinity heparin, representing about one-third of the total material, containing practically all the anticoagulant activity, and low affinity heparin with very little anticoagulant activity (7-9).

High affinity heparin strongly binds to antithrombin III, thereby inducing a conformational change in the antithrombin III molecule (10-12). Although antithrombin III apparently has two binding sites for heparin (10, 13), the binding of high affinity heparin to antithrombin III occurs in a 1:1 stoichiometry with a dissociation constant of approximately 10⁻⁶ M (14, 15). The heparin-antithrombin III complex inhibits a number of activated coagulation factors, including thrombin, factor IXa, factor Xa, factor XIa, and factor XIIa (16). The mucopolysaccharide acts as a catalyst of enzyme inactivation by antithrombin III since it can accelerate multiple rounds of enzyme-inhibitor complex formation (15, 17).

In purified systems, the specific activity of low molecular weight heparin (M, < 10,000) toward thrombin is lower than that of high molecular weight heparin (M, > 20,000) whereas the specific anticoagulant activity of heparin toward factor Xa is much less dependent on its molecular weight (18-20). This different behavior has been explained by the additional interaction between heparin and thrombin during the inactivation of thrombin by the heparin-antithrombin III complex (21), which probably is reduced during inactivation by low molecular weight heparin-antithrombin III complexes. Unlike thrombin, factor Xa does not bind heparin, and consequently, factor Xa inactivation is less influenced by the chain length of the heparin molecule. Low molecular weight heparin with a higher specific factor Xa-inhibiting activity might possess antithrombotic properties comparable to high molecular weight heparin but cause less bleeding (20, 22).

The half-life of the anticoagulant activity of heparin in plasma following intravenous injection in man varies between 23 min and 2.5 h (23), whereas that of antithrombin III is 2.7 days (24). At present, it is not clearly established whether the anticoagulant activity disappears from the blood due to clear- ing of the intact complex or to dissociation of the complex in the blood. Following intravenous injection of a mixture of heparin and ³⁹⁸I-labeled antithrombin III in a patient, we observed a marked difference in the disappearance rates of the anticoagulant activity and the label (24), suggesting that dissociation of the complex occurred. On the basis of this finding, we anticipated that stabilized active antithrombin III-heparin complexes might circulate longer in the blood and be cleared with a half-life between that of free heparin and free antithrombin III. The present study, undertaken to validate
this hypothesis, deals with the preparation and characterization of such stabilized complexes and their turnover in rabbits.

EXPERIMENTAL PROCEDURES

Materials

Picrilsulfonic acid, carbazole, tolylene-2,4-diisocyanate, and N,N'-diiallyltartardiamide were purchased from Aldrich; 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride, trifluoroacetic acid, and N,N-dimethyl-N-allylamine were obtained from Pierce; hexamethylenediamine, sodium tetra borate, BaCl₂, gelatin, sodium nitrate, trichloroacetic acid, pyridine, and dimethylulfoxide from E. Merck. Bovine serum albumin was from Poviet Producten (Os, The Netherlands), Folin phenol reagent from Hartman-Ledon (Philadelphia, PA), and [³⁵S]phenylisothiocyanate from Amersham. Dowex 50W-X8 (H⁺) (20 to 50 mesh) was purchased from Sigma and Sephadex products from Pharmacia. The synthetic substrates S-2238 and S-2222 were from Kabi (Stockholm, Sweden).

Thrombin was purified according to the method of Fenton et al. (25) with an average activity of 2,200 units/mg. Alternatively, thrombin was used, which was a gift from Kabi (1,800 NIH units/ml). Molar concentrations were calculated assuming a specific clotting activity of 2,800 NIH units/mg and a Mₛ of 36,500. Factor Xa was from Coatest kits (Kabi). The factor Xa concentration was determined by titration against antithrombin III of known activity and was in agreement with the rate of hydrolysis of the synthetic substrate S-2222, measured spectrophotometrically at 405 nm. Heparin activity was determined by affinity chromatography on heparin-Ultrogel (20) (approximately 5 mg of heparin/ml of gel, purchased from Pharmindustrie IBF, Clichy, France), followed by gel chromatography on Ultrogel AcA 150 (24). Antithrombin III was labeled with [¹²⁵I] by the method of McFarlane (27) and recycled on heparin-Ultrogel to a specific radioactivity of 0.5 to 2 µCi/mg of protein.

Some of the high affinity heparin used in this study was a stabilized heparin-antithrombin III-Sepharose 4B, followed by gel chromatography on Ultrogel AcA 150 (24). Antithrombin III was labeled with [¹²⁵I] by the method of McFarlane (27) when modified by N-desulfation in dimethylsulfoxide or by measuring the N-sulfate content through turbidimetry of the uronic acid content with the carbazole borate method as described by Bitter and Muir (29) when modified by N-desulfation in dimethy-l-L-arginine-p-nitroanilide in o-dichlorobenzene at 190 °C (28).

Methods

Heparin Determination—Heparin was determined by measuring the uronic acid content with the carbazole borate method as described by Bitter and Muir (29) when modified by N-desulfation in dimethylsulfoxide or by measuring the N-sulfate content through turbidimetric activity of the inorganic sulfate liberated after treatment of the sample with in o-dichlorobenzene at 190 °C. When indicated otherwise, the amount of heparin in the stabilized complexes was calculated from the specific radioactivity to partial thromboplastin time.

The anticoagulant activity of heparin in a plasma system was determined with a modified activated partial thromboplastin time (32). To 100 µl of aqueous heparin solution (0.5 to 2 units/ml) was added 200 µl of citrated normal human plasma, 100 µl of kaolin suspension (0.5 g/100 ml) and 100 µl of 20-fold diluted Thrombofax solution (Ortho Diagnostica). After 6 min incubation at 37 °C, 100 µl of 0.05 M CaCl₂ solution was added and the clotting time measured. Heparin was also determined by factor Xa inhibition using the chromogenic substrate S-2222, either in a plasma medium (33, 34) or in a purified system (18). The same procedures were used to monitor the disappearance rate of the anticoagulant activity of the stabilized heparin-antithrombin III complexes in rabbits. Heparin anticoagulant activity was expressed in units by comparison with the Third International Standard (Porcine) 65/69.

Antithrombin III Determination—Purified antithrombin III was measured spectrophotometrically using A₄₀₅ (280 nm) = 6.1, or by amino acid analysis. Concentrations determined by both methods agreed within 5%. Antithrombin III in the stabilized complexes was determined as described by Oprea et al. (35) using the N-terminus as standard or by Laurell immunoelectrophoresis (36) using antithrombin III in the presence of 100 units/ml of heparin as standard.

The antithrombin III concentration for kinetic measurements was determined by titration against thrombin. The purified preparations were used at 90% active. The concentration of active antithrombin III in the purified covalent complexes was likewise determined by titration against thrombin or factor Xa.

High Affinity Heparin—Heparin was separated into a low affinity and a high affinity fraction by the method of Anderson et al. (7). Between 1,000 and 1,500 units of heparin were applied to a column (2.5 x 15 cm) of antithrombin III-Sepharose (containing 3 mg of protein/milliliter of gel), equilibrated with 0.05 M NaCl, 0.05 M phosphate buffer, pH 7.5, at 4 °C and at a flow rate of 60 ml/h. The adsorbed fraction was eluted with 1.5 M NaCl, 0.05 M phosphate buffer, pH 7.5, and the column re-equilibrated with the starting buffer for reuse. Heparin Modification—In the N-desulfation reaction (31, 37), approximately 10,000 units of high affinity heparin were applied at 4 °C to a column (1.5 x 20 cm) containing 21 g of Dowex 50W and eluted with water. The effluent was neutralized with pyridine. After lyophilization, all the biological activity was recovered. Partial N-desulfation was performed by the method of Oprea et al. using 3.5 ml of dimethylulfoxide and vigorous stirring at room temperature for 35 min (31). The mixture was then diluted with an equal volume of water and the pH was adjusted to 9.5 by addition of NaOH. The solution was dialyzed for 2 days against water and lyophilized.

In the hexamethylenediamine substitution reaction (38, 39), approximately 10,000 units of high affinity heparin and 70 mg of hexamethylenediamine in 5 ml of H₂O were mixed and the pH was adjusted to 4.75. Then 55 mg of carbodiimide in 2.1 ml of H₂O, pH 4.75, was added and the mixture was stirred for 10 min at room temperature, while the pH was kept between 4.5 and 5.0 by addition of 0.1 M HCl. Finally, 15 ml of 1.5 M NaCl, 0.05 M disodium tetraborate buffer, pH 9.7, was added and the mixture was dialyzed against the same buffer for 1 day at room temperature, followed by dialysis against water for 2 days at 4 °C.

Primary amino groups in the heparin molecule were measured by the method of Yosizawa et al. in which a 1-ml solution containing 2 mg of heparin is reacted with picrilsulfonic acid (40). Absorbance at 348 nm was converted to concentration of NH₂ groups using a calibration curve constructed with norleucine or with hexamethylenediamine. Concentrations determined by both methods agreed within 5%. Antithrombin III in the stabilized complexes was determined with a modified activated partial thromboplastin time.

Heparin Coupling to Antithrombin III—Amino groups introduced in the heparin molecule were measured by the method of Oprea et al. in which a 1-ml solution containing 2 mg of heparin is reacted with picrilsulfonic acid (40). Absorbance at 348 nm was converted to concentration of NH₂ groups using a calibration curve constructed with norleucine or with hexamethylenediamine. The extent of coupling of heparin to antithrombin III was determined by titration against thrombin or factor Xa.

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Covalent Heparin-Antithrombin III Complexes

RESULTS

Covalent Coupling of Heparin to Antithrombin III—The coupling of heparin to antithrombin III was performed in three steps (Fig. 1b): introduction of amino groups in the heparin molecule (step 1), reaction of the amino groups with tolylene-2,4-diisothiocyanate (step 2), and reaction of the remaining isothiocyanate group with an amino group in the antithrombin III molecule (step 3). The introduction of amino groups in heparin was achieved in two ways: A, a solvolytic N-desulfation of the sulfamic acid derivative (compound 1) in polar medium; or B, coupling of the carboxyl group of uronic acid residues (compound 7) with an amino group of hexamethylenediamine (compound 9) using 1-ethyl-3-(3-dimethyl aminopropyl)-carbodimide (compound 8). The second step consisted in coupling of tolylene-2,4-diisothiocyanate (compound 3) to the modified heparin (compound 2 or 10) by reaction of one isothiocyanate group of the bifunctional reagent with an amino function of the mucopolysaccharide to form thiocarbamyl derivatives of the heparin molecule (compounds 4 and 11). Finally, the covalently stabilized heparin-antithrombin III complexes (compounds 6 and 12) were formed by reaction of the remaining isothiocyanate function in compounds 4 and 11 with an amino group in the protein (compound 5).

After N-desulfation (pathway A) approximately 65% of the activity was recovered. This modified heparin was used without further fractionation for coupling to antithrombin III and subsequent turnover studies. The heparin can however be separated into a low affinity and a high affinity fraction by chromatography on antithrombin III-Sepharose. The yields, specific activities, and the number of amino groups of the heparin fraction are presented in Table I. These results represent mean values of three to five independent preparations. In three preparations, addition of [35S]heparin was omitted in order to allow quantitation of the number of amino groups by [35S]phenylisothiocyanate incorporation. Four preparations of comparable size but with added [35S]heparin were made and used for coupling to antithrombin III and subsequent turnover studies. The recovery and specific activity (measured by APTT) of these preparations were comparable to those reported from the previously described methods.
followed by rechromatography, resulted in a reduction of the preparation.

Chromatography on an antithrombin III-Sepharose column, heparin, PITC, phenylisothiocyanate, revealed a recovery of about 85% of the original heparin activity of the starting material (about 56%). Measurement by APTT, results are expressed as percentage of the activity of the starting material (about 10,000 units of heparin in each preparation).

Purification of the Heparin-Antithrombin III Complexes—Before AT III-Septa-Dase A-35 with a linear gradient of NaCl, antithrombin III and the heparin-antithrombin III complex were only partially

TABLE I
Introduction of amino groups in heparin

<table>
<thead>
<tr>
<th>Recovery of activity*</th>
<th>Specific activity</th>
<th>Picrilsulfonic acid method</th>
<th>35S-labeled PITC method*</th>
</tr>
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<tbody>
<tr>
<td>High affinity heparin</td>
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<td></td>
</tr>
<tr>
<td>N-Desulfated heparin</td>
<td></td>
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</tr>
<tr>
<td>Before AT III-Sepharose</td>
<td>65 ± 5 (6)</td>
<td>191 ± 28 (6)</td>
<td>1.6 ± 0.4 (4)</td>
</tr>
<tr>
<td>Low affinity fraction</td>
<td>7 ± 6 (5)</td>
<td>33 ± 18 (5)</td>
<td>1.9 ± 0.3 (4)</td>
</tr>
<tr>
<td>High affinity fraction</td>
<td>45 ± 9 (5)</td>
<td>196 ± 26 (5)</td>
<td>1.4 ± 0.5 (4)</td>
</tr>
<tr>
<td>High affinity heparin</td>
<td>100 ± 0 (2)</td>
<td>100 ± 0 (2)</td>
<td>0.9 ± 0.2 (2)</td>
</tr>
<tr>
<td>Hexamethylenediamine-substituted heparin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before AT III-Sepharose</td>
<td>56 ± 7 (4)</td>
<td>153 ± 34 (3)</td>
<td>1.6 ± 0.6 (3)</td>
</tr>
<tr>
<td>Low affinity fraction</td>
<td>3 ± 3 (4)</td>
<td>22 ± 19 (3)</td>
<td>2.4 ± 0.8 (3)</td>
</tr>
<tr>
<td>High affinity fraction</td>
<td>39 ± 4 (4)</td>
<td>162 ± 25 (3)</td>
<td>1.4 ± 0.2 (3)</td>
</tr>
</tbody>
</table>

* Determined by APTT; results are expressed as percentage of the activity of the starting material (about 10,000 units of heparin in each preparation).

These results were obtained in preparations without use of [35S]heparin, PITC, phenylsulfoxycyanate.

ported in Table I. Following the hexamethylenediamine substitution reaction (pathway B) and lyophilization approximately 65% of the activity was recovered. The modified heparin was applied to antithrombin III-Sepharose and separated into a low affinity and a high affinity fraction. The latter was used in the coupling reaction with antithrombin III and the subsequent turnover experiments. Results of four independent preparations are given in Table I. Again, three preparations were made without addition of [35S]heparin and used for titration of amino groups with [35S]phenylisothiocyanate. Four preparations with added [35S]heparin were made with similar yields and specific activities and used for further studies.

The heparin used as starting material contained less than one NH2 group per molecule, but this number increased following modification as measured both with picrilsulfonic acid and phenylsulfoxycyanate (Table I). The substituted heparin could be fractionated by chromatography on antithrombin III-Sepharose into a low affinity fraction with a somewhat higher content of NH2 groups per molecule and a high affinity fraction with a slightly lower content of NH2 groups. The determinations of amino groups were, however, not very reproducible and the data obtained with both methods did not correlate very well.

SDS-gel electrophoresis of the mixture after the coupling reaction combined with 35S measurement in the sliced gel (Fig. 2) revealed that 25 to 30% of the heparin was coupled to antithrombin III. Total heparin recovery as judged from 35S measurement was 85 to 90%. Measurement by APTT also revealed a recovery of about 85% of the original heparin activity.

Purification of the Heparin-Antithrombin III Complexes—Chromatography on an antithrombin III-Sepharose column, followed by rechromatography, resulted in a reduction of the amount of uncoupled heparin in the unadsorbed fraction to approximately 10% in preparations in which the heparin had been rechromatographed on antithrombin III-Sepharose following modification to remove inactivated heparin molecules (hexamethylenediamine-substituted heparin) and to approximately 40% in preparations where this step had been omitted (N-desulfated heparin) (Fig. 3). The recovery of heparin-antithrombin III complex in this procedure was between 30 and 50% of that formed in the coupling reaction. This low yield was due to binding of a fraction of the heparin-antithrombin III complex to the antithrombin III-Sepharose column, as evidenced by SDS-gel electrophoresis and 35S measurements on the fraction eluted at high ionic strength (not shown).

On high performance liquid chromatography, the heparin-antithrombin III complexes eluted earlier than free antithrombin III and free heparin. In this way, a partial separation between complex and free materials could be obtained with a recovery of complex of about 50%.

When the coupling mixture was eluted from heparin-antithrombin III complexes eluted earlier than free antithrombin III and free heparin. In this way, a partial separation between complex and free materials could be obtained with a recovery of complex of about 50%. When the coupling mixture was eluted from DEAE-Sephadex A-25 with a linear gradient of NaCl, antithrombin III and the heparin-antithrombin III complex were only partially...
separated but were virtually free of heparin, which only eluted above 0.5 M NaCl as evidenced by SDS-gel electrophoresis. Chromatography of the mixture of heparin-antithrombin III complex and free antithrombin III on heparin-Ultrogel revealed that the complex eluted earlier and completely separated from free antithrombin III and from a small amount of a complex between 2 antithrombin III molecules with 1 heparin molecule (see below). The recovery of radioactivity in the first step is about 70% while virtually all the material is recovered in the second chromatography.

Crossed immunoelectrophoresis of the complexes in the presence and absence of heparin revealed that the covalent complex had the same electrophoretic mobility as the reversible complex (Fig. 4). Pure antithrombin III migrates as an α2-globulin in the absence of heparin (Fig. A1) and as a prealbumin when heparin is incorporated in the gel (Fig. B1). In the complex before purification, approximately 30% of the antithrombin III antigen migrates as prealbumin in the absence of heparin (Fig. A2), but the major part of the uncomplexed antigen can still react with heparin (Fig. B2). These results suggest that the coupling of heparin to antithrombin III has mainly occurred with a 1:1 stoichiometry since approximately 30% of the antithrombin III and 33% of the heparin are coupled to each other from a starting mixture with a 20% molar excess of antithrombin III. During purification on antithrombin III-Sepharose and gel filtration on the TSK 255-42 HPLC column, the uncoupled antithrombin III is removed from the sample (Fig. 4A3 and B3). Combination of DEAE-Sephadex-A-25 and heparin-Ultrogel chromatography also yielded a component which migrates as a prealbumin on crossed immunoelctrophoresis.

SDS-gel electrophoresis of purified heparin-antithrombin III complexes revealed a main peak, migrating in the antithrombin III region, and a smaller peak with a slower mobility (Fig. 3). In order to establish the identity of these complexes, the eluates from the Spherogel HPLC column were collected in an early fraction (±30% of the total complex, based on radioactivity measurements) and a late fraction (±80%). SDS-gel electrophoresis showed that the early fraction mainly corresponded to the slowly migrating material whereas the late fraction contained only material corresponding to the main peak. The stoichiometry of these two fractions, determined by measuring heparin (specific radioactivity) and antithrombin III (Lowry method) was 1:1.9 in the early fraction and 1:0.95 in the late fraction. These findings indicate that the slowly migrating material on SDS-gel electrophoresis is a complex of 1 molecule of heparin with 2 molecules of antithrombin III, whereas the main peak represents a 1:1 stoichiometric complex.

The content of tolylene-2,4-disothiocyanate in the complex was calculated from its absorbance at 280 nm, using an $A_{280}^0_{cm} = 1.280$, after correction for the content of antithrombin III ($A_{280}^0_{cm} = 6.1$). The 1:2 heparin-antithrombin III complex (early fraction of Spherogel column) contained 2.2 mol of bifunctional reagent/mol of antithrombin III and 4.3 mol/mol of heparin, while the 1:1 heparin-antithrombin III complex (late fraction) contained 1.3 mol of tolylene-2,4-disothiocyanate/mol of antithrombin III and 1.2 mol/mol of heparin. This indicates that most complexed molecules only contain 1 molecule of bifunctional reagent. The relatively low yield of the coupling reaction (25 to 50%) might therefore be explained by hydrolysis of the second isothiocyanate function after coupling of the bifunctional reagent to the few (average 1.5 to 2.6) available amino functions in the modified heparin molecule.

**Activity and Kinetic Properties of the Complex**—The inhibition kinetics of thrombin by heparin-antithrombin III is more complex than that of factor Xa (21). Therefore, the kinetics of the covalent heparin-antithrombin III complex (hexamethylene diamine-substituted) purified to homogeneity by DEAE-Sephadex-A-25 and heparin-Ultrogel chromatography was investigated using factor Xa. The concentration of functional complex was determined by titration against thrombin using the synthetic substrate S-2238.

Inactivation of factor Xa by antithrombin III revealed that the enzyme activity disappeared with a second order rate constant of $6.8 \times 10^7 \text{m}^{-1}\text{s}^{-1}$. Addition of heparin to the incubation mixture increased the inactivation rate by at least 2 orders of magnitude, which made it very difficult to measure the reaction rate under pseudo-first order conditions with manual sampling techniques. However, when the inhibition rate was measured in the presence of the synthetic substrate S-2222, the reaction rates were sufficiently slow to enable spectrophotometric recording of the hydrolysis rate of the substrate. In this way, factor Xa inactivation by antithrombin III was studied in the presence of an excess of heparin which saturates the inhibitor. Logarithmic plots of the residual enzyme concentration versus inactivation time resulted in monophasic disappearance rates for over 80% neutralization as shown in Fig. 5A. The inhibition is described by the following equilibria and reactions.

$$K_0 \quad A + H \quad \overset{k_0}{\underset{k_{-0}}{\rightleftharpoons}} \quad A \cdot H$$

$$Xa + S \quad \overset{k_S}{\underset{k_{-S}}{\rightleftharpoons}} \quad Xa \cdot S$$

$$K_c \quad A \cdot H + Xa \quad \overset{k_2}{\underset{k_0}{\rightleftharpoons}} \quad A \cdot H \cdot Xa \rightarrow AXa + H$$

with $A$, antithrombin III; $H$, heparin; $S$, synthetic substrate S-2222; $K_D$, dissociation constant of $A \cdot H$ complex; $K_c$, dissociation constant of $A \cdot H \cdot Xa$ complex; $k_0$, first order rate constant of irreversible inactivation; $K_m$, Michaelis constant.
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for S-2222 (0.3 mM); and by the following rate equation:

\[
v = \frac{d[Xa]}{dt} + \frac{d[Xa \cdot S]}{dt} = -k_2[A \cdot H \cdot Xa] = -k_2 \frac{[A \cdot H][Xa]}{K_a}
\]

which yields, upon transformation:

\[
\ln[\text{Xa}] = -\frac{k_2}{k_1/K_a - 1}[A \cdot H]t
\]

with \(k_2/K_a\) the second order rate constant of factor Xa inactivation; \([A \cdot H] = [A^+]\), the initial concentration of antithrombin III since the reaction is carried out under pseudo-first order conditions; \(t\), reaction time. The value of \(k_2/K_a\) can be calculated from the apparent inhibition constant \(k_{app} = (k_2/K_a)/(1 + [S^2]/K_m)\) and was determined at both varying substrate concentrations (0.2 to 0.6 mM) and varying inhibitor concentrations (3 to 12 nM). An average value of \(k_2/K_a\) = 7.0 ± 1.3 × 10^6 M⁻¹s⁻¹ was found for the inactivation of factor Xa by the noncovalent complex heparin-antithrombin III. In a similar way the inactivation of factor Xa by the covalent complex is described by:

\[
\ln[\text{Xa}] = -\frac{k_2}{k_1/K_a - 1}[C]^t
\]

with \(k_2/K_a\), the second order rate constant of factor Xa inactivation; \([C] \approx [C^+]\), initial concentration of covalent heparin-antithrombin III complex, since the reaction is carried out under pseudo-first order conditions. Linear semilogarithmic plots (Fig. 5b) were obtained for enzyme concentration versus reaction time, for different substrate concentrations (0.2 to 0.4 mM), and different concentrations of complex (4 to 8 nM). Factor Xa was inhibited by the covalent complex with a bimolecular rate constant \(k_2/K_a = 2.1 ± 0.1 × 10^6 \text{M}^{-1} \text{s}^{-1}\). This value is about 3 times lower than that obtained for factor Xa inhibition by the noncovalent complex, but 300 times higher than the value obtained with antithrombin III. These data indicate that heparin can be coupled chemically to antithrombin III resulting in a functional heparin-antithrombin III complex.

Addition of antithrombin III to the covalent complex in concentrations up to 0.3 μM did not significantly alter the \(k_{app}\) of the reaction. Addition of heparin (0 to 66 nM) resulted in a concentration-dependent increase in \(k_{app}\) to almost twice its initial value. This may be due to the fact that the purified covalent heparin-antithrombin III complex contains molecules in which the coupling does not occur in the functional configuration. This might explain the lower factor Xa inhibition constant of the covalent as compared to the reversible complex.

Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Heparin</th>
<th>Antithrombin III</th>
<th>Heparin-antithrombin III mixture</th>
<th>N-Desulfated heparin-antithrombin III complex</th>
<th>Hexamethylenediamine-substituted heparin-antithrombin III complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n_S)</td>
<td>Factor Xa inhibition</td>
<td>(n_T)</td>
<td>Xa inhibition</td>
<td>(n_S)</td>
</tr>
<tr>
<td>1</td>
<td>0.23</td>
<td>0.23</td>
<td>11.2</td>
<td>0.18</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
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<td>0.28</td>
<td>10.2</td>
<td>0.28</td>
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</tr>
<tr>
<td>3</td>
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<td>0.31</td>
<td>11.3</td>
<td>0.29</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>0.28</td>
<td>11.0</td>
<td>0.22</td>
<td>0.26</td>
</tr>
<tr>
<td>Mean</td>
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<td>0.28</td>
<td>11.0</td>
<td>0.22</td>
<td>0.26</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.03</td>
<td>0.04</td>
<td>0.4</td>
<td>0.05</td>
<td>0.05</td>
</tr>
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</table>

* \(n_T\) of second term.
inhibitory activity could be described by a single exponential term with a $t_{1/2}$ of $0.68 \pm 0.08$ h. The disappearance rate of $^{38}$S from plasma was biphasic: an initial faster disappearance of about 30% of the radioactivity and a slower disappearance rate of the remainder with a $t_{1/2} = 0.65 \pm 0.06$ h. This observation is consistent with the interpretation that the rapid component is due to removal of contaminating inactive uncoupled heparin and the slow component to removal of active complex. Following injection of hexamethylenediamine-substituted heparin-antithrombin III complex, both the radioactivity and factor Xa-inhibiting activity disappeared with a single $t_{1/2}$ of $0.89 \pm 0.26$ and $0.99 \pm 0.27$ h, respectively.

**DISCUSSION**

The aim of the present study was to couple heparin and antithrombin III covalently to form an active, stabilized complex and to study the turnover of this complex in the blood.

Since antithrombin III is a labile protein, coupling reactions were carried out at near neutral pH, in aqueous solutions, and at temperatures below 40 °C. Reactions involving primary amino groups can be carried out with reasonably good yields under those conditions. Since heparin is a stable mucopolysaccharide which can resist more extreme conditions of pH, temperature, and solvent composition, it was decided to perform primary modification reactions on the heparin molecule, with the aim of introducing a functional group with affinity for amino groups in antithrombin III. It was hoped that mixing of the modified heparin with antithrombin III would then lead to rapid formation of the reversible complex in the active configuration, followed by irreversible coupling.

Direct coupling of heparin after activation with CNBr at pH 10 (47) to antithrombin III failed. Although heparin apparently contains a small amount of amino groups (on average less than 1 mol/mol), attempts to couple heparin to antithrombin III via the bifunctional reagents listed below failed. This may be due to the fact either that the reactivity of heparin in the assays for primary amino groups is due to nonspecific interactions or that available amino groups are situated in unfavorable positions for coupling to antithrombin III. Therefore, attempts were made to introduce a limited number of amino groups in the heparin molecule.

Heparin is a polymer of repeating uronic acid and glucosamine disaccharide units with a varying degree of sulfation or acetylation of amino and hydroxyl groups (2–6). Introduction of amino groups may be achieved either by deblocking amino groups of glucosamine or by modification of reactive carboxyl groups. Both approaches could successfully be performed. By limited N-desulfation of sulfamic acid derivatives in polar groups, both approaches could successfully be performed. By limited N-desulfation of sulfamic acid derivatives in polar medium (31, 37) and by limited transformation of carboxylic groups through reaction with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and hexamethylenediamine (38, 39) on average 1 to 2 mol of amino groups were introduced per 15,000 g of heparin (average $M_r$ of heparin). These modifications, however, led to inactivation of some of the heparin (which was removed by affinity chromatography on antithrombin III-Sepharose) and a 30% decrease of the specific activity of the remaining fraction. It had, however, already been reported earlier that N-sulfate and carboxyl groups are of importance for the anticoagulant activity of heparin (48).

Coupling of the modified heparin to antithrombin III with the following bifunctional reagents under the experimental conditions described before failed: 1,5-difluoro-2,4-dinitrobenzene (49), 4-fluoro-3-nitrophenyl azide (50-52), and p-azidophenacyl bromide (53). Successful coupling was obtained with tolylene-2,4-diisocyanate when used essentially as described by Chuy et al. (54-56), but the extent of coupling was less than 5%. This yield decreased rapidly when the substituted heparin was preincubated on ice in borate buffer, pH 9.5, before addition of antithrombin III, probably as a result of hydrolysis of isocyanate groups. Therefore, tolylene-2,4-diisocyanate which is more resistant to hydrolysis, was prepared and used for coupling. When this bifunctional reagent was coupled to heparin using the conditions described by Edman (47), approximately 30% of the derivatized heparin was coupled to antithrombin III in 0.5 M NaHCO$_3$, pH 5.5, or 0.1 M NaHCO$_3$, pH 6.6.

When the complexes were chromatographed twice on antithrombin III-Sepharose to remove active uncoupled heparin, about 50% of the complex was lost due to interactions with the column. The final materials consisted of covalently bound complex, inactive free heparin, and free antithrombin III, which could be removed by chromatography on a Spherogel HPLC column, again resulting in some loss, however. Chromatography on DEAE-Sephadex A-25 on the contrary removed both active and inactive heparin with a relatively good recovery (70%) and subsequent chromatography on heparin-Ultrogel quantitatively separated complex and free antithrombin III.

The complex purified in this way showed functional antithrombin III activity and the stabilized complex inhibited factor Xa at a rate comparable to that of the noncovalent complex. From these data, it is concluded that high affinity heparin can be coupled chemically to its cofactor antithrombin III to yield a functional inhibitor in its active configuration. Factor Xa inactivation rates by the pure complex were not significantly influenced by the addition of antithrombin III to the reaction mixture, suggesting that the coupled heparin had lost its activity toward free antithrombin III. Addition of heparin increased the observed inhibition rate constant less than 2-fold, indicating that at least 50% of the heparin molecules were coupled in an active configuration.

The half-life of both the complexes purified by affinity chromatography on antithrombin III-Sepharose gel was determined following intravenous injection in rabbits and both were prolonged as compared to heparin. The N-desulfated heparin-antithrombin III complex had a $t_{1/2}$ that was 2.4 times longer than that of heparin, while the $t_{1/2}$ of the hexamethylenediamine-substituted heparin-antithrombin III complex was 3.5 times prolonged. The difference in turnover rate between the two complexes might be due to the fact that amino functional groups were introduced in a different manner in both complexes. The prolongation of the $t_{1/2}$ of both the complexes compared to the turnover of heparin indicates however that the normal clearance of the mucopolysaccharide occurs mainly but not entirely through its free form as was already anticipated (31) and essentially not in the form of a complex with its natural cofactor antithrombin III.

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