THE DISTRIBUTION OF 2-ACETAMIDO-2-DEOXY-D-GLUCOSE RESIDUES IN MAMMALIAN HEPARINS*

J. A. CIFONELLI AND J. KING

Departments of Pediatrics and Biochemistry, The La Rabida-University of Chicago Institute, and The Joseph P. Kennedy, Jr. Mental Retardation Center, University of Chicago, Chicago, Illinois 60637 (U. S. A.)

(Received May 19th, 1971; accepted for publication August 25th, 1971)

ABSTRACT

Highly purified, beef-mucosa heparin, shown to be free of contaminating glycosaminoglycans and having relatively high proportions of linkage-region components, was found to contain approximately three residues of 2-acetamido-2-deoxy-D-glucose per chain. The results of the examination of the fragments that were formed by treatment of this heparin with nitrites indicate that the 2-acetamido-2-deoxy-D-glucose residues are divided approximately equally between the linkage region and interior sections. Similar results were obtained with heparins from other sources, which suggests that approximately one-half of the non-reactive hexosamine residues may be distributed throughout the heparin molecule rather than be bound entirely to the linkage section. The results add further support to the proposal of a chemical relationship between heparins and heparan sulfates.

INTRODUCTION

The presence of 2-acetamido-2-deoxy-D-glucose residues in certain mammalian heparins has been reported by several workers[1–6], although other heparin preparations have been noted to lack such residues[5–7]. Recent studies have suggested, however, that most if not all mammalian heparin samples contain *N*-acetyl groups[8], although some may have as little as 1 or 2% of their hexosamine residues substituted with such groups.

Identification of 2-acetamido-2-deoxy-D-glucose as a structural component of heparin was accomplished by isolation of fragments containing this component after reaction of the polysaccharide with nitrous acid. Fractions containing 2-acetamido-2-deoxy-D-glucose residues joined to linkage-region residues were identified[4], and it was suggested that only such hexosamine residues and no 2-sulfamido-2-deoxy-D-glucose residues were involved in the linkage section.

*This work was supported by Grant No. AM-05996 from the National Institute for Arthritis and Metabolic Diseases and Grant No. HE-12927-02 from the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

Since nitrous acid reacts only with the 2-sulfamido-2-deoxy-D-glucose residues of heparin and heparan sulfate, investigation of the degradation products from this reaction affords a means for interpreting structural details of these molecules. Application of this reaction to heparan sulfate has been reported[9]. The present study describes the use of this degradative method for the determination of the relative distribution of 2-acetamido-2-deoxy-D-glucose residues within the heparin molecule.

EXPERIMENTAL

Materials. — The heparin samples used in this study were described previously[8]. They showed single, fast moving spots on examination by electrophoresis, indicating lack of detectable amounts of heparan sulfate or other acid glycosaminoglycans as contaminants[8]. Heparan sulfate was purified by fractionation on Dowex-1 resin, as reported earlier[9]. The fraction eluted with 1.25M sodium chloride was used in this study. 3-Methyl-1-butyl and butyl nitrites were purchased from Distillation Products, Inc., Rochester, N. Y., stored at −20°, and used without further purification.

Methods. — Methods for the determination of uronic acid, hexosamine, and amino acid residues, sulfate groups, and total carbohydrate components by the Technicon borate-column autoanalyzer have been reported earlier[8]. High-voltage electrophoresis was carried out in pyridine–acetic acid buffer (1:10:289, v/v, pyridine–acetic acid–water), pH 3.7. Prior to electrophoresis, fractions obtained by gel filtration were desalted by chromatography on Sephadex G-25 with water elution. The concentrated solutions were applied to Whatman No. 3MM paper and subjected to 3500–4000 V for 1 h in a Savant No. 5000 horizontal-plate apparatus. The spots were detected with a silver reagent or with ninhydrin. For preparative purposes, strips were eluted with 15% methanol solutions. The uronic acids present in the fractions obtained by preparative electrophoresis were identified by paper chromatography, after hydrolysis with 90% formic acid for 24 h, as described by Radhakrishnamurthy *et al.*[10].

The molecular sizes of the heparins used for this study were compared by gel filtration on Sephadex G-200, as described by Wasteson[11] and by Constantopoulos *et al.*[12], in a 107 × 0.8-cm column with 1.0M sodium acetate solution containing 12% ethanol as eluent, 3-ml fractions being collected every 80 min.

Determination of 2,5-anhydro-D-mannose formation. — Estimation of 2,5-anhydro-D-mannose formed after reaction of heparin or heparan sulfate with nitrous acid was performed by a modification of the Dische–Borenfreund method[13]. The solution of mucopolysaccharide containing 2–10 μg of hexosamine in 0.2 ml was mixed with freshly prepared 5% butyl nitrite in ethanol (0.2 ml) and 2M hydrochloric acid (0.2 ml). After incubation at room temperature for 1.0 h, ammonium sulfamate (12.5%, 0.2 ml) was added, and the mixture shaken occasionally for 1.0 h. After addition of 5% hydrochloric acid (1 ml) and 0.5% indole in ethanol (0.2 ml), the tubes were covered and heated for 5 min in a boiling-water bath. After being cooled, ethanol (1.2 ml) was added and the color formed was measured in the usual way[13]. Standard curves were prepared with beef-lung heparin containing 98–99% of the hexosamine

residues as the reactive *N*-sulfate derivative[8]. Determination of 2,5-anhydro-D-mannose in fractions obtained by gel filtration that contained heparin or heparan sulfate degradation products was done as just described, except that the reagent mixture made of nitrite, acid, and sulfamate was preincubated for 1 h before addition to the fractions.

Reaction of heparin or heparan sulfate with organic nitrites. — A solution of the sample (100 mg) in water (30 ml), cooled to 2°, was passed through a 1.6 × 7-cm column of Dowex 50 (8X, H^+, 200–400 mesh) at a rate of 2 ml per min, and the column was washed with water (20 ml). To this was added peroxide-free 1,2-dimethoxyethane (50 ml) and 3-methyl-1-butyl or butyl nitrite (2 ml), and the mixture was incubated for 4 h at −20°. Completion of the reaction was determined by removal of aliquots (0.05 ml) and estimation of the 2,5-anhydro-D-mannose formed, as just

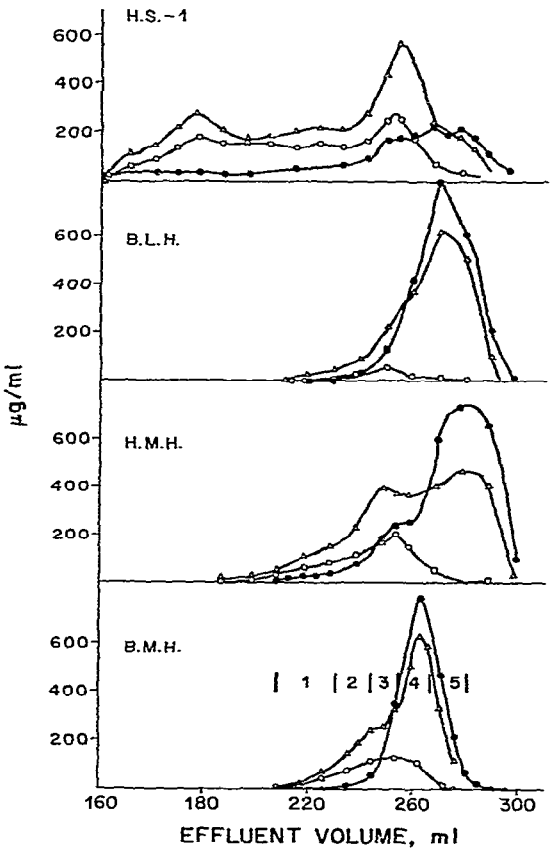

Fig. 1. Patterns of elution from Sephadex G-50 of the products of reaction of heparin and heparan sulfate preparations with butyl nitrite. H.S.-1, heparan sulfate isolated from beef-lung tissue; B.L.H., heparin from beef-lung tissue; H.M.H., heparin from hog-mucosa tissue; and B.M.H., heparin from beef-mucosa tissue: ○, hexosamine; ●, 2,5-anhydromannose; △, uronic acid. The fractions obtained from beef-mucosa heparin were pooled for further studies, as shown. The void volume was 110 ml.

described. After completion of the reaction, 10% sodium acetate (2 ml) was added, and the mixture concentrated *in vacuo* at 25° to approximately one-third of its volume. After addition of methanol (25 ml), the solution was concentrated to 3–4 ml for application to a Sephadex G-50 column (fine-graded, 1.6 × 200 cm). Elution of the column was performed with 0.2M sodium acetate in 12% ethanol and 5-ml fractions were collected. The void volume for this column was 110 ml and the sulfated tetrasaccharide obtained from chondroitin 4-sulfate by treatment with testicular hyaluronidase was eluted at approximately 250 ml. Fractions collected by gel filtration were analyzed for uronic acid, hexosamine, and 2,5-anhydro-D-mannose, and pooled as indicated in Fig. 1.

Recovery of L-*serine from treated heparin.* — A control reaction for the estimation of L-serine recovered after reaction of heparin with butyl nitrite was performed as follows: Beef-mucosa heparin (1.5 mg), containing approximately 90% of its amino acid content as serine, was dissolved in water (1 ml), and the solution was treated at −20° with 20% butyl nitrite in methanol (0.5 ml) and 2M hydrochloric acid (0.1 ml). After 4 h the mixture was extracted with ether (5 × 3 ml), and the aqueous solution was examined in the amino acid analyzer.

Chromatography of Fraction 4 on Dowex-1 resin. — A portion of pooled Fraction 4, obtained after gel filtration on Sephadex G-50, as illustrated in Fig. 1, was diluted with an equal volume of water, and the solution was applied to a 12 × 1.1-cm column of Dowex-1 (2X, Cl-, 200–400 mesh) resin. Elution was performed with 0.5, 0.75, 1.25, and 2M sodium chloride solutions, and approximately 50-ml fractions were collected with each eluent. Each eluate was concentrated to a few ml, one-fourth volume of ethanol added, and the supernatant solution decanted from the precipitated sodium chloride. This procedure was repeated once or twice to give a final volume of 3–4 ml that was desalted on a Sephadex G-25 column (fine-beaded, 1.4 × 40 cm). Fractions (6 ml) were collected at 40-min intervals and analyzed for uronic acid, hexosamine, and 2,5-anhydro-D-mannose. Some fractions were concentrated for high-voltage electrophoresis or for determination of the galactose and xylose content.

RESULTS

The heparin preparations used in this study were commercial samples purified either by precipitation with cetylpyridinium chloride in 1.2–1.4M sodium chloride or by fractionation on Dowex-1 columns, as described earlier[8,14]. Heparan sulfate was purified, as reported previously[9], by fractionation on Dowex-1 resin. These procedures were necessary to remove small amounts of low-sulfated contaminants generally accompanying commercial preparations[14]. Analytical data for the heparin samples used in this work have been previously reported[8].

The molecular weights of the samples used in this study were compared by the gel-filtration method on Sephadex G-200, as described by Wasteson[11]. The results

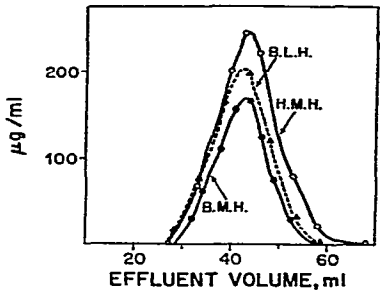

Fig. 2. Patterns of gel filtration on Sephadex G-200 of heparin preparations from hog mucosa (H.M.H.), beef mucosa (B.M.H.), and beef lung (B.L.H.) illustrating the similarity of molecular sizes.

(Fig. 2) indicated that the molecular weights were similar, and they were estimated at $11–12 \times 10^3$, on the basis of the viscosity data for the hog-mucosa preparation*.

Reaction of heparin with alkyl nitrites. — Previous studies have utilized solutions of nitrous acid at −20° for degrading heparin and heparan sulfate[9,15]. It has been found that alkyl nitrites are equally effective in cleaving these mucopolysaccharides. The specificity of the organic nitrites is similar to that shown by nitrous acid solutions at subzero temperatures[9], and reaction with compounds having free amino groups does not occur either at subzero or room temperatures. In control experiments, L-serine was completely recovered under the conditions used for reaction of heparin with alkyl nitrite.

The advantages of the present method over that using nitrous acid solutions, as applied formerly, include (a) elimination of the preparation of nitrous acid solutions, which required relatively cumbersome methods, and (b) stability of the alkyl nitrites, which does not limit the treatment to subzero temperatures, the reagents having been used with satisfactory results at room temperature. However, to eliminate the possibility of minor side-effects during reaction at higher temperatures, only subzero conditions were used in this study. Similar reactivities have been found for both 3-methyl-1-butyl and butyl nitrites, and the reaction rates of these two reagents are comparable to that of nitrous acid[9].

Gel filtration and composition of the products of reaction. — The cleavage products formed after reaction of heparin with alkyl nitrites were fractionated on Sephadex G-50. Either Sephadex G-25 or G-50 gave elution patterns from which nonreactive contaminating mucopolysaccharides could easily be distinguished from the reaction products. However, the cleavage fragments from heparan sulfate emerged partially near the void volume on gel filtration on Sephadex G-25, and the separation was better on Sephadex G-50 (Fig. 1). Detection of contaminant mucopolysaccharides was of primary importance in the evaluation of results from heparin, since even minor amounts of these could render assessment of 2-acetamido groups in heparin uncertain. The elution patterns shown in Fig. 1 indicate that the heparin preparations used for

*From data obtained on hog-mucosa heparin prepared as a standard reference substance (work supported by Grant 5-RO1-HE-11083 from the National Institutes of Health).

Carbohyd. Res., 21 (1972) 173–186

TABLE I

COMPOSITION OF THE FRACTIONS OBTAINED BY GEL FILTRATION OF THE PRODUCTS RESULTING FROM THE REACTION OF BEEF-MUCOSA HEPARIN WITH ALKYL NITRITE[a]

Pooled fraction[b]	*Uronic acid* (μg/ml)	*Hexosamine* (μg/ml)	*Fractions*					*Serine: Hexosamine* (*ratio*)
			Hexosamine (μ *moles*)	*Hexosamine* (%)	*Serine* (μ *moles*)	*Serine* (%)	*Xylose* (μ *moles*)	
1	53	27	3.4	14.3	1.2	19.6	1.0	0.36:1
2	170	86	6.5	27.4	3.4	55.7	2.8	0.52:1
3	299	124	6.2	26.2	1.0	16.4	0.9	0.16:1
4	760	94	7.0	29.5	0.50	8.2	0.4	0.07:1
5	210	8	0.6	2.5	<0.01	<0.1	0	0.01:1
Beef-mucosa heparin			150		6.6		5.5	0.044

[a]Analytical methods are given in the Experimental Section. [b]Pooled as illustrated in Fig. 1.

reaction with alkyl nitrite were devoid of unreactive acid mucopolysaccharides, since no products were detected in the void volume. Because of the enrichment of non-reactive polysaccharides in the void volume, the nitrous acid degradation technique is useful for detecting smaller amounts of contaminants than would otherwise be possible[16].

Examination of more than a dozen preparations of heparin from different sources showed that only the substance isolated from beef-mucosa tissue had a content of L-serine and D-xylose sufficient to indicate an intact carbohydrate–protein linkage in most or all molecules. For this reason, evidence for the partially random distribution of 2-acetamido-2-deoxy-D-glucose residues in heparin has been based primarily on results obtained with material obtained from beef mucosa.

Analyses of the pooled fractions from bovine-mucosa heparin (Fig. 1), showed that L-serine, D-xylose, and 2-acetamido-2-deoxy-D-glucose residues were present in significant amounts in the first four pooled fractions (Table I), Fractions 1 and 2 containing approximately 75% of the recovered L-serine, but only about 40% of the recovered hexosamine. In contrast, pooled Fraction 4 contained nearly 30% of the total nonreactive hexosamine but only 8% of the L-serine. Since L-serine was found to be unaffected by the conditions used, the high ratios of hexosamine to serine in the more retarded fractions suggest that a preponderant part of the hexosamine residues are located in the molecule at a position remote from the protein–carbohydrate linkage region. This suggestion is supported by the lack of an excess of D-xylose or D-galactose residues over hexosamine residues in pooled Fractions 4 and 5, which indicates that the effect of hydrolysis on the linkage region was not responsible for the results observed.

The L-serine to hexosamine ratio of 0.36:1 in pooled Fraction 1 suggests an average of approximately three hexosamine residues per degraded chain. The approximate molecular weight of this chain would be 2×10^3, which is consistent with the elution pattern from Sephadex G-50 (Fig. 1). Similary, the L-serine to hexosamine ratio of pooled Fraction 2 is consistent with a chain containing an average of two hexosamine residues bound to the protein-carbohydrate linkage region. In contrast, the ratio of 0.16:1 found for pooled Fraction 3 requires six repeating glycuronosyl-2-acetamido-2-deoxy-D-glucose residues per chain, if these residues originated entirely from the protein–carbohydrate linkage section, forming a product that should be eluted earlier from the Sephadex column. Furthermore, the approximately equivalent contents of D-xylose and L-serine in this fraction suggests that these constituents are present in linkage chains which, as judged from the gel-elution position, may contain a single glycuronosyl-2-acetamido-2-deoxy-D-glucose repeating residue. Therefore, the bulk of the 2-acetamido-2-deoxy-D-glucose units appear to be components of sections of the polysaccharide chain that are separated from the protein–carbohydrate linkage region by 2-deoxy-2-sulfoamido-D-glucose residues. Sections of this type would produce fragments, after treatment with nitrous acid, that contain nonreactive hexosamine residues and a 2,5-anhydro-D-mannose residue as reducing terminal. The increased concentration of 2,5-anhydro-D-mannose residues in pooled Fraction 3,

as compared to the two earlier fractions, supports the presence of fragments containing both 2-acetamido-2-deoxy-D-glucose and 2,5-anhydro-D-mannose residues. However, the reaction of heparin with nitrous acid produces a large proportion of glycuronosyl-2,5-anhydro-D-mannose residues, part of which are expected to be disulfated, which add to the difficulty of establishing the presence of 2,5-anhydro-D-mannose residues in fragments containing glycuronosyl-2-acetamido-2-deoxy-D-glucose residues.

The best evidence for the presence of randomly distributed 2-acetamido-2-deoxy-D-glucose residues in heparin is found in Fraction 4. In this pooled fraction, the L-serine to hexosamine ratio of 0.07:1, as well as the lack of excess D-xylose, relative to hexosamine, and the elution volume indicate that very few hexosamine residues are bound to the carbohydrate–protein linkage region. The elution volume of this fraction indicates that most of the material may consist of tetrasaccharides. The presence of a small concentration of 2,5-anhydro-D-mannose in Fractions 1 and 2 suggests the presence of minor amounts of products containing both 2-acetamido-2-deoxy-D-glucose and 2,5-anhydro-D-mannose residues.

Estimation of the L-serine and D-xylose residues recovered after gel filtration of the reaction products (see Table I) shows that approximately 90% of each component is accounted for. Since mechanical losses and analytical errors have not been taken into consideration, the recoveries are considered acceptable for the interpretation of the distribution of 2-acetamido 2-deoxy-D-glucose residues in heparin.

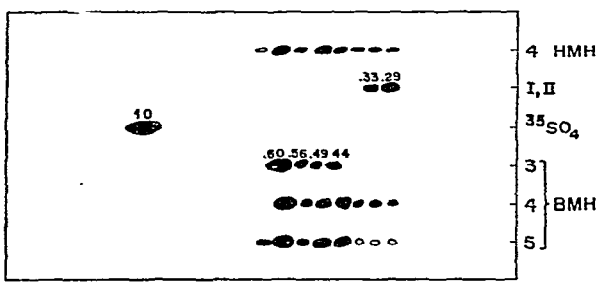

Fig. 3. High-voltage electropherogram of heparin-degradation fractions. Conditions are indicated in the Experimental Section. BMH-3, 4, 5 refer to fractions pooled as shown in Fig. 1. HMH-4 is a fraction from hog-mucosa heparin pooled as indicated for BMH-4. I and II are di- and tetrasaccharides prepared from chondroitin 4-sulfate by hydrolysis with 1.0M hydrochloric acid for 2 h, followed by *N*-acetylation and fractionation on Sephadex G-25. The tetrasaccharide fraction is the faster moving spot. $^{35}SO_4$ was applied as the sodium salt and detected by strip scanning.

High-voltage electrophoresis of gel-filtration fractions. — The fractions obtained by gel filtration (see Table I) were fractionated further by high-voltage electrophoresis (Fig. 3). Several spots were observed for the hexosamine-containing fractions after staining the electropherogram with a silver reagent. The mobilities of the two slower-moving degradation fractions of heparin were similar to those of di- and tetra-saccharides prepared by acid hydrolysis of chondroitin 4-sulfate, thus indicating that the former substances were desulfated. Two or three additional spots having

lower mobilities were noted for Fractions 1 and 2 after spraying with ninhydrin. These were found to contain L-serine, but since this report is concerned with the 2-acetamido-2-deoxy-D-glucose residues located in the interior of the heparin chain rather than in the protein-carbohydrate linkage region, these spots will not be further considered. Analyses of the fractions obtained by preparative electrophoresis of pooled Fractions 3 and 4 that showed spots having mobilities similar to those shown by spots of Fraction 5 indicated that these fractions contained both hexosamine and 2,5-anhydro-D-mannose residues. Therefore, distinction of products containing 2-acetamido-2-deoxy-D-glucose from those containing only uronic acid and 2,5-anhydro-D-mannose residues was not possible under these conditions, and other fractionation methods, as described in the following paragraphs were used prior to electrophoresis. Comparison of the electrophoretic patterns obtained with hexosamine-enriched fractions (Fraction 4) from beef-mucosa and hog-mucosa heparins showed striking similarities, which supports structural similarities between heparins from different sources.

Fractionation of Fraction 4 by ion-exchange chromatography and gel filtration. — Fraction 4 contains a proportion of products having both 2-acetamido-2-deoxy-D-glucose and 2,5-anhydro-D-mannose residues larger than do the other fractions (see Table I), and most of these nonreactive hexosamine residues are present in portions of the carbohydrate chain not directly united to the linkage-region residues. This Fraction 4, therefore, was chosen for further fractionation on a Dowex 1 (Cl^-) ion-exchange resin column, eluted with 0.5, 0.75, 1.25, and 3M sodium chloride. The eluates so obtained were desalted on Sephadex G-25 and several fractions, as

TABLE II

COMPOSITION OF THE FRACTIONS OBTAINED AFTER CHROMATOGRAPHY ON DOWEX-1 RESIN AND SEPHADEX G-25

Fraction[a]	*Uronic Acid* (μg/ml)	*Hexosamine* (μg/ml)	*2,5-Anhydro-D-mannose* (μg/ml)	*D-Galactose*[b] (μg/ml)	*D-Xylose*[b] (μg/ml)
0.5-a	68	38	19	21	11
-b	78	40	33	13	7
-c	92	20	88	traces	0
-d	117	0	77		
0.75-a	45	18	20	2	1
-b	109	25	155	traces	0
-c	45	17	68		
-d	146	0	237		
1.25-a	48	19	23		
-b	28	12	22		
3.0-a	36	21	18		
-b	14	4	6		

[a]Numerals refer to sodium chloride molarity of eluate from the Dowex-1 column and letters indicate the order of fractions obtained from the Sephadex G-25 column. [b]Determined with the Technicon Carbohydrate Autoanalyzer.

shown in Table II, were collected from each resin eluate. It should be noted that some fractionation of the products occurred during the desalting operation, although a relatively short column (1.4 × 38 cm) of Sephadex was used. D-Galactose and D-xylose were found mainly in Fractions 0.5-a and -b, indicating that the linkage section is almost entirely eluted with 0.5M sodium chloride from Dowex-1 resin. Fractions (a) from the 0.75, 1.25, and 3M eluates show approximately equivalent contents of 2-acetamido-2-deoxy-D-glucose and 2,5-anhydro-D-mannose, suggesting that 2-acetamido-2-deoxy-D-glucose may be present as interior residues in heparin. Fraction (d) from the 0.5 and 0.75M eluates contained uronic acid and 2,5-anhydro-D-mannose residues only, but these compounds were not found in significant amounts in the 1.25 and 3M eluates. The remaining fractions showed an amount of 2,5-anhydro-D-mannose in excess of that required to bind the 2-acetamido-2-deoxy-D-glucose residues present, indicating that these fractions contained a mixture of substances. The various ratios of uronic acid to 2,5-anhydro-D-mannose residues found in Fractions 0.5-d and 0.75-d suggest differences in sulfate content or uronic acid type and will be discussed further in the following paragraph.

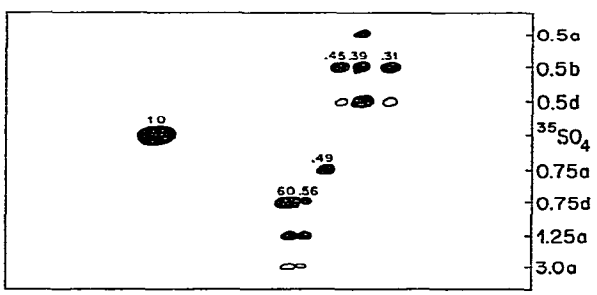

Fig. 4. High-voltage electropherogram of BMH-4 fractions obtained after fractionation on Dowex-1 resin and Sephadex G-25. The labeling of the fractions is similar to that given in Table II.

High-voltage electrophoresis of desalted fractions. — Electrophoretic mobilities of some of the fractions listed in Table II are illustrated in Fig. 4. Fraction 0.5-a showed one spot having a R_{SO_4} value of 0.39 after staining with a silver reagent, although after spraying with ninhydrin two serine-containing spots having R_{SO_4} values of 0.31 and 0.28 were detected. Since the 0.5-d and 0.75-d fractions contained only uronic acid and 2,5-anhydro-D-mannose residues, the variation in mobilities of the spots suggests differences in the sulfate content, the slower-moving fraction containing a ratio of sulfate group to 2,5-anhydro-D-mannose residue of approximately 1.0:1.0, the faster-moving material of about 2.0:1.0 (Table III). Furthermore, the ratio of uronic acid to 2,5-anhydro-D-mannose residues of these substances suggests a difference in type of uronic acid. Paper chromatography of the acid hydrolyzate of the two fractions showed that the more rapid component contained only iduronic acid, whereas the slower-moving fraction contained glucuronic acid and a much

TABLE III

COMPOSITION OF HIGH-VOLTAGE ELECTROPHORESIS FRACTIONS

Fraction[a]	$R_{SO_4}^{G}$	*Uronic Acid* (μg/ml)	*Hexosamine* (μg/ml)	*2,5-Anhydro-D-mannose* (μg/ml)	*Sulfate* (μ moles/ml)	*Suggested structures*[b]
0.5b	0.31	62	30	15	0	(HexA–GNAc)$_2$HexA–AnhMan
0.5b	0.39	49	31	18	[c]	(HexA–GNAc)$_2$HexA–AnhMan(–SO_3H)
0.5b	0.45	61	21	28	0.18	HexA–GNAc–HexA–AnhMan–SO_3H
0.5d	0.45	36	0	32	0.22	HexA–AnhMan–SO_3H
0.75a	0.49	65	32	29	0.24	HexA–GNAc–HexA–AnhMan–SO_3H
0.75d	0.60	36	0	77	0.91	HO_3S–HexA–AnhMan–SO_3H

[a]Fractions correspond to those shown in Fig. 4. [b]For abbrevations, see Fig. 5. [c]A fraction having a similar mobility, which had been isolated from hog-mucosa heparin fragments, showed a sulfate to 2,5 anhydro-D-mannose ratio of approximately 1.0:1.0 (unpublished results by the authors).

smaller amount of iduronic acid. This conforms with reports[17,18] that L-iduronic acid residues in heparin are sulfated, in contrast to D-glucuronic acid residues which are not sulfated; thus, the repeating disaccharide residues containing L-iduronic acid are trisulfated and those containing D-glucuronic acid are disulfated. Results from this study, as noted above, indicate that a small proportion of the disaccharide residues containing L-iduronic acid are disulfated, although it is not known whether the L-iduronic acid residues of such moieties are sulfated.

The results of the preparative high-voltage electrophoresis of some of the hexosamine-containing fractions are shown in Table III. None of the fractions listed, except for 0.5-b, contained significant amounts of D-galactose or D-xylose, indicating that a preponderance of the products have 2,5-anhydro-D-mannose as reducing terminals. Lack of material did not permit the examination of the neutral sugar components of the 0.5-b subfraction that showed an electrophoretic mobility of 0.31, and therefore it is not possible to state whether some material derived from the linkage section was present. However, fractions having similar mobility, but lacking D-galactose or D-xylose have been isolated from hog-mucosa heparin (Table III, footnote *b*), which suggests that such products, originating from the interior of the molecule, may also occur in beef-mucosa heparin.

Tentative structural formulas drawn from the results given in Table III are shown in the last column of the table and illustrate the spectrum of fragments derived from interior sections of the heparin molecule.

DISCUSSION

The results, obtained in the study of the chemical structure of heparin and heparan sulfate[6,9], of the degradation products formed after reaction with nitrous acid indicated a chemical interrelationship for these substances[14]. The finding that 2-acetamido-2-deoxy-D-glucose residues were distributed in heparan sulfate partially in random fashion suggested that similar studies of the location of these residues in heparin would support more directly the relationship between these two types of acid mucopolysaccharides. For this purpose, a heparin sample containing intact protein–carbohydrate linkage residues would be highly desirable, since only such a preparation could provide, after reaction with nitrous acid, cleavage fragments representative of the native structure. Furthermore, a preparation possessing complete protein–carbohydrate linkage sections with a theoretical content of serine would have the advantage of possessing groups which may be easily detected in the presence of other cleavage products, thus simplifying the identification of the degradation fragments as well as the interpretation of the results.

The absence of contaminating heparan sulfate is a further requirement for the study of the chemical structure of heparin. Because of the occurrence of both 2-acetamido-2-deoxy-D-glucose and 2-deoxy-2-sulfamido-D-glucose residues in heparan sulfate, the fragments produced after reaction of heparan sulfate with nitrous acid are similar to those formed from heparin. Although the range of products formed from the former polysaccharide permits its distinction from heparin, the overlap of degrada-

tion fragments, as shown in Fig. 1, would introduce difficulties in the interpretation of the results when such contamination exists. However, the presence of mucopolysaccharides other than heparan sulfate would not be as objectionable, since these substances would emerge in the void volume during the gel filtration of the products of reaction of heparin and would be removed from the cleaved material. For the present study, the heparin preparations have been purified by procedures shown to be efficient in the removal of heparan sulfate[8,14], and products associated with heparan sulfate were not detected in the degradation material formed by the reaction of nitrous acid with the heparin samples.

Under the subzero conditions used for the reaction of heparin with alkyl nitrites, the serine residues were not affected. This was demonstrated by the recovery of over 90% of the original L-serine in the gel-filtration fractions. Since no L-serine or D-xylose was present in the most retarded gel-filtration fraction (see Table I), hydrolysis during the degradation reaction was not a factor, and the results obtained were regarded as reliable for estimating the extent and distribution of the nonreactive hexosamine residues in heparin.

More direct evidence for the occurrence of interior residues of 2-acetamido-2-deoxy-D-glucose was obtained from electrophoretic data. Preparative isolations of the cleavage products formed after reaction of beef- or hog-mucosa heparin with nitrites indicated that most of the nonreactive hexosamine residues in pooled Fractions 3 and 4 occurred singly, although smaller amounts of degradation fragments containing two and possibly three hexosamine residues were found. These findings are consistent (*a*) with the elution volumes of the gel filtration of fractions containing both 2-acetamido-2-deoxy-D-glucose and 2,5-anhydro-D-mannose residues and (*b*) with the fact that about half of the approximately three residues of 2-acetamido-2-deoxy-D-glucose occurring in beef-mucosa heparin is estimated to be attached to the linkage region, leaving an average of approximately one and one-half of such moieties to be distributed elsewhere in the molecule.

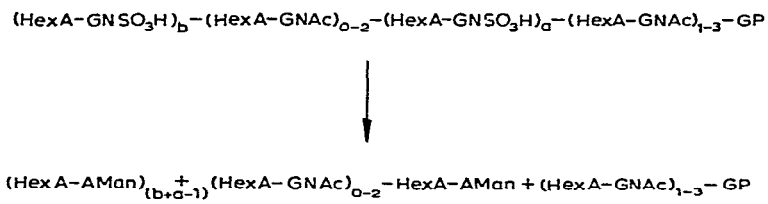

Fig. 5. Proposed chemical structure of beef-mucosa heparin and reaction with nitrous acid showing sections containing both 2-acetamido-2-deoxy-D-glucose and 2-deoxy-2-sulfamido-D-glucose residues. Abbreviations: HexA, hexuronic acid; AMan, 2,5-anhydro-D-mannose; GNAc, 2-acetamido-2-deoxy-D-glucose; $GNSO_3H$, 2-deoxy-2-sulfamido-D-glucose; GP, linkage-region residue.

Based on these results, a simplified structure, shown in Fig. 5, may be drawn for beef-mucosa heparin. This structure bears a close similarity to that reported for heparan sulfate[9], although the latter polysaccharide possesses a more extended

distribution of both single and multiple 2-acetamido-2-deoxy-D-glucose residues, in keeping with the much larger proportion of this constituent in heparan sulfate. The structure shown in Fig. 5 contains at least a single residue of 2-acetamido-2-deoxy D-glucose at the linkage region. This is based on the results of gel filtration, Dowex-1 chromatography, and preparative electrophoresis of the fractions obtained from degradation of beef-mucosa heparin. These fractions which contain D-xylose and L-serine have not been found devoid of hexosamine, thus suggesting that most or all heparin molecules have 2-acetamido-2-deoxy-D-glucose residues uniting the protein–carbohydrate linkage section to the main chain of heparin. Similar conclusions have been reported previously for hog-mucosa heparin[4] and heparan sulfate[9,15].

The similarities in the detailed molecular structures of heparin and heparan sulfate[19] is of particular interest in relation to the evidence of the biosynthesis of these glycosaminoglycans from a 2-acetamido-2-deoxy-D-glucose-containing "precursor substance"[20]. The results from the chemical studies are in accord with the suggestion of a biosynthetic mechanism[20,21] whereby *N*-acetyl groups are replaced by sulfate groups.

ACKNOWLEDGMENTS

We wish to thank Dr. H. H. R. Weber, Wilson Laboratories and Dr. L. L. Coleman, The Upjohn Co., for the heparin and heparan sulfate fractions used in this study, and Mrs. L. Roth and Miss M. L. Spach for excellent technical assistance.

REFERENCES

1 B. RADHAKRISHNAMURTHY, E. R. DALFERES, JR., AND G. S. BERENSON, *Anal. Biochem.*, 26 (1968) 61.
2 T. KOTOKU, Z. YOSIZAWA, AND F. YAMAUCHI, *Arch. Biochem. Biophys.*, 120 (1967) 553.
3 L. B. JACQUES, L. KAVANAGH, AND A. LAVALLEE, *Arzneim.-Forsch.*, 17 (1967) 774.
4 U. LINDAHL, *Biochim. Biophys. Acta*, 130 (1966) 368.
5 A. S. PERLIN, M. MAZUREK, L. B. JACQUES, AND I. W. KAVANAGH, *Carbohyd. Res.*, 7 (1968) 369.
6 J. A. CIFONELLI, *Fed. Proc.*, 24 (1965) 354.
7 M. L. WOLFROM, S. HONDA, AND P. Y. WANG, *Carbohyd. Res.*, 10 (1969) 259.
8 J. A. CIFONELLI AND J. KING, *Carbohyd. Res.*, 12 (1970) 391.
9 J. A. CIFONELLI, *Carbohyd. Res.*, 8 (1968) 233.
10 B. RADHAKRISHNAMURTHY, E. R. DALFERES, JR., AND G. S. BERENSON, *Anal. Biochem.*, 24 (1968) 397.
11 A. WASTESON, *Biochim. Biophys. Acta*, 177 (1969) 154.
12 G. CONSTANTOPOULOS, A. S. DEKABAN, AND W. R. CARROLL, *Anal. Biochem.*, 31, (1969) 59.
13 Z. DISCHE AND E. BORENFREUND, *J. Biol. Chem.*, 184 (1950) 517.
14 J. A. CIFONELLI AND L. RODÉN, *Biochem. Prep.*, 12 (1968) 12.
15 J. KNECHT, J. A. CIFONELLI, AND A. DORFMAN, *J. Biol. Chem.*, 242 (1967) 4652.
16 J. A. CIFONELLI AND J. KING, *Biochim. Biophys. Acta*, 215 (1970) 273.
17 U. LINDAHL AND O. AXELSSON, *J Biol. Chem.*, 246 (1971) 74.
18 M. L. WOLFROM, P. Y. WANG, AND S. HONDA., *Carbohyd. Res.*, 11 (1969) 179.
19 J. A. CIFONELLI, in E. A. BALAZS (Ed.), *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 2, Academic Press, London, 1970, p. 961.
20 J. E. SILBERT, *J. Biol. Chem.*, 242 (1967) 5153.
21 A. S. BALASUBRAMANIAN, N. S. JOUN, AND W. MARX, *Arch. Biochem. Biophys.*, 128 (1968) 623.