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Metachromatic Activity of Heparin and Heparin Fragments

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Heparin of an average molecular weight of 13,000 was fractionated on the basis of size into five fractions of different weight-average molecular weight ranging from 8500 to 20,000. The heparin was also degraded using microbial heparinase resulting in products ranging from a disaccharide of molecular weight 500 to an oligosaccharide of molecular weight 3100. These products were also size fractionated. The individual heparin fractions and products were tested for metachromatic activity with Azure A. The metachromatic activity of the heparin fractions was independent of molecular weight, while the metachromatic activity of the products was dependent on molecular weight. Metachromatic activity was found in a fragment as small as a tetrasaccharide. Anticoagulant activity was found in fragments of tetrasaccharide or larger by a Factor Xa clotting assay and in fragments of hexasaccharide or larger by a Factor Xa amidolytic chromogenic assay.

KEY WORDS: heparin; heparinase; metachromasia; Azure A; anticoagulation.

The metachromatic properties of Azure A have long been used to determine the presence or quantity of heparin in simple solutions and in plasma (1–3). The metachromatic reaction of heparin with Azure A has been most extensively studied by Jaques (1,4,5). Our laboratory has used Azure A to follow heparinase production by *Flavobacterium heparinum* (6) and, in conjunction with anticoagulant activity assays, to examine the degradation of heparin by heparinase (7,8).

To use heparin's metachromasia, either alone or in conjunction with anticoagulation assays, to study the structure of heparin as well as its *in vivo* metabolism and activity requires a clear understanding of the relationship between heparin's molecular weight and its metachromatic activity. Shanberge *et al.* found that high-molecular-weight heparin with high sulfate content had a greater metachromasia with Azure A than low-molecular-weight heparin fractions (9). Dietrich reported that di- and tetrasaccharides produced from partially purified microbial heparinase showed little or no metachromatic activity, while hexasaccharides of the same origin retained full metachromatic activity (10). An in-depth investigation of these relationships was undertaken to determine whether metachromasia is a function of the molar or weight concentration of heparin and heparin fragments and to establish standards for the metachromatic activity of these compounds.

In this study, commercial porcine mucosal heparin was size fractionated into five groups with different average molecular weights. The metachromatic activity with Azure A in each molecular weight fraction was examined. Heparin was also enzymatically degraded with a microbial heparinase (6). Heparinase (EC 4.2.2.7) is an eliminase which cleaves specifically at α-glycosidic linkages between the *N*-sulfated-D-glucosamine 6-sulfate (the 6-*O* sulfate may not be required) and L-iduronic acid-

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2-sulfate (11). Heparinase-generated heparin fragments are simply chain-shortened heparin fragments with minor end group modification (heparinase cleavage results in a Δ-4,5 site of unsaturation in the terminal uronic acid residue (12)). Heparin digestion products were also size fractionated. Fragments ranging from a disaccharide to an oligosaccharide of molecular weight 3100 were isolated and tested for metachromatic activity. In addition, the anticoagulant activity of the products was examined by two assays.

MATERIALS AND METHODS

Materials. Azure A, certified biological stain, total dye content 71%, was obtained from Fisher Scientific Company. Heparin, sodium salt from porcine intestinal mucosa, from Hepar Industries, Franklin, Ohio (157 USP units/mg) was used for the degradation product studies, and from Sigma Chemical Company (Grade II, 151 USP units/mg) for the whole heparin studies. Blue Dextran, Sephadex G-75, Factor Xa, and rabbit brain cephalin in anticoagulant-free bovine plasma (used for clotting measurement of heparin-potentiated inhibition by Factor Xa) were also from Sigma. Factor Xa, substrate S-2222 (Benzoyl–Ile–Glu–Gly–Arg–*p*-nitroanilide), antithrombin III, and human normal plasma (used for amidolytic measurement of the heparin-potentiated inhibition of Factor Xa) were obtained from Kabi Diagnostica, Stockholm. Fractogel TSK HW40(S) and (F) were obtained from MCB Manufacturing Chemists, Gibbstown, New Jersey. Spectropore dialysis tubing was obtained from Spectrum Medical Industries, Los Angeles, California. Polyethylene glycols of defined molecular weights were obtained from Polysciences, Inc., Warrington, Pennsylvania. ^{3}H-labeled di-, tetra-, and octasaccharide standards were the generous gift of Dr. Robert Rosenberg, Whitaker College of Health Sciences, Technology and Management, MIT. All inorganics were reagent grade or better.

Heparinase preparation. Heparinase was produced fermentatively as described in (6). The enzyme was purified using batch hydroxylapatite chromatography and chromatofocusing (13). This purified heparinase contained no contaminating activites (i.e., sulfatase, sulfamidase, glycuronidase) capable of acting on heparin or heparin degradation products (13,14). This preparation was dialyzed overnight at 4°C through 50,000 molecular weight cutoff dialysis tubing (no heparinase activity is lost at this cutoff) against 0.05 M sodium phosphate. The enzyme concentration was approximately 90 μg/ml and its activity was about 6 units/mg protein, where one unit of activity is defined as 1 μmol of bonds cleaved per minute (the activity of this enzyme could also be considered as 462 units/mg protein if one unit were defined as 1 mg heparin degraded/mg protein/h (7,8,14)).

Heparin fractionation. Porcine mucosal heparin was applied to a G-75 Sephadex column (1.5 × 50 cm), with a flow rate of 0.16 ml/min. The solvent was 1.0 M NaCl and the void volume was 26.1 ml as determined using Blue Dextran. The fractionated heparin was combined into five groups of different average molecular weight, which were exhaustively dialyzed against distilled water using 1000 molecular weight cutoff dialysis tubing; all samples were then lyophilized. They were then separately reapplied to the column for more accurate sizing, followed by dialysis and lyophilization.

Preparation of heparin degradation products. The substrate solution contained heparin at 100 mg/ml in 25 mM sodium acetate, 0.25 mM calcium acetate, pH 7.0. To 1 vol of substrate solution, 1 vol of the heparinase preparation was added. This mixture was incubated at 30 ± 0.1°C. The percentage degradation was measured by the uv 232 and metachromatic assays as described in (7). After incubating for 9 h the digestion was complete and the solution was freeze dried.

Separation of heparin degradation products. The freeze-dried degradation products (approximately 400 mg in 4 ml) were first applied to a Fractogel TSK-HW40(F) (particle size 32–63 μm) column of dimensions 2.5 × 87

cm, flow rate of 0.82 ml/min, and void volume of 144 ml. The solvent was 1.0 M ammonium acetate. Fractions were collected at 90-s intervals. Separated products were lyophilized and individually applied (approximately 60 mg in 1 ml) to a Fractogel TSK HW40(S) (particle size 25–40 μm) column of dimensions 1.5 × 168 cm, with a flow rate of 0.11 ml/min and a void volume of 101 ml. Fractions were taken at 7-min intervals. Presence of products was determined directly by measuring the absorbance of each fraction at 254 nm. This wavelength was chosen instead of 232 nm to avoid interference from the ammonium acetate buffer. The molecular weight standards, ^{3}H-labeled, heparin-derived di-, tetra-, and octasaccharides, had been prepared as described by Shively and Conrad (15,16). All chromatography was performed at room temperature.

Metachromatic activity assay. One gram of Azure A dye was dissolved in 1.0 liter distilled water and stored at 4°C for up to 2 weeks. This concentrated solution was then diluted to 0.02 g/liter shortly before use. The fractionated and degraded heparin samples were exhaustively freeze-dried, weighed, dissolved, and serially diluted in distilled water to obtain concentrations of 1 to 8 mg/ml. Ten-microliter aliquots were added to 10.0 ml of the azure solution. The absorbance at 620 nm was measured within 30 min.

Anticoagulant activity assays. Factor Xa clotting time and Factor Xa inhibition assays were performed as previously described (7).

RESULTS

The porcine mucosal heparin used for sizing had a molecular weight distribution from 5000 to 26,000 with an average of 13,000 as determined using gel-permeation chromatography with polyethylene glycol molecular weight standards. The elution profile of the whole heparin and the five different molecular weight fractions applied to the G-75 Sephadex column is shown in Fig. 1. The fractions were chosen such that each contained an equal mass of heparin. Unfractionated heparin as well as each heparin subfraction were tested for their metachromatic activity with Azure A. Standard curves of absorbance at 620 nm vs heparin concentration (0 to 8 μg/ml) were prepared since this range represents the linear region of the curve (1,17). The slopes of the standard curves for unfractionated heparin and each of the subfractions were identical (Table 1).

A purified microbial heparinase was used to completely digest heparin to a mixture of small heparin fragments. The characteristic profile obtained when heparin degradation products are applied to a Fractogel TSK HW-40(S) column is shown in Fig. 2. To further

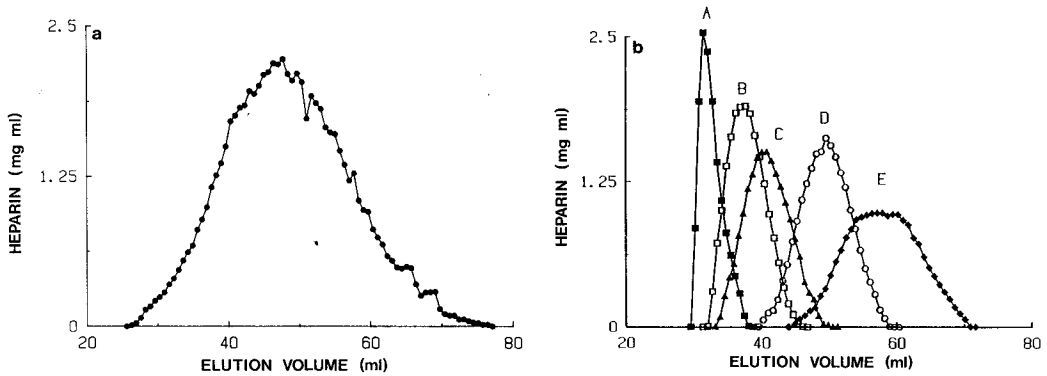

FIG. 1. G-75 Sephadex elution profile of (a) 50 mg porcine mucosal heparin, and (b) size fractionated heparins. The average molecular weights of the five regions appear in Table 1. A 10-μl aliquot from each eluted fraction was added to 10 ml of the dilute Azure A solution and the absorbance was measured at 620 nm. The mg heparin/ml solution were calculated from the standard curve of unfractionated heparin.

TABLE 1

METACHROMATIC ACTIVITY OF FRACTIONATED HEPARIN

Sample	K_{av}[a]	Molecular weight[b] (weight average)	Metachromatic activity[c,d]
Unfractionated heparin	0.32	12,600	0.177 ± 0.001
Heparin fraction			
A	0.11	20,000	0.176 ± 0.002
B	0.20	16,500	0.175 ± 0.003
C	0.25	15,000	0.176 ± 0.002
D	0.40	11,000	0.179 ± 0.002
E	0.51	8,500	0.177 ± 0.001

[a] The heparin was eluted from a G-75 Sephadex column. K_{av} is defined as $(V_e - V_o)/(V_t - V_o)$ where V_e = elution volume, V_o = void volume, and V_t = column volume.

[b] The molecular weight was determined by comparing the K_{av} values with those of polyethylene glycol standards of known molecular weight.

[c] Metachromatic activity is expressed as the negative slope of the standard curve of absorbance at 620 nm vs heparin concentration (μg/ml) in a dye solution of 0.02 g/liter. The intercept of all the curves was 1.97 ± 0.01.

[d] Each point was done in triplicate to produce one standard curve for each molecular weight region.

purify the products each fraction was reapplied to the column. The results of these elutions are shown in Fig. 3. The molecular weights of the fragments were determined both by gel-permeation chromatography using tritiated heparin fragments of known molecular weight as standards, and by absorbance (λ_{max} = 232 nm, $\epsilon = 5.5 \times 10^3$ M^{-1} (18)).

The metachromatic activities of the heparin products are shown in Table 2. The activity is reported both as the slopes of the standard curves and as the ratio of the slope of the product standard curve to that of the original heparin, which represents the relative product activity. The metachromatic activity of the products is dependent on their molecular weight. As product size increases beyond that of a tetrasaccharide to an oligosaccharide of molecular weight 3100, the metachromatic activity increases from 40% to 87% of heparin's activity.

The products were tested by a Factor Xa clotting time and a Factor Xa amidolytic assay (7), and the results are given in Table 2. The disaccharide had neither metachromatic nor anticoagulant activity. The tetrasaccharide, which showed substantial metachromasia, only possessed extremely low levels of anticoagulant activity and then only by one of the two Factor Xa assays. The differences between the amidolytic and coagulation assay observed in the smaller tetra- and hexasaccharide fragments are noteworthy and warrant further investigation.

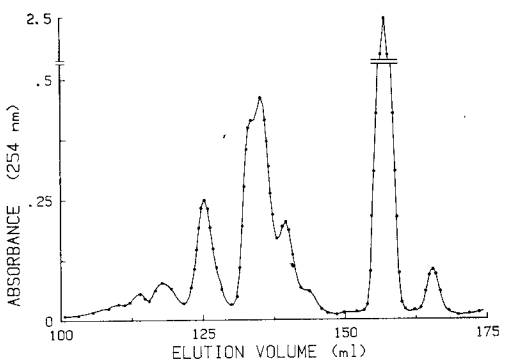

FIG. 2. Fractogel TSK-HW40(S) elution profile of heparin degradation products. The elution volume (ml) of the product peaks was oligosaccharide, 105; decasaccharide, 112; octasaccharide, 117; hexasaccharide, 125; tetrasaccharide, 134; and disaccharide, 156.

DISCUSSION

Several models of dye–heparin interaction have been proposed. Wollin and Jaques (19) combined the dye stacking model of Bradley and Wolf (20) with the pairing concept of

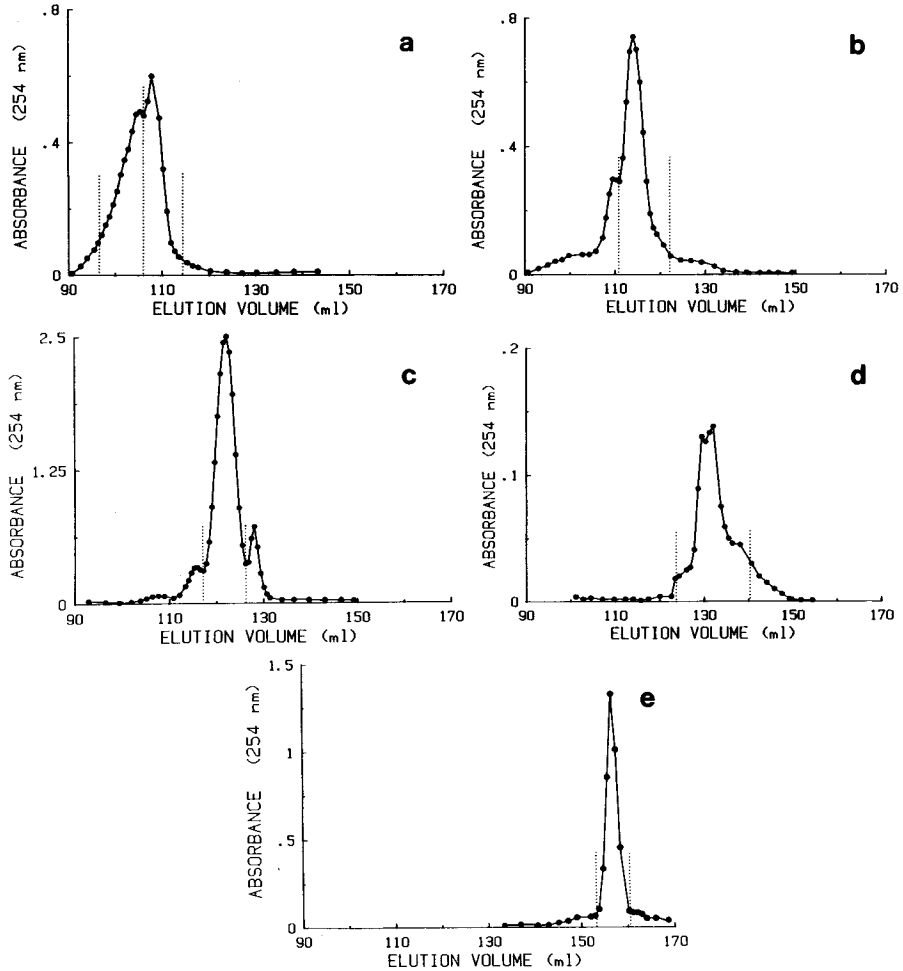

FIG. 3. Fractogel TSK-HW40(S) elution profiles of (a) oligosaccharide from 97 to 107 ml, decasaccharide from 107 to 114 ml; (b) octasaccharide; (c) hexasaccharide; (d) tetrasaccharide; (e) disaccharide. The region between the dotted lines represents the fractions used.

Young *et al.* (21) to suggest that metachromasia is produced by a dye dimer reacting with the polyanionic heparin in a loose electrostatic manner. The dimerization results in a decrease in the π electron delocalization of the dye molecules which is observed as a shift in the absorbance maxima from 620 to 530 nm. The exceptional strength of Azure A–heparin binding is due to the suitable arrangement of heparin's anionic sites for interaction with the dye dimer, which can bridge between successive charges on alternate sugars (5,22). Although these models predict a decrease in metachromasia resulting from extreme shortening of the heparin chain, little attention has been paid to the relationship between the molecular weight of the heparin and the amount of metachromasia. This may be due to the scarcity of size fractionated heparin and heparin degradation products. Until now, a systematic investigation of this relationship had never been performed.

If the Azure A assay is insensitive to the molecular weight of the heparin in solution, then the slopes of the standard curves presented in Table 1 (absorbance vs heparin

TABLE 2

METACHROMATIC ACTIVITY OF ENZYMATICALLY DEGRADED HEPARIN

Sample	K_{av}[a]	Molecular weight[b]	Metachromatic activity[c,d,e]	Relative metachromatic activity[c,d]
Unfractionated heparin	—	13,000	0.199 ± 0.002	1.0
Oligosaccharide	0.021	3,100	0.174 ± 0.004	0.87
Decasaccharide	0.042	2,800	0.125 ± 0.004	0.62
Octasaccharide	0.067	2,250	0.099 ± 0.003	0.50
Hexasaccharide	0.109	1,650	0.096 ± 0.005	0.48
Tetrasaccharide	0.160	1,150	0.080 ± 0.004	0.40
Disaccharide	0.285	500	0.007 ± 0.002	0.03

[a] The products were eluted from a Fractogel TSK-HW40(S) column.

[b] The product molecular weight was determined by comparing their K_{av} values with those of standard di-, tetra-, and octasaccharides. The molecular weight measured from the absorbance (λ_{max} = 232 nm, ϵ = 5.5 10^3 M^{-1}) of each of the sized heparin fragments agree to within 20%.

[c] Metachromatic activity is expressed as the negative slope as in Table 1, and as activity relative to that of the unfractionated heparin (slope sample/slope heparin). The intercept of all the curves was 2.04 ± 0.03.

[d] These data represent the average of results for at least two separately derived product samples of each size. The points for each standard curve were performed in duplicate.

[e] The product clotting and amidolytic anticoagulant factor Xa activities (USP units/mg, ±1 unit/mg) were, respectively, Di—0.4, 0.07; tetra—5, 0.21; hexa—45, 8; octa—54, 55; deca—79, 54; oligosaccharide not determined, 74; and heparin 154, 154.

weight concentration) should be identical for each molecular weight fraction. However, if the assay is sensitive to the number of moles of heparin in solution, then the slopes of these standard curves should vary with the molecular weight of the heparin used. Since the slopes are equal, we conclude that there is no correlation between metachromatic activity with Azure A and molecular weight for heparin of this molecular weight range. Instead, the amount of metachromasia is directly proportional to the mass of heparin in solution, regardless of its molecular weight.

These results are consistent with current models of dye–heparin interaction (5,19). Many dye dimers can "stack," even along a relatively short heparin chain of 5000 Da or 20 saccharide units, so that end effects are minimized (20,22). For heparin chains of this and greater length, the total number of anionic sites with which the dye can interact, and not the number of chains, will determine the amount of metachromasia. Assuming a homogeneous distribution of charge, the number of sites is related only to mass, whereas number of chains is actually dependent on molecular weight. Therefore, our results relating metachromasia to weight rather than molar concentration are in agreement with the current theories of dye binding. However, our results do not agree with those of Shanberge *et al.* (9) which indicate a molecular weight dependence on the metachromasia of heparin.

Contrasting the independence of heparin's metachromatic activity on its molecular weight, the results presented in Table 2 indicate a direct relationship between metachromasia and molecular weight for heparin fragments ranging from 500 to 3100. Metachromatic activity persists with a product as small as a tetrasaccharide, while the disaccharide shows no activity at all. Dietrich also found that the disaccharide is without activity, but reported that the tetrasaccharide has only 7% of heparin's activity while the hexasaccharide retained full activity (10). This may be a result of differences in our enzyme preparations. Our enzyme is catalytically pure (13,14), while the previous study used freeze-dried cells that probably contained impurities

such as sulfatases and sulfamidases which would have significantly altered the structure of the resulting degradation products.

The small size of the disaccharide probably prevents its interaction with a dye dimer. Although a single dye molecule might interact with the disaccharide, this is not sufficient to produce the metachromatic shift. The larger tetrasaccharide, however, has sufficient anionic groups to bind a dye dimer and this binding capacity is responsible for the dramatic increase in metachromatic activity between the di- and tetrasaccharide.

The precise nature of the relationship between the product's size and its ability to produce metachromasia is complex. Stone has reported that one of the optical effects caused by dye–heparin interaction is induced circular dichroism in the metachromatic band (22). This occurs when many dimers occupy adjacent sites along the heparin chain. The helical arrangement of heparin in solution allows for maximum separation of charges along the heparin chain, and this positioning of anionic sites may facilitate dye dimer–heparin interaction. For small heparin fragments, this helicity should be dependent upon fragment size and thus will affect observed metachromatic activity. The total number of anionic sites is therefore not the only variable affecting the interaction of the dye with small heparin fragments.

The minimum molecular weight at which a heparin fragment exhibits full activity cannot be precisely determined from our data. However, based on the large increase in activity between the octasaccharide and decasaccharide, as well as between the decasaccharide and oligosaccharide (molecular weight 3100) this minimal molecular weight is probably about 4000. Isolation of heparin products in this molecular weight range will be necessary before such a minimal molecular weight can be precisely determined.

In conclusion, we have demonstrated that the metachromatic activity of commercial porcine mucosa heparin is independent of the molecular weight of the heparin. This is in sharp contrast to the dependence of heparin's anticoagulant activity on its molecular weight (23). The metachromatic activity of heparin degradation products (molecular weight <3100) is dependent on molecular weight. Preliminary results indicate that all of the enzymatically produced heparin fragments which had metachromatic activity also possessed some anticoagulant activity as measured by one or both Factor Xa assays. Fragments as small as a hexasaccharide produced by chemical depolymerization of heparin using nitrous acid have been reported to have anticoagulant activity (24,25). Continuing studies are aimed at examining the precise structural requirements for dye binding to these products as well as any correlations between metachromatic and anticoagulant activities.

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