Heparin of an average molecular weight of 13,000 with known polydispersity was degraded using microbial heparinase. The kinetics of this degradation were followed by four assays which measured the anticoagulant activity of the heparin digestion products. Both clotting and amidolytic chromogenic assays were used to measure heparin-potentiated inhibition of both thrombin and Factor Xa. These assays showed different profiles throughout the digestion and were related to the average molecular weight of the digestion products. The final products of this enzymatic digestion were fractionated on the basis of size and their anticoagulant activities were measured. Fragments causing Factor Xa inhibition but not thrombin inhibition were isolated. Anticoagulant activity was found in a fragment as small as a tetrasaccharide.

Heparin, an α,β-glycosidically linked highly sulfated copolymer of uronic acid and glucosamine (1), has been used clinically as an anticoagulant for half a century (2). Despite its importance and widespread use, both the exact structure of heparin and the precise nature by which it acts in blood anticoagulation have not been elucidated. Much of the difficulty in determining the structure of heparin is because it is not a homogeneous substance. Heparin is polydisperse with a molecular weight range from 5,000 to 40,000 (3). Within a given chain, there are also structural variations such as varying degrees of sulfation (1), N-acetylation (1), and C-5 epimerization in the uronic acid residue (1). Equally complex is the anticoagulant activity of heparin. The anticoagulant activity of heparin is thought to be derived primarily through its binding to antithrombin III (4). It has also been suggested that the anticoagulant activity of heparin may involve either the direct binding of thrombin to heparin (5) or the co-binding of ATIII and thrombin to heparin (6, 7). Although ATIII alone acts as an inhibitor of several coagulation factors (8), heparin accelerates inhibition of these factors (8). In addition to thrombin, other activated coagulation factors such as Factor IXa, Xa, XIa, and XIIa (6, 9-11) are inhibited by the ATIII-heparin complex.

The relationship of the structure of heparin to its activity has been a source of much study. The ATIII binding site on heparin has been characterized as a short partially desulfated polysaccharide sequence (12, 13). The relationship of the molecular weight of heparin to the potentiated inhibition of thrombin and of Factor Xa by ATIII is different (14, 15), indicating that these inhibitions may not be entirely due to the simple interaction of heparin with ATIII (15).

This study makes use of a microbial heparinase which we have prepared (16), purified of contaminating activities (17, 18), characterized (17, 18), and immobilized (17, 19). Heparinase (E.C.4.2.2.7) is an eliminase which cleaves specifically at the α-glycosidic linkages between N-sulfated-D-glucosamine 6-sulfate (the 6-O-sulfate is not required) and l-iduronic acid 2-sulfate (20). The average molecular weight of the products indicates that approximately half of the α-glycosidic linkages in porcine mucosal heparin (those linkages having the substitution described above) are cleaved by heparinase in a random endolytic fashion (17). We have examined the kinetics of this enzymatic degradation by six assays, including four which measure the anticoagulant activity of heparin. In addition, the anticoagulant activity and the distribution of that activity have been examined in the final product mixture.

**MATERIALS**

**Chemicals**

Heparin, sodium salt from porcine intestinal mucosa (Grade II, 151 USP units/mg), thrombin (for amidolytic measurement of the heparin-potentiated inhibition of thrombin), Factor Xa, and rabbit brain cephalin in anticoagulant free bovine plasma (used for clotting measurement of heparin-potentiated inhibition by Factor Xa) were purchased from Sigma. Activated thromboplast reagent-optimized (for measuring aPTT) was purchased from Ortho Diagnostic Systems, Inc. ( breach, NJ). Factor Xa, substrate S-2222 (Benzoyl-Ile-Glu-Gly-Arg-pNA) (used for amidolytic measurement of the heparin-potentiated inhibition of Factor Xa), and substrate S-2238 (H-D-Phe-Pip-Arg-pNA) (used for amidolytic measurement of the heparin-potentiated inhibition of thrombin) were obtained from Kabi Diagnostica, Stockholm. Purified thrombin and ATIII (used in place of NHP in the amidolytic thrombin assay) were the generous gift of Dr. Robert Rosenberg of the Harvard Medical School. Human blood was collected over citrate from paid donors at Children's Hospital Medical Center Blood Bank (Boston, MA). Cyanogen bromide activated Sepharose 4B was purchased from Pharmacia Fine Chemicals. All inorganics were reagent grade or better.

**Equipment**

All spectrophotographic measurements were made with a Gilford model 1084 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Freeze-drying was done in a Virtils model 10-104 LD freeze-drier. All assays were performed in Boroassilicate glass culture tubes (12 × 75 mm).

**METHODS**

**Heparinase Preparation and Immobilization**

Heparinase was produced fermentatively from Flavobacterium heparinum (16) and was purified using batch hydroxylapatite chromatography (18, 21). This preparation was assayed for contaminating sulfatase, sulfamidase, and glucuronidase activity (17). Immobilization...
to cyanogen bromide activated Sepharose 4B was performed by the method described in (17) at pH 7.0 in 0.2 M sodium phosphate buffer at a protein concentration of 0.5 mg/ml and in the presence of 60 mg of heparin/mg of protein.

Preparation of Heparin Degradation Products

The substrate solution contains heparin at 50 mg/ml in 0.025 M sodium acetate, 0.25 mM calcium acetate adjusted with acetic acid to pH 7.0 (a sample was taken at this point to generate standard curves). To 1 volume of substrate solution, 0.4 volumes of the same buffer (without heparin), containing a 50% suspension of Sepharose-immobilized enzyme having a protein concentration of 0.6 mg of protein/ml bed, were added. This suspension was incubated at 30 ± 1 °C and 200-μl aliquots were removed at various times over a 30-h period and filtered to remove the beads. After 30 h, the remaining suspension was filtered and then freeze-dried.

Chromatography

Heparin was sized by gel permeation chromatography by the method of Laurent et al. (3). Heparin digestion products were sized as described in (17).

Assays for Heparinase Activity

Ultraviolet 232-nm Assay

Aliquots (20 μl) were removed over the 30-h digestion and added to 2 ml of 30 mM hydrochloric acid and the absorbance was measured at 232 nm (18).

Metachromatic Assay

Aliquots (5 μl) were removed over the 30-h digestion and added to 20 ml of 0.02 g/liter of azure A dye solution and the absorbance was measured at 620 nm (18).

Preparation of Normal Human Plasma

NHP was prepared from citrated blood (1.9, v/v, whole blood to 3.8% weight trisodium citrate) by centrifugation at 2500 x g for 20 min. After centrifugation, the NHP was removed from the formed blood components and stored in 4-m1 polyethylene vials at −40 °C.

Preparation of Samples Used for Measuring Anticoagulant Activity

Aliquots were removed over the 30-h digestion and were serially diluted. The data appears in the following order: assay, dilution factor, clotting factor, and heparin concentration in the initial sample (units/ml): aPTT, 14,410 X, normal human plasma, 0.52; Factor Xa clotting, 1510 X, 20 mM trizma maleate (pH 7.5); S. thrombin amidolytic, 15,100 X, distilled water, 0.5; Factor Xa amidolytic, 37,880 X, 50 mM Tris, 7.5 mM EDTA (pH 8.4), 0.2.

Fractionated heparin degradation products were prepared for Factor Xa clotting assay using the same buffer and concentrations.

Assays for Measurement of Heparin Potentiated Inhibition of Thrombin and Factor Xa

Clotting Methods

Activated Partial Thromboplastin Time (aPTT)—A test tube containing activated thromboplastin reagent and a second containing 20 mM calcium chloride were warmed in a bath at 37.0 ± 0.1 °C. To a third tube, 15 μl of NHP (stored at 30 °C during the assay) and 85 μl of sample are added. Premixed activated thromboplastin reagent (100 μl) was added and the tube was mixed. After 4 min, 100 μl of 200 mM calcium chloride solution was added (t = 1). After 20 s, an innoculating loop was drawn through this mixture (2 times/s) until a clot formed and the time was recorded (t = t). A standard curve generated from the initial sample (0.0-50 units of heparin/tube) gave a correlation of \( r^2 = 0.98 \).

Factor Xa Clotting Time—During the assay, NHP and Factor Xa (0.4 units/ml with 10 mg/ml of bovine serum albumin in 20 mM trizma maleate buffer, pH 7.5) were stored at 4 °C while the rabbit brain cephalin reagent, the trizma maleate buffer, and the 25 mM calcium chloride solution were stored at 37 °C. To a prewarmed tube, in a 37.0 ± 0.1 °C bath, 100 μl of NHP, 325 μl of trizma maleate buffer, and 25 μl of sample were added. After 1 min, 50 μl of Factor Xa was added and the tube was gently mixed. Ninety seconds later, 100 μl of this mixture was transferred to a prewarmed tube. After another 20 s had elapsed, 100 μl of calcium chloride solution was added and 10 s later, 200 μl of cephalin reagent was added (t = 4). An innoculating loop was drawn through this mixture (2-3 times/s) until a clot formed and the time was recorded (t = t). A standard curve generated from the initial sample (0.0-198 units of heparin/tube) gave a correlation of \( r^2 = 0.98 \).

Amidolytic Methods

Thrombin Inhibition—During this assay, all the reagents were stored at 20 °C. To a prewarmed tube (in a bath at 37.0 ± 0.1 °C), 160 μl of buffer (consisting of tris (50 mM), EDTA (7.5 mM) and sodium chloride (175 mM), pH 8.4), 30 μl of sample, and 300 μl of diluted NHP (1 volume of NHP and 9 volumes of Tris/EDTA/NaCl buffer) were added. After 3 min, 100 μl of thrombin (15 NIH units/ml in 0.2 M sodium chloride and 67 mM calcium chloride with 15 μg/ml of bovine serum albumin at pH 7.2) was added. After 30 s, 300 μl of substrate solution (containing 0.47 mg/ml of S-2238 and 0.53 mg/ml of polybrene in distilled water) was added. After 30 s, 300 μl of 50% acetic acid (v/v) was added and the absorbance of the mixture was measured at 405 nm. A standard curve generated from the initial sample (0.0-2 units of heparin/tube) gave a correlation of \( r^2 = 0.99 \). Purified ATIII used in place of NHP (by the procedure described in Ref. 22) gave identical results (see Fig. 1).

Factor Xa Inhibition—During the assay, the sample, Factor Xa (7 nmol of S-2222 acted on/s/ml), distilled water, and the 50% acetic acid (v/v) were stored at 20 °C; the antithrombin III (1 unit/ml) and the HNP were stored at 4 °C; the substrate solution (0.75 mg/ml of S-2222 in distilled water) was stored at 37 °C. To a prewarmed tube (in a bath at 37.0 ± 0.1 °C), 800 μl of sample, 100 μl of ATIII, and 100 μl of HNP were added. To 2 prewarmed tubes (a “test” and a “blank” tube), 200 μl of this mixture was transferred. These tubes were incubated 3 min and to the test tube, 100 μl of Factor Xa was added and after 30 s, 200 μl of S-2222 was also added to this tube. After an additional 3 min, 300 μl of 50% acetic acid was added to both the test and blank tubes. At this point, 300 μl of distilled water was added to the blank tubes. The absorbance of the test tube was measured against the blank tube at 405 nm. A standard curve generated from the initial sample (0.0-17 units of heparin/tube) gave a correlation of \( r^2 = 0.92 \).

RESULTS

The porcine mucosal heparin used in this study had a molecular weight distribution (3) of from 5,000 to 40,000 with an average molecular weight of 13,000. The immobilized enzyme preparation was found to be free of impurities which would show activity against heparin or the products derived from heparinase action on heparin (e.g. glucuronidases, sulfatases, and sulfamidases (17, 23)). The resultant products were therefore simply chain shortened heparin fragments with minor end group modification (heparin eliminase results in a Δ-4,5 site of unsaturation in the terminal uronic acid residue (24)).

The results of the enzymatic digestion of heparin are shown in Fig. 1 as a plot of per cent reaction completion (as measured by the appearance of Δ-4,5 sites of the unsaturation by the UV at 232 nm assay) versus per cent of activity of heparin (this is a relative measurement of the activity of the product compared with that of the starting material at the same concentration). The two assays measuring the heparin potentiated inhibition of thrombin, the aPTT clotting assay and the amidolytic thrombin assay, show rapid deactivation of anticoagulant activity with remarkably similar profiles, until almost no anticoagulant activity remains at the end of the enzymatic digestion of heparin. The two assays measuring the heparin-potentiated inhibition of Factor Xa, the Factor Xa clotting assay and the amidolytic Factor Xa assay, show a slower rate of anticoagulant activity deactivation giving a higher level of residual activity but strikingly different kinetic profiles. Lastly, the loss of metachromatic activity during enzymatic digestion of heparin most closely parallels that of Factor Xa activity loss as measured by clotting assay.
The ATIII-heparin complex has been proposed as the single major species inhibiting thrombin and Factors IXa through XIIa in the coagulation cascade (6, 9–11). The results of this study suggest that heparin anticoagulation is the result of a heparin-ATIII complex in which the exact nature of the heparin fragment (i.e. size, structure) determines the degree to which a given factor is inhibited. If heparin contains an ATIII-binding site, as has been proposed (12, 13), and the ATIII-heparin complex inhibits the various coagulation factors and thrombin (all serine proteases (25)) in a similar fashion, then no differences should be displayed by Factor Xa and thrombin when inhibited by ATIII potentiated with heparin digested to varying degrees with heparinase. The specific (amidolytic) assay for Factor Xa inhibition should not differ substantially from the clotting assay for Factor Xa, which involves “multiple roles” (14). A similar conclusion was arrived at by Andersson et al. (14) after examining the differences between specific assays and multiple role assays when measuring the molecular weight dependence of the anticoagulant activity of heparin. Andersson (14) suggests that the molecular weight itself might not be an important parameter in determining anticoagulant activity and that this relationship might be merely an artifact of the manufacturing or processing of mucosal heparin. Our studies, however, show the fundamental nature of the relationship of molecular weight to heparin anticoagulant activity.

A different relationship of anticoagulant activity to molecular weight is observed in this study and by Andersson et al. (15), as measured by Factor Xa clotting (multiple role) and Factor Xa amidolytic (specific) assay. This may be due to the different activity of low molecular weight heparin towards coagulation factors acting subsequent to Factor Xa (i.e. prothrombin and thrombin). The action of these factors is measured in the clotting assay but not in the amidolytic assay.

The heparin-potentiated inhibition of Factor Xa, as measured by both Factor Xa clotting and amidolytic assay, is still present even after complete enzymatic digestion of heparin to an average molecular weight of 900. The average molecular weight can be calculated either from the molar absorptivity of the A4,5 unsaturated end group (24) or from the distribution obtained in gel permeation fractionation of the degradation products (Fig. 2). The similarity of the profile of metachromatic activity and that of Factor Xa activity as a function of molecular weight is expected as such metachromatic activity is exhibited by the dye binding of heparin fragments of hexasaccharide size or larger (28). Some anticoagulant activity, by Factor Xa clotting assay, is found in fragments as small as tetrasaccharides. Until now, hexasaccharide fragments, pre-
pared from nitrous acid cleavage of heparin, were the smallest reported units possessing Factor Xa anticoagulant activity (27). The tetrasaccharide unit corresponds to the size of the ATIII-binding site proposed by Rosenberg and Lam (12) and is considerably smaller than that proposed by Lindahl et al. (13). Additionally, the lowered degree of sulfation in the ATIII-binding site proposed by Rosenberg and Lam (12) would protect it from being degraded by heparinase on the basis of the specificity requirements of the enzyme (20). These specificity requirements and the retention of the activity of these fragments against Factor Xa indicate that the ATIII heparin binding site remains intact throughout the enzymatic digestion.

The loss of the thrombin inhibition capability of fully degraded heparin, as measured by the thrombin assays, indicates a requirement for more than just an ATIII-binding site (sufficient for Factor Xa inhibition) in order for heparin potentiated inhibition of thrombin. It has been proposed (6, 7) that both ATIII and thrombin co-binding to a heparin chain might be required for optimal inhibition. The mixture of short fragments produced in the enzymatic digestion of heparin may not contain the necessary thrombin-binding site while still containing the ATIII-binding site. Alternatively, the thrombin and ATIII-binding sites might both be present but only on separate fragments. Factor Xa binding (27) to heparin is known to be weaker than thrombin binding (28) and hence, as has been suggested (15), such an additional binding site may not be an absolute requirement for heparin-potentiated inhibition of Factor Xa.

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