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Structure of the antithrombin-binding site in heparin

(anticoagulant activity/nonsulfated iduronic acid/nitrous acid deamination/periodate oxidation/disaccharide units in heparin)

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ABSTRACT **Heparin preparations from pig intestinal mucosa and from bovine lung were separated by chromatography on antithrombin-Sepharose into a high-affinity fraction (with high anticoagulant activity) and a low-affinity fraction (with low anticoagulant activity). Antithrombin-binding heparin fragments (12–16 monosaccharide units) were prepared, either by digesting a high-affinity heparin–antithrombin complex with bacterial heparinase or by partial deaminative cleavage of the unfractionated polysaccharide with nitrous acid followed by affinity chromatography on immobilized antithrombin. Compositional analysis based on separation and identification of deamination products reduced with sodium boro[^{3}H]hydride showed that nonsulfated L-iduronic acid occurred in larger amounts in high-affinity heparin than in low-affinity heparin; furthermore, this component was concentrated in the antithrombin-binding regions of the high-affinity heparin molecules, amounting to approximately one residue per binding site. It is suggested that nonsulfated L-iduronic acid is essential for the anticoagulant activity of heparin. The location of the nonsulfated uronic acid in the antithrombin-binding site was determined by periodate oxidation of antithrombin-binding fragments containing a terminal 2,5-anhydro-D-[1-^{3}H]mannitol unit. Tentative structures for antithrombin-binding sequences in heparin are proposed, including some structural variants believed to be compatible with, but not required for, activity.**

Heparin impedes the coagulation of blood by accelerating the rate at which antithrombin, a plasma protein, inactivates the proteases of the so-called coagulation cascade (1). Binding of heparin to antithrombin appears to be an essential part of the mechanism (1, 2). The structural basis for such binding is unclear. Only about one-third of the molecules in heparin preparations bind with high affinity to antithrombin and this fraction (in the following denoted "high-affinity heparin") accounts for most of the anticoagulant activity of the unfractionated material (3–5). Characterization of fragments obtained on digesting a heparin-antithrombin complex with bacterial heparinase indicated that the antithrombin-binding site in heparin molecules involves a sequence of about 12–16 monosaccharide units (6). Recently, Rosenberg *et al.* (7) claimed that a tetrasaccharide sequence with a *N*-sulfated glucosamine residue at its reducing end, a *N*-acetylated internal glucosamine unit, and one residue each of D-glucuronic and L-iduronic acid would represent a critical structural element required for anticoagulant activity.

The present study involves the structural characterization of antithrombin-binding regions from two different types of heparin, isolated from pig intestinal mucosa and from bovine lung, respectively.

MATERIALS AND METHODS

Materials. Heparin (stage 14) from pig intestinal mucosa was obtained from Inolex Pharmaceutical Division (Park Forest South, IL). Heparin from bovine lung was kindly donated by the Upjohn Company (Kalamazoo, MI). Both preparations were purified by repeated precipitation with cetylpyridinium chloride from 1.2 M NaCl (8). Samples (50 mg) were fractionated by affinity chromatography on a 75-ml column of antithrombin covalently linked to Sepharose (9). A typical separation of mucosal heparin is illustrated in Fig. 1; lung heparin gives a similar picture. Some analytical data for the isolated materials are shown in Table 1. Gel chromatography on Sephadex G-100 of the mucosal and lung heparin fractions having high affinity for antithrombin gave peak partition coefficient, K_{av}, values of 0.19 and 0.23, respectively, corresponding to molecular weights of about 15,000–20,000 (9).

Antithrombin-binding heparin fragments were isolated after digesting high-affinity heparin fractions with bacterial heparinase in the presence of antithrombin, essentially as described (6). Heparin (about 5 mg) bound to 5 ml of antithrombin-Sepharose (5–7 mg of protein per ml) was exhaustively digested with 1 mg of enzyme. The isolated (6) enzyme-resistant fragments showed narrow, single peaks on Sephadex G-100 chromatography, with a K_{av} value of 0.62 (same for mucosal and lung heparin fragments). This value indicates a molecular weight in the order of 3500–5000, corresponding to six to eight disaccharide units (see ref. 6).

Alternatively, antithrombin-binding fragments were isolated by affinity chromatography after random partial depolymerization of heparin with nitrous acid. The procedure involved treatment of unfractionated pig mucosal heparin with nitrous acid as described by Cifonelli and King (10), except that the reaction was carried out at +10°C and was interrupted after 2 min. The products precipitable with 10 vol of ethanol were fractionated by affinity chromatography on antithrombin-Sepharose (9). Most of the material consisted of oligosaccharides that were not retained by antithrombin-Sepharose. However, a significant fraction was absorbed to the gel; on gradient elution a peak of uronic acid-containing components, corresponding to 2–3% of the starting material,[§] appeared in the elution position of high-affinity heparin. A portion of the material eluting at ≥1 M NaCl was reduced with sodium boro[^{3}H]hydride (11), yielding a product (specific activity, 1.0×10^5 cpm per μg of uronic acid) with terminal 2,5-anhydro-[1-^{3}H]mannitol units. The labeled fragments retained a high affinity for antithrombin (Fig. 1). Gel chromatography showed

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[§] In view of the presumably random nature of the deaminative depolymerization process this yield, recorded in two separate preparations, is unexpectedly high. No obvious explanation can be given at present.

Table 1. Analytical data for heparin fractions

Preparation	Uronic acid,* %	Hexosamine,* %	Sulfate/ disaccharide†	Anticoagulant activity,‡ units/mg	
Pig mucosal heparin					
High-affinity fraction§	33.7	21.5	1.94	259	
Subfraction I	—	—	—	—	307
Subfraction II	—	—	—	—	401
Subfraction III	—	—	—	—	431
Low-affinity fraction	36.4	24.4	1.74	29	
Bovine lung heparin					
High-affinity fraction	28.7	21.5	2.13	237	
Low-affinity fraction	28.2	22.4	2.20	32	

* Percent of sample weight, not corrected for moisture. The hexosamine values are not corrected for losses during hydrolysis.

† Molar ratios with uronic acid as 1.00.

‡ Determined by the *British Pharmacopoeia* whole-blood assay (left column), or by the colorimetric antithrombin-activation assay (right column).

§ The terms "high-affinity" and "low-affinity" refer to the interaction of heparin with antithrombin. The separation of the two types of heparin by affinity chromatography on immobilized antithrombin is shown in Fig. 1. The subfractions of high-affinity heparin were obtained by combining effluent fractions eluting at NaCl concentrations between 0.4 and 0.9 M (subfraction I), 0.9 and 1.2 M (subfraction II), and 1.2 and 1.7 M (subfraction III), respectively.

a monodisperse component (see Fig. 4); the K_{av} on Sephadex G-100 was 0.68, in reasonable agreement with the value (0.62) recorded for antithrombin-binding fragments obtained by the enzymatic procedure.

Glycuronosyl→2,5-anhydro-[1-^{3}H]mannitol disaccharides with *O*-sulfate groups in various positions were obtained as described (11).

Purified α-L-iduronidase [specific activity about 120 units (12) per mg of protein] from human kidney was kindly given to us by Leonard H. Rome (National Institutes of Health, Bethesda, MD). The enzyme degraded iduronosyl→2,5-anhydro-[1-^{3}H]mannitol 6-sulfate to a labeled product that migrated like anhydromannitol 6-sulfate on paper electrophoresis (11) but had no detectable effect on either glucuronosyl→anhydro-[1-^{3}H]mannitol 6-sulfate or iduronosyl 2-sulfate→anhydro-D-[1-^{3}H]mannitol 6-sulfate.

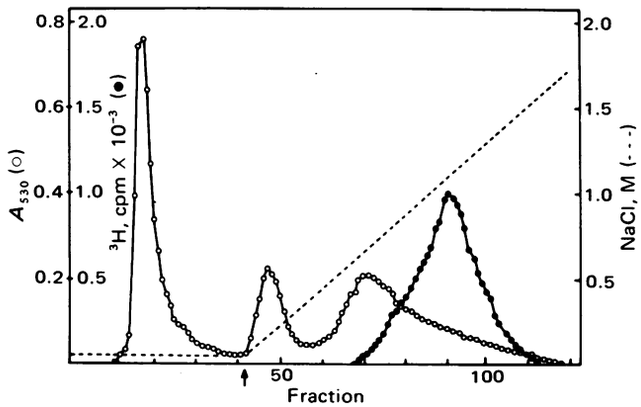

FIG. 1. Affinity chromatography on antithrombin-Sepharose (9) of 50 mg of pig mucosal heparin (○) mixed with 3 μg of ^{3}H-labeled antithrombin-binding fragment (●), obtained by partial random depolymerization of the same heparin with nitrous acid (see the text). Effluent fractions were analyzed for uronic acid (○) or for radioactivity (●). The salt gradient (starting at arrow) used for elution is indicated by the broken line. In preparative experiments the first two peaks of uronic acid-containing material (one of nonadsorbed material and one eluting at less than 0.4 M NaCl), representing heparin species essentially devoid of anticoagulant activity (9), were combined into one low-affinity fraction, whereas material eluting at greater than 0.4 M NaCl was considered to be of high-affinity type.

Analytical Methods. Methods for the determination of uronic acid, hexosamine, sulfate, and radioactivity have been described (13). Gel chromatography of poly- or oligosaccharides was carried out as described in refs. 6 and 14.

Anticoagulant activities were determined by the *British Pharmacopoeia* whole-blood assay (15). Alternatively, the antithrombin-activating potency was determined as described by Laurent *et al.* (9) and related to that of a heparin preparation (3rd International Standard) of known activity.

The disaccharide composition of heparin preparations was determined as described in ref. 11 and outlined in the legend to Table 2.

Periodate Oxidation. Periodate oxidation followed by alkali treatment and reduction was carried out as described (16).

RESULTS AND DISCUSSION

In a previous study from this laboratory the presumed antithrombin-binding site of heparin was isolated after digestion of heparin-antithrombin complexes with bacterial heparinase (6). In the present investigation antithrombin-binding fragments were also prepared by a different method, involving partial, random depolymerization of heparin with nitrous acid followed by affinity chromatography on antithrombin-Sepharose. The size of the smallest antithrombin-binding fragment obtained by this method was similar to that of the fragments obtained by the enzymatic procedure, corresponding to six to eight disaccharide units. These results thus confirm our previous conclusions regarding the size of the antithrombin-binding site in heparin (6, 9).

Evidence presented below indicates that the antithrombin-binding site in heparin does not consist of a single, unique oligosaccharide sequence but has a variable structure. However, certain regions within this sequence appear to be nonvariable and should therefore be of critical importance to the interaction between heparin and antithrombin. One of these nonvariable components has been identified as nonsulfated L-iduronic acid, tentatively allocated to position 3 of the binding sequence, position 1 being the nonreducing terminal unit. This is illustrated in Fig. 2, which depicts the antithrombin-binding site as a tetradecasaccharide sequence (see the legend to the figure). The evidence for the proposed structure is based on two types of experiments. First, analysis of di- and tetrasaccharides formed on deamination with nitrous acid showed a prepon-

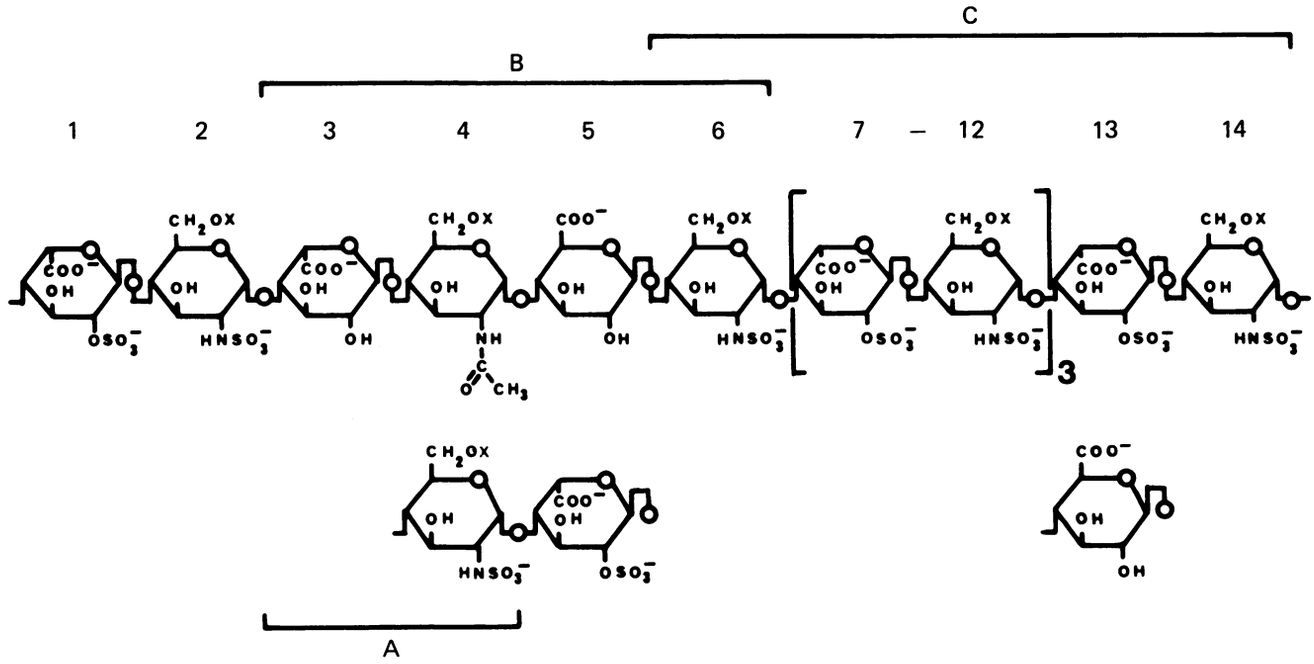

FIG. 2. Tentative structure for the antithrombin-binding site of pig mucosal heparin. X = H or SO_3^-. The continuous sequence represents the majority of the binding sites, whereas the isolated sugar units below (at positions 4, 5, and 13) represent structural variants compatible with (but not required for) anticoagulant activity. The variant unit in position 13 has been indicated as D-glucuronic acid, although it has been identified only as a nonsulfated uronic acid residue (see the legend to Fig. 4). The total number of disaccharide units per antithrombin-binding site has been set at seven, on the basis of gel chromatography data (see Fig. 4 and the text), but may instead be six or eight; such deviations from the structure shown should presumably affect only the portion of the sequence to the right of position 7. The labeled antithrombin-binding fragments obtained after partial deaminative cleavage of heparin contain 2,5-anhydro-[1-^{3}H]mannitol in position 14. For additional details, including explanation of the segments A, B, and C, see the text.

derance of nonsulfated L-iduronic acid in the antithrombin-binding site, as compared to other regions of the heparin molecules. Second, the position of nonsulfated uronic acid residues was determined by periodate oxidation of antithrombin-binding fragments having ^{3}H-labeled 2,5-anhydromannitol end groups (in position 14).

Composition of Antithrombin-Binding Fragments

Preparations of heparin or of antithrombin-binding fragments were subjected to compositional analysis as outlined in the legend to Table 2. The deamination products obtained were largely glycuronosyl→2,5-anhydro[^{3}H]mannitol disaccharides, with the di-*O*-sulfated species, iduronosyl 2-sulfate→anhydromannitol 6-sulfate predominating (Table 2). In addition, three different monosulfated disaccharides occurred in the deamination products of all samples. One of these disaccharides, iduronosyl→anhydromannitol 6-sulfate, was consistently obtained in larger amounts from high-affinity heparins than from low-affinity heparins. Furthermore, the antithrombin-binding fragments gave higher yields of this particular disaccharide than did the corresponding high-affinity heparins. These results suggest that the L-iduronosyl→*N*-sulfo-D-glucosaminyl 6-sulfate disaccharide unit is concentrated in the antithrombin-binding regions of heparin molecules.

In addition to disaccharides, the deamination products, especially those derived from mucosal heparin, contained appreciable amounts of tetrasaccharide (Table 2). These tetrasaccharides should have the general structure glycuronosyl→*N*-acetylglucosaminyl→glycuronosyl→anhydro[^{3}H]-mannitol (ref. 10; however, see footnote‡ in Table 2). On incubation with purified α-L-iduronidase such tetrasaccharides, isolated from mucosal high-affinity heparin, were converted to labeled trisaccharides (Fig. 3*B*) to a larger extent than were the corresponding tetrasaccharides derived from low-affinity heparin (Fig. 3*C*). The α-L-iduronidase-susceptible tetrasaccharide, like the iduronosyl→anhydromannitol 6-sulfate disaccharide, was obtained in higher yield (about 70% of the total tetrasaccharide) from the antithrombin-binding fragment (Fig. 3*A*) than from the parent high-affinity heparin. Because the enzyme preparation contained neither β-glucuronidase nor iduronate 2-sulfatase activity, such tetrasaccharides must have nonsulfated iduronic acid residues in the nonreducing terminal position. The total nonsulfated iduronic acid, as represented by the sum of the iduronosyl→anhydromannitol 6-sulfate disaccharide and the α-L-iduronidase-susceptible tetrasaccharide (See Table 2), equaled or exceeded one residue per antithrombin-binding site in mucosal heparin.¶ This applied probably also to lung heparin, judging from the amounts of iduronosyl→anhydromannitol 6-sulfate and tetrasaccharide, respectively, formed on deamination of isolated antithrombin-binding fragment (Table 2); however, the effect of α-L-iduronidase on the tetrasaccharide could not be properly evaluated, due to poor separation of the labeled digestion products.

The presence of a nonsulfated L-iduronic acid residue, presumably in each antithrombin-binding site, and the relative lack of such residues in heparin molecules having low affinity for antithrombin (see Table 2 and Fig. 3) strongly suggest that nonsulfated L-iduronic acid is essential to the anticoagulant activity of heparin. Exhaustive digestion of antithrombin-binding fragment with α-L-iduronidase did not significantly affect its affinity for antithrombin, as shown by subsequent affinity chromatography (as in Fig. 1), suggesting an internal rather than terminal location for the essential nonsulfated iduronic acid. For reasons discussed below this residue will be

¶ Total amount of disaccharide containing nonsulfated iduronic acid = [4.3 + (0.7 · 34/2)]/100 = 16% of total disaccharide units. One such disaccharide unit per tetradecasaccharide-binding site would correspond to 14% of the total disaccharide units.

Table 2. Products formed on deamination of heparin fractions

Preparation	% of disaccharide units recovered as*†				
	Tetrasaccharide‡	Iduronosyl 2-sulfate→anhydromannitol 6-sulfate	Glucuronosyl→anhydromannitol 6-sulfate	Iduronosyl→anhydromannitol 6-sulfate	Iduronosyl 2-sulfate→anhydromannitol
Mucosal heparin					
Antithrombin-binding fragment§ (nitrous acid)	35 (1.2)	48 (3.4)	5.9 (0.4)	4.9 (0.3)	6.2 (0.4)
Antithrombin-binding fragment§ (heparinase)	34 (1.2)	49 (3.5)	6.9 (0.5)	4.3 (0.3)	5.3 (0.4)
High-affinity fraction	27	56	7.9	2.8	6.1
Low-affinity fraction	27	50	11.0	1.8	10.0
Lung heparin					
Antithrombin-binding fragment§ (heparinase)	17 (0.6)	65 (4.5)	5.9 (0.4)	7.2 (0.5)	5.0 (0.4)
High-affinity fraction	17	68	5.1	4.6	6.3
Low-affinity fraction	15	72	4.8	1.9	6.2

* The method used to determine the disaccharide composition of heparin preparations is described in detail elsewhere (11). Briefly, polysaccharide (10–50 μg of uronic acid) is treated with nitrous acid, under conditions resulting in conversion of *N*-sulfated glucosamine residues to 2,5-anhydromannose units, with cleavage of the glucosaminidic linkages. The samples are reduced with sodium boro[^{3}H]hydride and the labeled di- and oligosaccharides are fractionated by a combination of gel chromatography, high-voltage paper electrophoresis, and paper chromatography. In the present experiments no significant amounts of oligosaccharides larger than tetrasaccharide were observed. Nonsulfated disaccharide invariably amounted to less than 1% of the total disaccharides.

† Values within parentheses indicate moles per mole of antithrombin-binding fragment, assuming seven disaccharide units per molecule.

‡ Deaminative cleavage of heparin-like polysaccharides yields tetrasaccharides where isolated *N*-acetylated disaccharide units occur surrounded by *N*-sulfated units (10). However, tetrasaccharides may also be formed by an anomalous (deaminative ring contraction) reaction in which *N*-sulfated glucosamine residues are converted into 2-aldehydo-D-pentofuranoside units without cleavage of the glycosidic bond (17, 18). The tetrasaccharide contents given should therefore represent maximal values.

§ The antithrombin-binding fragments analyzed were isolated after partial depolymerization of heparin with nitrous acid or with bacterial heparinase, as indicated. For further experimental details, see the text.

allocated to position 3 in the sequence shown in Fig. 2. The adjacent glucosamine residue (position 4) can apparently be either *N*-sulfated or *N*-acetylated; after deamination–reduction the *N*-sulfated residues were recovered (as anhydromannitol) in iduronosyl→anhydromannitol 6-sulfate disaccharides (corresponding to segment A in Fig. 2), whereas the analogous *N*-acetylated units were included in tetrasaccharides (segment B in Fig. 2). When the glucosamine residue at position 4 is *N*-acetylated the following sugar unit (no. 5 in Fig. 2) will be predominantly or exclusively D-glucuronic acid (19), because a glucuronic acid residue bound to C-1 of a *N*-acetylated (rather than *N*-sulfated) glucosamine unit is a poor substrate (or not a substrate) for the epimerase that catalyzes the formation of iduronic acid units during biosynthesis of heparin (unpublished data). When the glucosamine residue in position 4 is *N*-sulfated the following uronic acid unit (position 5) may probably be either glucuronic or iduronic acid.

Periodate oxidation of antithrombin-binding fragments

Taken together, the results presented above indicated that the majority of antithrombin-binding sites in the mucosal heparin contained the iduronosyl→*N*-acetylglucosaminyl→glucuronosyl→*N*-sulfoglucosaminyl sequence recovered as a tetrasaccharide after deamination with nitrous acid. The location of the glucuronosyl moiety, and hence of the entire tetrasaccharide sequence (segment B in Fig. 2) in the antithrombin-binding site was determined by periodate oxidation. Antithrombin-binding fragment, isolated after partial deaminative cleavage of mucosal heparin and ^{3}H-labeled at the reducing end, was subjected to periodate oxidation at pH 7, 37°C, for 3 hr, followed by scission in alkali. This treatment should result in cleavage of any nonsulfated uronic acid residues present (16). Analysis of the products by gel chromatography (Fig. 4) showed that the material had been degraded and appeared as essentially two major peaks of radioactivity. The less retarded fraction consisted of a distinct, sharp peak at the elution position of a nona- or undecasaccharide, along with a smaller amount of somewhat larger components. The corresponding antithrombin-binding fragments must thus have included a terminal sequence of four (as assumed in Fig. 2) or five consecutive disaccharide units containing 2-sulfated (periodate-resistant) iduronic acid residues, followed by a disaccharide unit containing a nonsulfated (periodate-susceptible) uronic acid residue. The periodate-resistant region would correspond to segment C in Fig. 2. In order to comply with the periodate cleavage pattern the glucuronosyl moiety of the tetrasaccharide structure referred to above would have to be allocated to position 5. The iduronosyl moiety would then be in position 3, leaving positions 1 and 2 to be occupied by a terminal *N*-sulfated disaccharide unit.

The additional products of periodate oxidation, besides the major component at 103 ml effluent volume (Fig. 4), presumably reflect variations in the structure of the antithrombin-binding site. The shoulder around 90 ml may thus represent antithrombin-binding fragments cleaved at position 3 only, due to the presence of a sulfated iduronic acid residue at position 5. Analysis of the retarded peak (at 140 ml) is described in the legend to Fig. 4.

Concluding remarks

At least seven different disaccharide units are present in heparin (11). Due to the specificities of the enzymes involved in the biosynthesis of heparin (20) these units are linked to each other in a fashion that is only partly random; certain combinations are favored whereas others are precluded. However, even with these inherent restrictions the number of tetradecasaccharide sequences likely to occur in heparin molecules must be exceedingly large. The number of such sequences capable of binding to antithrombin is obviously smaller (9) but may still

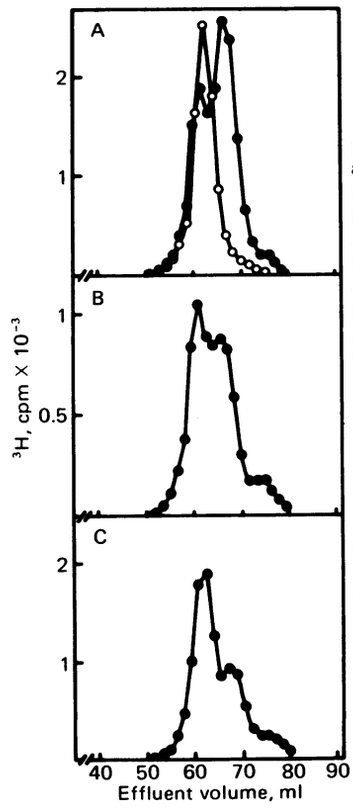

FIG. 3. Gel chromatography on Sephadex G-15 of anhydro[^{3}H]-mannitol-labeled tetrasaccharides, after incubation with α-L-iduronidase, active (●) or heat-inactivated (○) enzyme. The tetrasaccharides were derived, as outlined in Table 2, from (heparinase-produced) antithrombin-binding fragment (*A*), high-affinity fraction (*B*), and low-affinity fraction (*C*) of pig mucosal heparin. Samples (20,000–30,000 cpm, about 50 ng of uronic acid) were digested with 0.15 unit of enzyme at 37°C for 12 hr, in 0.05 ml of 0.05 M sodium acetate buffer, pH 4.3, containing 0.1 M NaCl, 0.003 M NaN_3, and 0.05% Triton X-100. The incubation products were analyzed by chromatography on a column (1 × 190 cm) of Sephadex G-15, eluted with 0.2 M NH_4HCO_3. The undegraded tetrasaccharides all gave identical elution patterns.

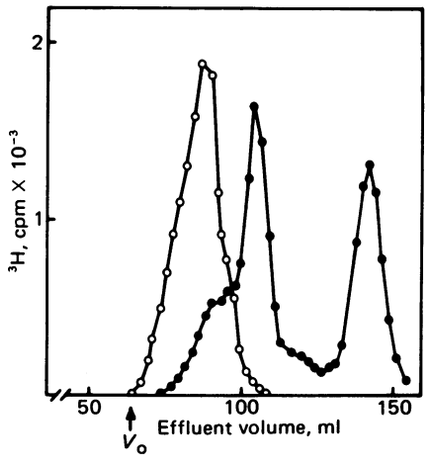

FIG. 4. Gel chromatography on Sephadex G-50 (14) of antithrombin-binding fragment from mucosal heparin before (○) and after (●) periodate/alkali treatment. V_o, void volume. The fragment had been obtained by limited depolymerization of mucosal heparin with nitrous acid and contained a ^{3}H-labeled, terminal anhydromannitol residue (see the text). Comparison with reference oligosaccharides derived from heparin-like polysaccharides (unpublished data) indicated that the undegraded material is a tetradecasaccharide and the major degradation product (peak elution volume, 103 ml), a nona- or undecasaccharide. Prolonging the reaction time with periodate to 16 hr did not affect the elution pattern. Analysis of the retarded peak (at 140 ml) by paper electrophoresis and paper chromatography (11) indicated 30% anhydromannitol 6-sulfate and about 20% anhydromannitol, in addition to unidentified components. Apparently some of the antithrombin-binding sites must have contained a nonsulfated uronic acid residue in position 13 (see Fig. 2).

be considerable, as suggested by the present structural analysis of antithrombin-binding heparin fragments. The structural variability is probably the reason for the extended elution pattern of high-affinity heparin (as defined in Fig. 1) on affinity chromatography. It is not possible at present to fully define the variable (in addition to positions 4, 5, and 13) and nonvariable (in addition to position 3) regions in the antithrombin-binding site. Obviously, the nonsulfated iduronic acid in position 3 cannot represent the only structural feature that is essential for the high-affinity binding of heparin to antithrombin, because such residues occur also in the low-affinity fraction (Table 2). Additional nonvariable regions must therefore occur elsewhere in the sequence.

Our conclusions regarding the structural requirements for anticoagulant activity differ from those of Rosenberg *et al.* (7). The tetrasaccharide sequence (see introduction) implicated in their study appears to be essential only to the extent that it contributes a nonsulfated iduronic acid residue; the *N*-acetylglucosaminyl-glucuronosyl component would rather represent one of the structural variants (at positions 4 and 5 in Fig. 2) compatible with, but not required for, activity. The preferential association of nonsulfated iduronic acid with this disaccharide sequence can be rationalized in terms of the biosynthetic mechanisms (see above and ref. 20). Although the data available do not exclude that the glucuronic acid component may also be required for anticoagulant activity, there is at present no reason to postulate such a requirement.

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