Structural Characteristics of Heparan Sulfates with Varying Sulfate Contents†

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ABSTRACT: Structural properties of heparan sulfate preparations from hog mucosa and beef lung sources were obtained by application of Smith degradation and nitrous acid reactions. Products formed by these reactions indicated that most of the iduronic acid present in these mucopolysaccharides is ester sulfated, whereas *N*-sulfated glucosamine residues are ester sulfated much less frequently. Repeating units with sulfated iduronic acid were found to occur almost entirely in single sequences. Furthermore, the iduronic acid moieties may be bound to either *N*-acetylated or *N*-sulfated glucosamine units, with these occurring at either end of the uronic acid unit.

The variable composition of heparan sulfate preparations has been recognized for many years (Cifonelli and Dorfman, 1960; Linker and Hovingh, 1968) and, more recently, heterogeneity in chemical structure has been demonstrated (Linker and Hovingh, 1973; Hovingh and Linker, 1974; Cifonelli, 1968, 1970). These properties show a striking parallelism with those of heparin (Cifonelli and King, 1975). Distinctions between the two classes of compounds involve mainly differences in ester and *N*-sulfate contents, in iduronic and glucuronic acid proportions, and in the distribution of these components in the molecule. The variations noted in both heparan sulfate and heparin fractions have added to the difficulty in establishing definitive structural formulations for these compounds. Because of the apparent importance of these substances in cellular metabolism (Kraemer, 1971; Kleinman and Silbert, 1975; Margolis and Atherton, 1972; Buonassissi, 1973), in hemostasis (Damus and Rosenberg, 1973), and in clinical therapy (Wessler, 1975), clarification of the chemical and structural characteristics of the two mucopolysaccharides, as well as the correlation of these parameters to biological activity, has been of much interest.

Previous studies, using chemical degradation of heparan sulfates with nitrites (Cifonelli, 1968), demonstrated that segments with multiple sequences of *N*-acetylglucosamine residues were present in heparan sulfates and that increasing sulfation was correlated with decreased chain size of sections containing these residues (Cifonelli, 1970). Similar results were obtained by Linker and Hovingh (1968; 1973) in an elegant illustration of the usefulness of enzymatic techniques for revealing structural features of heparan sulfate fractions obtained from beef lung tissues.

The present investigation is an extension of earlier chemical studies on these mucopolysaccharides and has been directed toward improved understanding of the distribution of sulfated iduronic acid and contiguous hexosamine units within the heparan sulfate molecule.

Experimental Procedure

Materials

Beef lung by-products from the preparation of commercial heparin were generously provided by Dr. L. L. Coleman, The Upjohn Co., Kalamazoo, Mich. Similar by-products from the preparation of hog mucosal heparin were kindly supplied by Dr. H. H. R. Weber, Wilson Laboratories, Chicago, Ill.

Methods

Analytical procedures for the estimation of uronic acid, hexosamine, *N*-sulfated hexosamine, total sulfate, amino acids, and neutral carbohydrates have been given previously (Cifonelli and King, 1975). Carbazole to orcinol ratios have been determined according to Hoffman et al. (1956) and *N*-acetylhexosamine estimations, after acetylation, by the method of Levvy and McAllan (1959).

Isolation of Heparan Sulfate Preparations. The heparan sulfate fractions from beef lung by-products were obtained after preliminary fractionation from Dowex 1 chloride columns as described in detail by Rodén et al. (1972). The forerun fraction obtained by elution with 1.25 M sodium chloride was further purified by fractionation with ethanol (Meyer et al., 1956) in order to remove nucleic acids which generally contaminate this fraction. Ten grams of heparan sulfate was dissolved in 200 mL of 0.1 M sodium chloride and 20 mL of 5% calcium acetate in 0.5 M acetic acid was added. Addition of 70 mL of ethanol precipitated most of the nucleic acid, but little of the acid mucopolysaccharide material. The supernatant solution was mixed with 40 mL of ethanol and the flocculant precipitate amounted to 3.5 g, of which approximately 10% was nucleic acid. A further addition of 75 mL of ethanol to the supernatant solution from above gave 4.5 g of material (HSI) with negligible nucleic acid content and was used for this study.

HSII was isolated from Dowex 1 as the midfraction after elution with 1.5 M sodium chloride (Rodén et al., 1972). Because of molecular size heterogeneity, this sample was fractionated further from a column of Sephadex G-75 (3.3 × 143 cm), as described previously (Cifonelli and King, 1973). Fractions eluted between 350–510 mL were pooled and concentrated fivefold, and three volumes of ethanol was added to obtain the fraction used in this investigation.

Fraction HSIII was isolated from hog mucosal by-products after elimination of dermatan sulfate by complexing with copper, as described earlier (Cifonelli and King, 1973). The purified product was then fractionated from Dowex 1 chloride and the midfraction (Rodén et al., 1972) eluted with 1.25 M sodium chloride was isolated.

Smith Degradation of Heparan Sulfate Preparations. Smith degradations of the heparan sulfate samples were per-

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TABLE I: Composition of Heparan Sulfate Preparations.

Sample	Uronic acid (%)	Hexosamine (%)	Sulfate[a] Ester	Sulfate[a] Amino	Iduronic acid/total uronic acids
HSI	38.3	21.3	0.52	0.40	0.40
HSII	42.7	27.7	0.65	0.52	0.41
HSIII	37.8	23.7	0.48	0.45	0.27

[a] Given as molar ratios to hexosamine.

formed as reported previously (Cifonelli and King, 1975), except that hydrolysis was conducted at pH 1.5 for 15 min. After removal of borate, the oxidation and reduction steps were repeated to ensure completion of reaction and to avoid the possibility of anomalously low consumption of periodate, as observed for hexuronic acid residues of alginate (Painter and Larsen, 1970). However, under the conditions of Wolfrom et al. (1969) as used here, there appears to be little difference in the gel filtration pattern of the products between a single or double Smith degradation.

Reaction products were fractionated by gel filtration on a 1.5 × 234 cm column of Sephadex G-50 (fine grade). The column was eluted with 0.2 M sodium acetate in 12% ethanol and fractions of 4.5 mL were collected each 40 min. These were analyzed for uronic acid, hexosamine, and *N*-sulfated glucosamine contents and in some instances for *N*-acetylglucosamine or total sulfate.

Further hydrolysis of pooled fractions was performed at pH 1.0 for 15 min, and after neutralization and the hydrolysates were fractionated on Sephadex G-25 (1.1 × 186 cm), as described above.

Reaction of the Smith-degraded products with nitrous acid and gel filtration of the fragments was accomplished as reported earlier (Cifonelli and King, 1975).

The recovery in terms of hexosamine in the three heparan sulfate samples was variable. The best data available are for HSI, where 63% of the hexosamine was recovered after the double oxidation step. There was no loss of material after hydrolysis at pH 1.0, and a 77% recovery of hexosamine after reaction with nitrous acid. Recoveries of hexosamine in HSII and HSIII were comparable, but slightly lower.

Results

The preparations used in this investigation were isolated from beef lung or hog mucosal heparin by-products. Fractions were chosen which varied in proportions of *N*-sulfate and ester sulfate and which could be obtained in adequate amounts. Two preparations (HSI and HSII) were obtained from beef lung by-products after chromatography on Dowex 1 chloride by elution with 1.25 and 1.5 M sodium chloride solutions, respectively. A preliminary ethanol fractionation of HSI was necessary to remove nucleic acid, present as a significant contaminant in the beef lung material. Heparan sulfate obtained from hog mucosal by-products was similarly purified by chromatography from Dowex 1 and the fraction eluted with 1.25 M sodium chloride (HSIII) was used for these studies.

The compositions of the preparations are given in Table I. Each sample showed a single spot on electrophoresis and a single gel filtration peak from Sephadex G-75. Molecular weights, estimated from peak elution volumes, ranged from approximately 12 to 15 × 10^3. *N*-Sulfate groups were calculated to be present on 40 to 52% of the hexosamine residues and ester sulfate varied from approximately 0.5 to 0.65 M equiv per repeating unit. Iduronic acid, calculated from previous data (Taylor et al., 1973) on similar fractions, was estimated to vary between 27 and 41% of the total uronic acid contents.

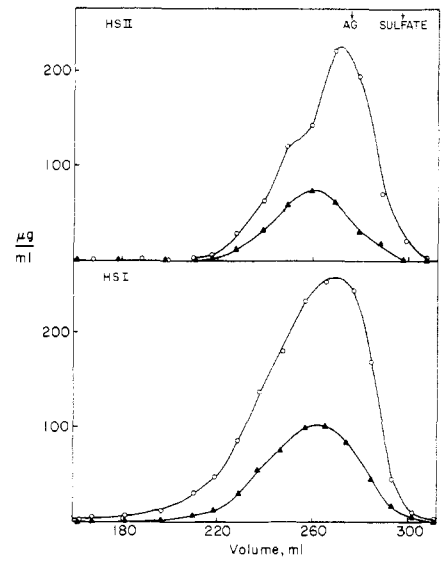

FIGURE 1: Sephadex G-50 diagram of Smith-degradation fragments from HSI and HSII. The oxidized and reduced samples were partially hydrolyzed at pH 1.5 for 15 min before gel filtration, as described previously (Cifonelli and King, 1975). The void volume was 115 mL and the total volume was 295 mL. The elution volume of trisaccharide was 265 mL. Elution markers indicated are: AG, *N*-acetylglucosamine and inorganic sulfate. Analyses include: (○) hexosamine; (▲) uronic acid.

Smith Degradation of Heparan Sulfate. Reaction of the heparan sulfate preparations with periodate followed the conditions reported for oxidation of nonsulfated uronic acids in heparin (Wolfrom et al., 1969), except for a 24–48 h longer reaction period. The Smith-degradation procedure was performed twice on each fraction to avoid the possibility of hemiacetal formation (Painter and Larsen, 1970) or other inhibition effects (Leonard and Richards, 1975) leading to decreased oxidation. However, it was noted that a single Smith degradation of HSI led to products similar to those from a double Smith degradation of this substance, suggesting that under the conditions chosen single-step oxidation suffices for reaction of most of the nonsulfated uronic acid residues present in heparan sulfates.

Gel diagrams from Sephadex G-50 of the Smith-degraded products from HSI and HSII are shown in Figure 1. The gel filtration pattern for HSIII reaction products was similar to that from HSI and is not illustrated in the Figure. Since oxidation of N-substituted hexosamines cannot occur during reaction of heparan sulfate with periodate ions, fragments are produced, after Smith degradation, having hexosamine units at both the reducing and nonreducing ends of the oxidized fragments. Comparison of the results for hexosamine and uronic acid, shown in Figure 1 for both HSI and HSII, with the analogous data from heparan sulfate of umbilical cords reported previously (Cifonelli and King, 1975), indicates that the former compounds have up to 10 or 15% more nonoxidized uronic acid than the substance from umbilical cords. Since the uronic acid containing cleavage products in all instances appear from the elution volumes to be predominantly trisaccharides, the heparan sulfates used in this study possess a wider distribution of sulfated iduronic acid residues throughout the molecule than are found in the umbilical cord sample and, thus,

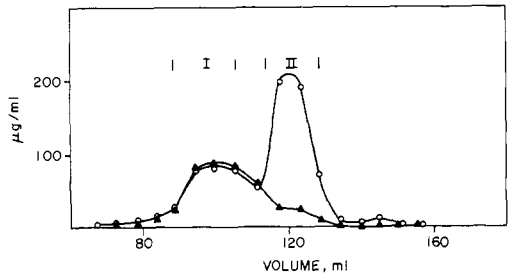

FIGURE 2: Gel filtration pattern from Sephadex G-25 of HSI pooled fractions (219–257 mL) shown in Figure 1 after additional hydrolysis at pH 1.0 for 15 min. Elution volume of markers include: void volume, 65 mL; trisaccharide, 95 mL; and inorganic sulfate, 130 mL. Analyses include: (○) hexosamine; (▲) uronic acid.

TABLE II: Composition of Partially Hydrolyzed Fragments from HSI.[a]

Fraction	Hexosamine (μg/mL)	*N*-Sulfated glucosamine (μg/mL)	*N*-Acetyl hexosamine (μg/mL)	Sulfate (μmol/mL)	Carbazole/orcinol
I	66	40	0	0.28	
I (reduced)	37	22			
II	187	28	60	Inorganic	1.4

[a] Fractions are illustrated in Figure 2. Conditions of hydrolysis are given in this text.

show closer structural similarities to heparins than does the latter.

Hydrolysis conditions (pH 1.5 for 15 min) to complete Smith degradation were chosen to ensure cleavage of oxidized units contiguous to sulfated uronic acid residues while not causing excessive release of ester sulfate groups. Although, under the conditions used, Smith-degraded *N*-acetylhexosamine-containing chains had undergone appreciable hydrolysis in comparison to hydrolysis at pH 3.0, as used previously for umbilical cord heparan sulfate oxidized products (Cifonelli and King, 1975), this did not interfere with assessment of ester sulfated iduronic acid moieties.

Determination of the proportion of uronic acid containing fragments as compared to Smith-degraded hexosamine cleavage products was made after further hydrolysis of the peak fractions shown in Figure 1. An illustration of the effect of hydrolysis at pH 1.0 for 15 min is given in Figure 2 for HSI (pooled fractions 219–257 mL). Previous data showed that, under the above conditions, Smith-degraded hexosamine sections are cleaved to monosaccharide units, whereas uronic acid containing fractions are unaffected and ester sulfate groups remain largely uncleaved (Cifonelli and King, 1975). The first peak shown in Figure 2 contains the major portion of uronic acid and nearly half of the hexosamine. This peak is eluted at the volume for trisaccharides, showing that sulfated iduronic acid is present in this preparation mainly in single sequences. Earlier reported results (Cifonelli and King, 1975), as well as data discussed above, indicate that these are separated by nonsulfated uronic acid units (primarily glucuronic acid), predominantly in multiple sequences.

Analytical data for the two fractions, pooled as indicated in Figure 2, are given in Table II. A ratio of *N*-sulfated hexosamine to total hexosamine in pooled fraction I of approximately 0.6, in contrast to a ratio for these components in pooled fraction II of 0.15, indicates that *N*-sulfated hexosamine units in this preparation are associated predominantly with sulfated iduronic acid rather than nonsulfated uronic acids.

If a ratio of hexosamine to uronic acid of 2 is assumed for pooled fraction I, the sulfate content suggests that this is present on little else besides uronic acid and the expected small proportion of *N*-sulfated hexosamine which escaped hydrolysis.

The uronic acid in peak II probably lacked ester sulfate, since this peak coincides with the elution volume for desulfated disaccharide from chondroitin sulfate. The carbazole to orcinol ratio for this fraction of 1.4 is indicative of the presence of bound, rather than free, iduronic acid, supporting the oligosaccharide nature of this material (Cifonelli and King, 1975). The hydrolysis conditions used, furthermore, were effective for cleaving the oxidized uronic acid fragments from one-third or more of the hexosamine moieties in peak II, as judged by results from Morgan–Elson determinations. Reduction of fraction I with sodium borohydride decreased the hexosamine nearly 50%, about half of which was the *N*-sulfated derivative.

The similar gel-filtration patterns observed for hydrolysates of HSII and HSIII provided further evidence for the occurrence of single sequences of sulfated uronic acid residues as a common structural feature of heparan sulfate preparations. In addition, uronic acid containing cleavage products found in the Smith-degraded heparan sulfate fractions, corresponding to more than 20% of the original uronic acid, indicate that a major proportion of the iduronic acid in these samples is ester sulfated.

Reaction of Smith-Degraded Fragments with Nitrous Acid. The presence of both *N*-sulfated and *N*-acetylated hexosamine residues in Smith-degraded fragments from heparan sulfate indicates that reaction of the cleavage products with nitrous acid should be useful for assessing the distribution of hexosamine units contiguous to sulfated iduronic acid. This is apparent from inspection of possible trisaccharide structures for the uronic acid containing fragments obtained after the Smith-degradation procedure:

$$\begin{array}{c} SO_4 \\ | \\ \text{GlcNR–IdUA–GlcNR}^1 \end{array}$$

Thus, when R = sulfate, the products after reaction with nitrites will include free anhydromannose and a disaccharide composed of sulfate iduronic acid and either *N*-acetylglucosamine or anhydromannose, depending on whether R^1 = sulfate or acetyl. Obviously, when R = acetyl, glycosidic bond cleavage cannot occur and the trisaccharide structure will remain unchanged or will have anhydromannose at the reducing end of the instances where R^1 = sulfate.

The products resulting from reaction with nitrites of Smith-degraded HSI and HSII gave the results from Sephadex G-25 shown in Figure 3. The decreased uronic acid to hexosamine proportions apparent in the diagrams is explainable by the fact that compounds containing iduronic acid bound to anhydromannose give considerably lower carbazole color yields than substances having this uronic acid linked to glucosamine, as occurs in Smith-degraded fragments (Cifonelli and King, 1975). The results from Figure 3, as well as from Tables III and IV, therefore, suggest that most of the iduronic acid in these fractions has anhydromannose bound to its reducing end. The analytical data for the nitrite-produced cleavage products from HSI and HSII (Tables III and IV) conform with this

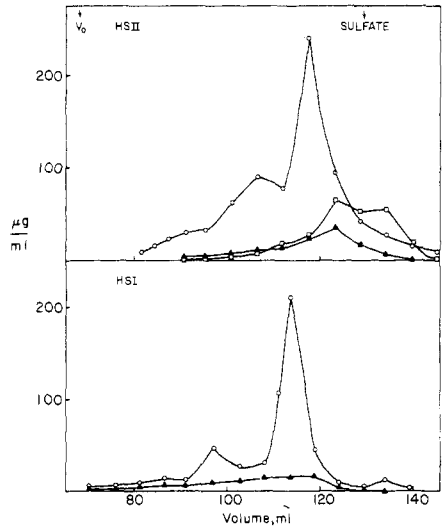

FIGURE 3: Gel filtration pattern from Sephadex G-25 of products obtained after reaction of HSI (pooled fractions 229–298 mL, Figure 1) and HSII (pooled fractions 219–289 mL, Figure 1) with nitrous acid as noted under Methods. Analyses and symbols are the same as for Figure 2. Analyses include: (○) hexosamine; (▲) uronic acid; (□) anhydromannose.

TABLE III: Composition of Cleavage Fragments from Smith Degraded HSI after Reaction with Nitrous Acid.[a]

Fractions (pooled vol) (mL)	Uronic acid (μg/mL)	Hexosamine (μg/mL)	Anhydromannose (μg/mL)	Carbazole/orcinol
(1) 92–108	8	31	11	0.36
(2) 108–119	16	134	22	0.16
(3) 129–139	0	9	14	

[a] Fractions are illustrated in Figure 3.

conclusion and show carbazole to orcinol ratios for the smaller-sized fragments characteristic of free iduronic acid (Rodén et al., 1972) or that bound to anhydromannose. However, the less retarded fractions, emerging in the volume for trisaccharides, show slightly higher ratios, suggesting that a minor portion of the iduronic acid in these fractions may have *N*-acetylated glucosamine bound to it at both ends.

The gel filtration data also indicate that most of the Smith-degraded segments containing only hexosamine and oxidized uronic acid residues are not cleaved further during reaction with nitrites, in accord with previous results from umbilical cord heparan sulfate (Cifonelli and King, 1975). Results of the totally included fractions from HSI and HSII indicate that free anhydromannose, indicative of *N*-sulfated glucosamine linked at the nonreducing end of uronic acid, corresponds to approximately one-fourth of the total anhydromannose, in agreement with the suggestion above that the majority of *N*-sulfated hexosamine residues in these compounds are attached to the reducing end of sulfated iduronic acid. Similar results were found for HSIII. The sulfate content in HSII pooled fraction 1 suggests that both iduronic acid and anhydromannose may be sulfated, if it is assumed on the basis of previous evidence (Cifonelli and King, 1975) that *N*-acetylhexosamine units in heparan sulfate are sulfated to a minor extent only.

High-voltage electrophoresis of fractions 1 and 2 (Table IV)

TABLE IV: Composition of Cleavage Fragments from Smith-Degraded HSII after Reaction with Nitrous Acid.[a]

Fraction (pooled vol) (mL)	Uronic acid (μg/mL)	Hexosamine (μg/mL)	Anhydromannose (μg/mL)	Sulfate (μmol/mL)	Carbazole/orcinol
(1) 95–112	11	88	8	0.23	0.39
(2) 112–123	22	202	31		0.21
(3) 123–134	15	23	54		0.21
(4) 134–146	0	9	17		

[a] Fractions are illustrated in Figure 3.

showed major spots at the origin and at the location of monosulfated disaccharides. Minor spots were observed with mobilities characteristic of free glucuronic acid and of disulfated disaccharide units from heparin. Similar electrophoretic patterns were observed for fractions from HSI. These data are in harmony with the conclusion that the ester sulfate present in heparan sulfates resides mainly on iduronic acid units. Further support for this was obtained by reaction of HSI with alkali as described by Sampson and Meyer (1971) for conversion of sulfated hexosamine to the anhydro derivative. The low order of anhydroglucosamine formation accorded with that given by heparan sulfate from umbilical cords (Cifonelli and King, 1975).

Discussion

The close chemical and structural relationship between heparin and heparan sulfate has made a clear distinction between these difficult. Although variations in ester and *N*-sulfate contents and in proportion of iduronic acid distinguish these compounds, these differences are not always large enough to provide a sharp differentiation between the substances. Similarly, defining the two classes of compounds by means of anticoagulant activities is complicated by the occurrence of partial biological activities for compounds fitting into each category when based on chemical considerations (Cifonelli and King, 1975). Since the distribution of components in these compounds may influence the biological properties, it is evident that structural parameters may be of importance in distinguishing heparan sulfate and heparin and in clarifying relationships between these polysaccharides.

The scope of the present study was concerned with establishing further insights into the relationships between heparin and heparan sulfate with the ultimate aim of correlating biological activity with specific parameters of these compounds. In conformity with previous data from umbilical cord heparan sulfate, the present results from preparations with varying sulfate contents also indicate that ester sulfate occurs predominantly on iduronic acid moieties rather than on *N*-sulfated hexosamine units. Furthermore, the ester sulfated uronic acid groups have both *N*-acetylglucosamine and *N*-sulfated glucosamine residues bound to it. Although the proportion of *N*-sulfated glucosamine in some of the Smith-degraded trisaccharide fractions indicated that this component was present to some extent at both ends of the sulfated iduronic acid units, the data did not establish whether *N*-acetylglucosamine could simultaneously occur at the reducing and nonreducing termini of the uronic acid residues. None of the heparan sulfate fractions examined possessed multiple sequences of sulfated iduronic acid in significant concentration. Thus, all heparan sulfate and heparin samples investigated in this laboratory had

ester sulfate on most of their iduronic acid residues, with such units occurring mainly in single sequences, except in compounds containing approximately 2 or more M equiv of sulfate per repeating unit (Cifonelli and King, 1975). In contrast, the frequency of ester sulfation of hexosamine units in heparan sulfates appears to be considerably less than for iduronic acid units. These observations may have implications for the sequencing of sulfate groups during conversion of glucuronic acid to iduronic acid in the biosynthesis of heparin (Hook et al., 1974).

Based on a combination of present and previous results (Linker and Hovingh, 1973; Cifonelli and King, 1975), some distinctions between heparan sulfate and heparin may be noted. (1) The former compounds have repeating units containing *N*-acetylglucosamine and uronic acid residues which are predominantly nonsulfated and occur largely in multiple sequences, whereas such repeating units occur predominantly in single sequences in heparins. (2) Repeating units with iduronic acid in heparan sulfates are predominantly disulfated, in contrast to the prevalence of trisulfated repeats in heparins. (3) Heparan sulfates contain ester-sulfated iduronic acid residues almost completely in single sequences, while heparin may have up to five or six such units in consecutive order. (4) Most of the iduronic acid residues in both types of compounds appear to be ester sulfated, whereas *N*-sulfated glucosamine moieties are ester sulfated in major proportion only when sulfate contents are high, such as occur in some of the heparins (Danishefsky et al., 1969; Perlin et al., 1971). (5) *N*-Sulfated hexosamine is associated with nonsulfated uronic acid in multiple sequences only to a minor extent, if at all. (6) Sulfated iduronic acid in heparan sulfate may be bound to *N*-acetyl or *N*-sulfated hexosamine at either the reducing or nonreducing end, although in heparins the uronic acid moiety is predominantly bound to *N*-sulfated hexosamine.

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