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Identification of Iduronic Acid as the Major Sulfated Uronic Acid of Heparin*

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SUMMARY

Oxidation of purified heparin with periodate destroyed about 30% of the total uronic acid and almost all of the glucuronic acid residues, suggesting that glucuronic acid was essentially nonsulfated.

Heparin was degraded with nitrous acid at low temperature, and the resulting products were separated by gel chromatography followed by paper electrophoresis. Four fractions were isolated and identified as nonsulfated uronic acid monosaccharide (B), monosulfated uronosylanhydromannose disaccharide (C), disulfated uronosylanhydromannose disaccharide (D), and sulfated uronic acid monosaccharide (E), respectively. Fractions B and C contained both glucuronic acid and iduronic acid, whereas iduronic acid constituted almost all of the uronic acid present in Fractions D and E. The results obtained do not support a recent claim that treatment of heparin with nitrous acid causes a conversion of glucuronic acid residues to iduronic acid via epimerization at C-5.

It is concluded that iduronic acid is the major sulfated uronic acid of heparin.

Heparin is generally depicted as a sulfated polymer of alternating D-glucosamine and D-glucuronic acid units, joined by α-1 $\rightarrow$ 4 glycosidic linkages (2–4). However, more recent work clearly shows that a considerable amount of L-iduronic acid is also present (5–9). The relative proportions of D-glucuronic acid and L-iduronic acid in heparin are not yet known.

Although there has been some controversy regarding the sulfate content of heparin, the currently accepted structure involves five sulfate groups per tetrasaccharide unit. The locations of these sulfate residues have not been definitely established. Most of the glucosamine residues carry sulfamino groups (2), the remainder being largely *N*-acetylated (7, 10, 11). The majority of the glucosamine units are sulfated also at C-6 (2, 12–14), whereas the hydroxyl group at C-3 seems to be essentially unsubstituted (13, 15). Periodate oxidation of *N*-desulfated heparin indicated that approximately half of the uronic acid units were sulfated (16). After conversion of the same polymer to sulfated uronosylanhydromannose disaccharides by deaminative cleavage with nitrous acid, all the uronic acid was destroyed by periodate; it was therefore concluded that the uronic acid-bound sulfate groups were located at C-2.

The present investigation was conducted in order to obtain information on the identity of the sulfated uronic acid moieties in heparin. The techniques employed have involved periodate oxidation of intact heparin as well as structural studies on mono- and disaccharides obtained by degradation of heparin with nitrous acid. While this work was in progress Wolfrom, Wang, and Honda (14) described periodate oxidation experiments similar to those carried out in the present study.

EXPERIMENTAL PROCEDURE

Materials

Heparin (stage 14), which had been isolated by mild procedures from hog intestinal mucosa, was purchased from the Wilson Laboratories, Chicago, Illinois, and was purified further by repeated precipitation with cetylpyridinium chloride from 1.2 M NaCl (17). Analysis of the purified material indicated (expressed as percentage of dry weight) hexosamine (not corrected for losses during hydrolysis), 21.2; uronic acid, 32.4; sulfur, 9.1; molar ratio of sulfate to hexosamine, 2.4. The anticoagulant activity was 255 U.S.P. units per mg.

A sample of 3-*O*-β-D-glucuronosyl-D-galactose was kindly provided by Dr. L. Rodén, Department of Pediatrics, University of Chicago, Chicago, Illinois. Crystalline hexaacetates of galactitol, D-mannitol, D-glucitol, and L-iditol were generous gifts from Dr. P. Haglund, Stora Kopparbergs Bergslags AB, Falun, Sweden. L-Iduronic acid was prepared from dermatan sulfate as described previously (7).

Analytical Methods

Uronic acid was determined by the method of Bitter and Muir (18) or by a procedure differing from the latter by the omission of borate from the reaction mixture. The two methods will in the following be referred to as the carbazole-borate and carbazole methods, respectively. In both assays D-glucuronolactone was used as standard. Anhydromannose was estimated by the indole method of Dische and Borenfreund (19); the deamination procedure was omitted. Hexosamine was determined by a modification (20) of the Elson-Morgan procedure, after hydrolysis of saccharides in 4 M HCl at 100° for 14 hours. Glucosamine to galactosamine ratios were determined with an amino acid analyzer.

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Uronic acids were identified by gas-liquid chromatography after conversion to the corresponding alditol acetates. Samples of 10 to 20 μg in 0.2 ml of distilled water were adjusted to pH 7.0 to 7.5 with 0.1 M ammonia, and 0.4 mg of potassium borohydride was added. After 2 hours at room temperature acetic acid was added to destroy the excess borohydride, and the reaction mixtures were passed through small columns of Dowex 50 (H^+). The effluents were repeatedly evaporated to dryness with additions of methanol followed by a final evaporation from distilled water. The resulting aldonic lactones were reduced to alditols by the procedure of Frush and Isbell (21), with Dowex 50-X8 (H^+) and boric acid as buffers. Acetylation of the alditols and the separation by gas-liquid chromatography of the alditol acetates have been described previously (22). The relative detector response of D-glucitol acetate as compared to that of L-iditol acetate was determined by chromatography of known amounts of alditol acetate standards.

High voltage electrophoresis was carried out with 47-cm strips of Whatman No. 3MM paper, in 1.2 M pyridine-0.17 M acetic acid, pH 6.0. Paper chromatography was performed using Whatman No. 1 papers, which were developed with ethyl acetate-acetic acid-water (3:1:1). Papers were stained by a silver dip procedure (23).

Periodate Oxidation of Heparin

A sample (200 mg) of purified heparin in 25 ml of distilled water was mixed with an equal volume of 0.1 M sodium metaperiodate and left in the dark at room temperature for 96 hours. After the addition of 0.25 ml of ethylene glycol, the reaction mixture was kept in the dark for another hour and was then treated with a total amount of 400 mg of potassium borohydride over a period of 14 hours. Following destruction of excess borohydride by the addition of acetic acid, the material was concentrated and desalted on a column of Sephadex G-25. The combined carbazole-positive effluent fractions were lyophilized, yielding 183 mg of material (dry weight).

The uronic acid components of the original and the periodate-treated heparin preparations were identified by paper chromatography after acid hydrolysis of the polysaccharides. The hydrolysis procedure of Wolfrom, Honda, and Wang (8) was employed, involving degradation of the polysaccharide with sulfuric acid followed by adsorption of cationic material to Dowex 50. When applied to the preparations used in the present study, this procedure yielded 7% and 9% of the total uronic acid contents of untreated and periodate-oxidized heparin, respectively, in a non-cationic form. In order to increase these yields the cationic components of the hydrolysates were recovered by elution of the ion exchange resin with 0.25 M ammonia, *N*-acetylated by treatment with acetic anhydride (4), and rehydrolyzed. By combining the Dowex 50 effluents from the two successive hydrolyses the yields given above were increased to 15% and 26%, respectively. Prior to paper chromatography the liberated uronic acids were purified by preparative paper electrophoresis at 90 volts per cm for 1 hour.[1]

Degradation of Heparin with Nitrous Acid

Fractionation of Degradation Products—Purified heparin (2 g) was deaminated with nitrous acid at −15°, according to the method of Cifonelli (24). The details of the procedure employed in the present study have been described previously (7). The reaction mixture was applied to a column (2 × 110.5 cm) of Sephadex G-25 (fine grade, bead-polymerized) and eluted with distilled water at a rate of 12 ml per hour. Effluent fractions were analyzed for uronic acid by the carbazole-borate method. The retarded fractions of the main peak (Fig. 1*A*) were combined, concentrated in a rotatory evaporator, and applied to a second column (2 × 186 cm) of Sephadex G-25, which was eluted with 10% ethanol at a rate of 10 ml per hour. Analysis of the effluents showed a distribution of uronic acid corresponding to at least three incompletely separated fractions (denoted as *I*, *II*, and *III* in Fig. 1*B*). Fractions I, II, and III showed hexosamine to uronic acid molar ratios of 1:12, 1:20, and 1:50, respectively.

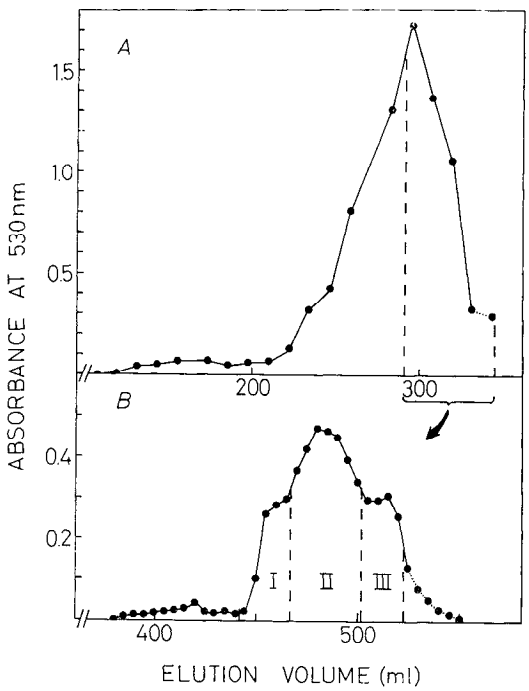

FIG. 1. *A*, gel chromatography on Sephadex G-25 of heparin deamination products; *B*, gel chromatography of low molecular weight portion of heparin deamination products. Effluent fractions were analyzed for uronic acid by the carbazole-borate method and combined as indicated by the *vertical lines*. Fractions corresponding to the *dashed portions* of the elution curves contained inorganic salts. For further details, see the text.

Each of Fractions I, II, and III was separated further by preparative paper electrophoresis at 56 volts per cm for 105 min.

Characterization of Degradation Products—Desulfation of the isolated deamination products was effected by treatment of the lyophilized materials with 0.06 M HCl in dry methanol (25) for 12 hours at room temperature. The reaction mixtures were concentrated to a small volume in a rotatory evaporator at low temperature, diluted with distilled water, and finally lyophilized. The desulfated materials were subsequently de-esterified by treatment for 15 hours with 0.5 M ammonia at room temperature. After evaporation to dryness the resulting products were subjected to paper electrophoresis at 56 volts per cm for 105 min.

The uronic acid constituents of the isolated compounds were identified by paper chromatography or, following derivatization, by gas-liquid chromatography. Prior to analysis samples were hydrolyzed in sealed tubes with 2 M trifluoracetic acid at 100° for 5 hours. After evaporation of the hydrolysates to dryness, the

[1] At pH 6 glucuronic acid and iduronic acid migrate at similar rates.

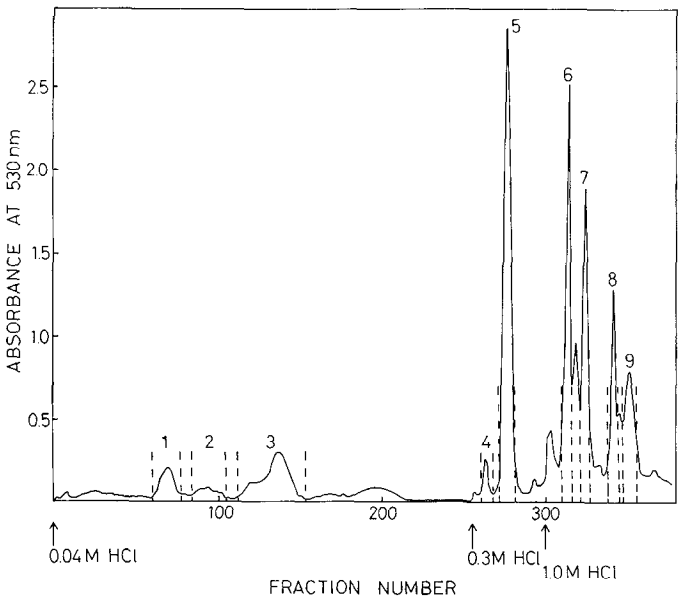

FIG. 2. Chromatography on Dowex 50 of heparin hydrolysate. The concentration of the HCl eluant was increased in a stepwise manner, as indicated by the *arrows*. Effluent fractions were analyzed for uronic acid by the carbazole-borate reaction and combined as indicated by the *vertical lines*.

liberated uronic acids were isolated by preparative paper electrophoresis, as described above.

Degradation of Heparin by Acid Hydrolysis

Fractionation of Degradation Products—A solution of 3.0 g of purified heparin in 15 ml of 72% sulfuric acid was kept at room temperature, under occasional stirring, for 2 hours (8). After dilution to an acid concentration of 3% the hydrolysate was heated at 100° for 3 hours, cooled, and adjusted to pH 5 to 6 by the addition of barium carbonate. Precipitated barium sulfate was removed by centrifugation, and the supernatant was passed through a column (2.2 × 50 cm) of Dowex 50W-X4 (H^+) which was washed exhaustively with distilled water. The column was then eluted in a stepwise manner with 0.05 M, 0.3 M, and 1.0 M HCl, respectively, as indicated in Fig. 2. Fractions of 10 ml were collected at a rate of about 30 ml per hour, analyzed for uronic acid by the carbazole-borate method, and finally pooled as indicated in Fig. 2. After elution with 1 M HCl the resin was washed with distilled water and eluted with 0.25 M ammonia. The alkaline eluate was discarded after analysis for uronic acid.

The pooled fractions (Fig. 2) were either lyophilized or desalted by passage through a column of Sephadex G-15 after neutralization with sodium hydroxide. By the latter procedure Fraction 7 was separated into two subfractions, one of which (Fraction 7a) appeared as a distinct peak at the void volume of the Sephadex column, while the other (Fraction 7b) was retarded. Some of the fractions from the Dowex 50 eluate showed obvious inhomogeneity on paper chromatography and were therefore purified by preparative paper chromatography on washed sheets of Whatman No. 3MM filter paper. Only subfractions corresponding to distinct spots were recovered, yielding Fractions 3a and 4a from Fractions 3 and 4, respectively, and Fractions 5a and 5b from Fraction 5.

Characterization of Degradation Products—Reduction of oligosaccharides was carried out by mixing samples (0.2 ml) containing 10 to 50 μg of uronic acid with 0.1 ml of 1% potassium borohydride. The solution was kept at room temperature for 1 hour and another 0.1-ml portion of 1% borohydride was added. After a total reaction time of 2 hours, 0.1 ml of 4 M acetic acid was added to decompose the excess borohydride, and the reduced samples were subjected to hexosamine or uronic acid (carbazole-borate method) analysis. Controls were prepared by treating the borohydride with acetic acid prior to addition of the sample. Hexosamine analyses of reduced materials were carried out after acid hydrolysis as described above; the liberated amino sugars were recovered by adsorption to columns (1 × 3 cm) of Dowex 50 (H^+) followed by elution with 2 M HCl.

The uronic acid composition of oligosaccharides was examined in a semiquantitative manner as follows. Oligosaccharides corresponding to 50 to 300 μg of uronic acid were *N*-acetylated (4), passed through columns of Dowex 50-X8 (H^+), and hydrolyzed with 2 M trifluoracetic acid at 100° for 5 hours. The liberated uronic acid monosaccharides were isolated by preparative paper electrophoresis, as described above, and were separated by paper chromatography. The relative amounts of the various uronic acids were estimated by the intensities of the spots revealed by staining with silver nitrate.

Deaminative cleavage of oligosaccharides with nitrous acid was performed according to the procedure of Dische and Borenfreund (19), modified to effect complete degradation of *N*-desulfated heparin (26). Samples containing 50 to 300 μg of uronic acid in 0.25 ml of water were mixed with equal volumes of 5% $NaNO_2$ and 33% acetic acid and were then left at room temperature for 3 hours. After the addition of 1 ml of methanol the reaction mixtures were concentrated to near dryness in a rotatory evaporator at low temperature; this procedure was repeated twice. After passage through columns (1 × 3 cm) of Dowex 50-X8 (H^+) the samples were fractionated by high voltage paper electrophoresis as described above, and the isolated uronic acid monosaccharides were subjected to paper chromatography. The nitrous acid deamination products were also analyzed for uronic acid by the carbazole and carbazole-borate methods. Since the removal of nitrite was critical for the latter assays the samples were passed through columns of Dowex 50 equilibrated with 0.54 M formic acid-1.30 M acetic acid (pH 1.85) prior to evaporation with methanol.

RESULTS

Treatment of Heparin with Periodate—The uronic acid content of the periodate-treated heparin was 24% (dry weight basis), indicating that approximately 30% of the uronic acid residues of the original material had been destroyed. Paper chromatography of the uronic acids liberated from the intact heparin by acid hydrolysis showed spots corresponding to glucuronic acid and iduronic acid and to their lactones. Judging from the staining intensities, iduronic acid was present in somewhat larger amounts than glucuronic acid. In contrast, the periodate-treated heparin gave distinct spots of iduronic acid and iduronolactone, whereas only traces of glucuronic acid were observed. This observation is in accord with the results of Wolfrom *et al.* (14) who reported preferential destruction of glucuronic acid during periodate oxidation of heparin. The latter investigators also showed that no L-iduronic acid was formed by inversion at C-5 of D-glucuronic acid during the hydrolysis with sulfuric acid.

Isolation of Heparin Deamination Products—Gel chromatography of the low molecular weight degradation products yielded

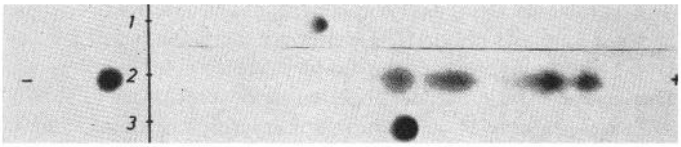

FIG. 3. High voltage electrophoresis at pH 6.0 of mono- and disaccharides liberated during degradation of heparin with nitrous acid. *1*, Glucuronosylgalactose; *2*, Fraction III of the gel chromatogram shown in Fig. 1*B*; *3*, D-glucuronic acid. The subfractions isolated by preparative paper electrophoresis are denoted A, B, C, D, and E in the text, mentioned in the order of increasing mobility.

three poorly resolved fractions (I, II, III; Fig. 1*B*) which were separated further by preparative paper electrophoresis. The three fractions showed similar patterns on electrophoresis (Fig. 3), differing only with respect to the relative amounts of the various components (Table I). The five distinct subfractions obtained are denoted A to E in the order of increasing mobility. The slight migration of Fraction A toward the cathode is due to electroendosmosis. Fraction B migrated similar to glucuronic (or iduronic) acid, whereas Fractions C, D, and E showed higher migration rates. Re-electrophoresis of each isolated fraction showed a slight admixture of Fractions B and E with Fractions C and D, respectively, whereas the two latter fractions appeared to be homogeneous under these conditions.

The $R_{galactose}$ values observed on paper chromatography of Fractions B to E are given in Table I. Fractions B and C both yielded two major spots, while each of Fractions D and E showed essentially one single component. The two spots of Fraction B had migrated similar to glucuronic acid and iduronic acid, respectively. Fraction A migrated close to the solvent front.

The total yields of Fractions B to E are shown in Table I. Fraction D was obtained in the highest yield, followed by Fractions C, E, and B, in the order mentioned. Fraction A contained neither uronic acid nor anhydromannose and was therefore not investigated further.

Characterization of Heparin Deamination Products—Analytical data for the Fractions B to E are shown in Table I.

Fractions C and D, which were obtained in highest yields, were assumed to represent mono- and disulfated uronosylanhydromannose disaccharides, *i.e.* the major expected deamination products of heparin (see under "Discussion" and Fig. 7). Both fractions contained approximately equimolar amounts of uronic acid and anhydromannose.[2] Hexosamine was absent.

Attempts to determine the sulfate contents of the isolated heparin deamination products were unsuccessful due to the presence of inorganic sulfate in the eluates obtained by preparative paper electrophoresis. However, the degree of sulfation could be estimated indirectly by the effect of desulfation on the electrophoretic properties of the various fractions (Fig. 4). After treatment with methanolic hydrogen chloride, Fractions C and D thus showed identical electrophoresis patterns involving four components which had migrated like nondesulfated Fraction C, uronic acid monosaccharide, glucuronosylgalactose, and an uncharged compound, respectively. The latter component, presumably anhydromannose, and the uronic acid monosaccharide were probably formed by cleavage of uronidic linkages during the desulfation or subsequent de-esterification of Fractions C and D.

[2] Anhydromannose determinations were performed by the indole reaction, with deaminated glucosamine as standard (19). The effect of uronic acid substituents on the color yield of anhydromannose is not known. However, it may be observed that the anhydromannose values obtained for heparan sulfate after treatment with nitrous acid considerably exceeded the theoretical yield (24). The anhydromannose values given by Fractions B and E may reflect the low specificity of the indole reaction, as well as the incomplete separation of these fractions from Fractions C and D, respectively.

TABLE I

Yields, chromatographic properties, and analytical data of Fractions B to E obtained by deamination of heparin

The analytical data refer to components isolated from Fraction III of the gel chromatogram shown in Fig. 1*B*.

Fraction	Yield[a]	Distribution[b] of Fraction B to E between			$R_{galactose}$[c]	Molar ratio of anhydromannose to uronic acid[d]	Ratio[e] of carbazole to carbazole-borate	Ratio[f] of glucitol acetate to glucitol acetate-iditol acetate
		Fraction I	Fraction II	Fraction III				
	mg uronic acid	%						
B	14.7	11	65	24	1.01; 1.29	0.50	0.77	0.47
C	26.6	15	69	16	0.15; 0.19	1.43	0.74	0.37
D	74.4	28	64	8	0.08	1.23	0.58	<0.08
E	24.7	9	45	46	0.45	0.29	0.60	<0.07

[a] Determined after preparative paper electrophoresis of Fractions I to III. Uronic acid was assayed by the carbazole-borate method; no correction was applied for the difference in color yield of glucuronic acid and iduronic acid (18).

[b] Expressed as percentage of the total yield of each of Fractions B to E, determined by the carbazole-borate method. Fractions I to III refer to the gel chromatogram shown in Fig. 1*B*.

[c] Mobility on paper chromatography, related to that of D-galactose.

[d] Uronic acid, determined by the carbazole-borate method, as 1.00. No correction was applied for unspecific color yield in the indole reaction.

[e] Ratio of uronic acid contents determined by the two methods, with D-glucuronolactone as standard.

[f] Determined by gas-liquid chromatography after acid hydrolysis and conversion of uronic acids to alditol acetates. No attempt was made to estimate the extent of derivatization by determining the yields of glucitol acetate and iditol acetate from the corresponding uronic acid standards.

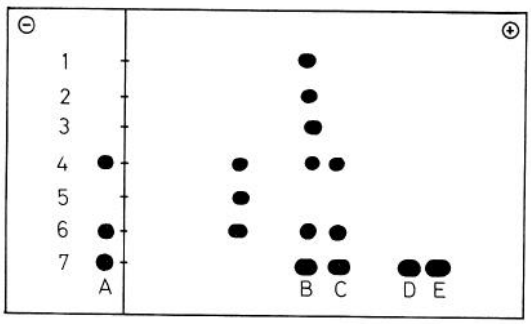

FIG. 4. Effect of desulfation on the electrophoretic migration rates of heparin deamination products. *1*, Glucuronic acid; *2*, desulfated Fraction B; *3*, desulfated Fraction E; *4*, desulfated Fraction C; *5*, glucuronosylgalactose; *6*, desulfated Fraction D; *7*, Fraction III of the gel chromatogram shown in Fig. 1*B* (consisting of subfractions A to E, as indicated).

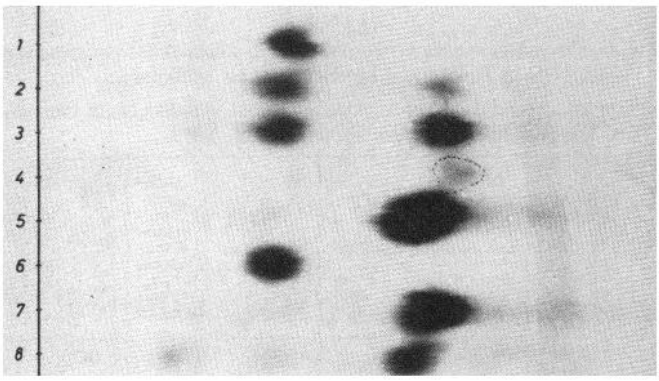

FIG. 5. Paper chromatography of uronic acids liberated from heparin deamination products by acid hydrolysis. *1* and *6*, D-glucuronic acid; *2*, Fraction B; *3*, Fraction C; *4* and *8*, L-iduronic acid; *5*, Fraction D; *7*, Fraction E. The figure gives a false impression of the relative migration distances, since the paper was cut to facilitate reproduction.

The presence in the desulfated fractions of a nonsulfated uronic acid-containing disaccharide (mobility similar to glucuronosylgalactose) supports the contention that the two parent fractions are similar in nature but differ with respect to sulfate contents. Since Fraction D showed the higher electrophoretic mobility it is concluded that this fraction contains a disulfated and Fraction C a monosulfated uronosylanhydromannose disaccharide.

The behavior of Fraction B on paper chromatography (Table I) and on paper electrophoresis (Fig. 3) suggested the presence of nonsulfated uronic acid monosaccharides. Accordingly, analysis of this fraction showed uronic acid to be the major carbohydrate constituent[2] (Table I). Also Fraction E contained uronic acid as the only sugar present in significant amounts (Table I). The identity of this fraction with sulfated uronic acid monosaccharide, as indicated by the electrophoretic mobility (Fig. 3), was confirmed by paper electrophoresis of the desulfated material (Fig. 4). The electrophoresis patterns of the desulfated Fractions B and E were identical, showing one single spot corresponding to uronic acid monosaccharide. Desulfation thus affected Fraction E only, in support of the view that Fractions B and E correspond to nonsulfated and monosulfated uronic acid monosaccharides, respectively.

The behavior of Fractions B to E on gel chromatography is in fair agreement with the structures assigned to their components. As seen from Table I, the distributions of Fractions B to E among the Fractions I, II, and III obtained by gel chromatography (Fig. 1*B*) indicate that Fractions C and D (disaccharides) consist of larger molecules than do Fractions B and E (monosaccharides).

Uronic Acid Composition of Heparin Deamination Products—The uronic acid components of Fractions B to E were identified by paper chromatography and by gas-liquid chromatography. Prior to analysis the fractions were degraded by acid hydrolysis. Paper electrophoresis of the hydrolysates indicated extensive degradation of Fractions C, D, and E to nonsulfated monosaccharides. A paper chromatogram of the liberated uronic acids is shown in Fig. 5. Fractions B (uronic acid monosaccharide) and C (monosulfated uronosylanhydromannose disaccharide) yielded a mixture of glucuronic acid and iduronic acid. In contrast, Fractions D (disulfated uronosylanhydromannose disaccharide) and E (sulfated uronic acid monosaccharide) showed iduronic acid as the only significant component, glucuronic acid occurring in trace amounts only. These findings were confirmed by gas-liquid chromatography of the alditol acetates obtained by reduction and acetylation of the liberated monosaccharides (Table I). The hexaacetates of galactitol, D-mannitol, D-glucitol, and L-iditol were clearly separated in the chromatographic procedure employed. Fractions B and C showed two peaks of about equal size with the retention times of D-glucitol hexaacetate and L-iditol hexaacetate, respectively. The alditol acetates derived from Fractions D and E were almost entirely of the *ido* configuration, although trace amounts of glucitol hexaacetate were also present. As shown by two independent methods, the uronic acid component of Fractions D and E was thus almost exclusively iduronic, whereas Fractions B and C contained glucuronic acid and iduronic acid in comparable amounts. This conclusion is further supported by the relative color yields of Fractions B to E in the carbazole and carbazole-borate reactions. Since iduronic acid shows a higher color yield relative to glucuronic acid in the carbazole-borate assay than in the original method (18), the carbazole to carbazole-borate ratios shown in Table I suggest higher glucuronic acid to iduronic acid ratios for Fractions B and C than for Fractions D and E.

Fractionation and Partial Characterization of Oligosaccharides Obtained by Acid Hydrolysis of Heparin—Fractionation of the heparin hydrolysate on Dowex 50 is illustrated by the chromatogram shown in Fig. 2. Material corresponding to 7% of the uronic acid content of the starting material emerged with the water wash and contained glucuronic and iduronic acid monosaccharides, as evidenced by paper electrophoresis and paper chromatography. The total uronic acid contents of the fractions eluted with hydrochloric acid of varying concentration (Fig. 2) and with 0.25 M ammonia comprised 42% and 5%, respectively, of that of the starting material. It seems reasonable to assume that the losses were at least partly due to destruction of uronic acid residues during hydrolysis. Additional losses occurred during the subsequent fractionation of the acidic eluate, yielding the subfractions listed in Table II.

Analytical data for the various oligosaccharide fractions are shown in Tables II and III. Whereas most of the fractions seemed to consist of larger oligosaccharides, as judged from paper chromatography (Table II), Fractions 4 and 5 contained considerable amounts of disaccharides with reducing uronic acid units. The two disaccharide components (5a and 5b) of Fraction 5 were isolated by preparative paper chromatography and were characterized in more detail (Table III). Both disaccharides contained glucosamine as the only amino sugar, apparently in nonreducing terminal position. Fraction 5a contained glucosamine and uronic acid (determined as glucuronic acid) in almost equimolar amounts, whereas Fraction 5b gave a glucosamine to uronic acid molar ratio of 0.7. However, these ratios should be evaluated with caution, as neither of the fractions contained glucuronic acid; furthermore, the effect on the carbazole-borate reaction of the glucosamine substituents is unclear (see under "Discussion"). Paper chromatography of the uronic acid constituent of Fraction 5b showed a single spot that had migrated like iduronic acid. No glucuronic acid was detected, whether nitrous acid deamination or acid hydrolysis of the *N*-acetylated disaccharide had been employed for the liberation of uronic acid monosaccharide. It is concluded that Fraction 5b contained the disaccharide, glucosaminyliduronic acid. By the same procedures Fraction 5a yielded a component, presumably a uronic

TABLE II

Yields and analytical data of oligosaccharides obtained by acid hydrolysis of heparin

Fraction	Yield[a]	$R_{GlcUA \rightarrow Gal}$[b]	Ratio[c] of reducing terminal uronic acid to total uronic acid	Ratio[d] of carbazole to carbazole-borate		Uronic acid composition[e]			Uronic acid monosaccharide liberated by nitrous acid[e]		
				Before HNO_2 treatment	After HNO_2 treatment	GlcUA	IdUA	X	GlcUA	IdUA	X
	%										
1	1.2	0.08	0.48	1.32	0.54	−	++	−	−	++	−
2	0.7	0.46; 0.73	0.34	1.23	0.92	++	+	−	++	++	−
3a	1.7	0.34	0.51	1.24	0.80	++	++	−	−	++	−
4a	0.3	0.84[f]	0.83			++	++	++	++	++	++
5a	0.9	0.76	0.79	1.16	1.38	−	−	++	−	−	++
5b	4.3	1.14	0.95	1.27	0.67	−	++	−	−	++	−
6	6.2	0.05	0.25	1.38		++	++	−	++	++	−
7a	1.2	<0.05	0.13	1.22	0.78	++	++	−	++	++	−
7b	2.1	0.08	0.40	1.35	0.84	++	++	−	+	++	−
8	1.1	0.07	0.19	1.19	0.69	++	+	−	+	++	−
9	3.2	0.07	0.22	1.26	0.67	++	++	−	++	++	−

[a] Expressed as percentage of total uronic acid content of starting material.

[b] Mobility on paper chromatography related to that of 3-*O*-β-D-glucuronosyl-D-galactose. Only major components are indicated.

[c] Determined by reduction with potassium borohydride as described in detail in the text.

[d] Ratio of uronic acid contents determined by the two methods, with D-glucuronolactone as standard.

[e] Determined by paper chromatography after degradation of the oligosaccharides as described in detail in the text. The relative amounts of uronic acid monosaccharides are indicated by ++, +, or −, the latter symbol denoting trace amounts or absence of the corresponding uronic acid. X refers to the uronic acid or uronic acid derivative migrating faster than L-iduronic acid.

[f] Elongated spot; value refers to the midpoint.

TABLE III

Analytical data of disaccharides obtained by acid hydrolysis of heparin

Fraction	Hexosamine[a]	Uronic acid[a]	Ratio[b] of reducing terminal uronic acid to total uronic acid	Ratio[b] of reducing terminal hexosamine to total hexosamine
	%			
5a	60	61	0.79	0.17
5b	31	49	0.95	0.07

[a] Percentage of lyophilized material. Uronic acid contents were determined by the carbazole-borate method. Weight recoveries were corrected by subtracting the weights of appropriate paper blanks; the high values of Fraction 5a probably reflect the uncertainty of such corrections when applied to materials obtained in low yield (see Table II).

[b] Determined by reduction with potassium borohydride, as described in detail in the text.

acid or uronic acid derivative, which migrated somewhat faster on paper chromatography than did iduronic acid.

Judging from the patterns obtained on paper chromatography of liberated uronic acid monosaccharides (Fig. 6), Fraction 4a was largely a mixture of three disaccharides, with uronic acid located at the reducing terminal position (Table II). Glucuronic and iduronic acids were detected and in addition, a component which had migrated similar to the uronic acid constituent of Fraction 5a. Fraction 4a thus contains hexosaminylglucuronic acid and in addition, two disaccharides containing iduronic acid and the unknown, fast migrating uronic acid, respectively. The structural relationship between these latter disaccharides and those isolated from Fraction 5 is unclear. Since Fractions 4 and 5 emerged closely together from the Dowex 50 column (Fig. 2), the occurrence in the two fractions of identical components cannot be excluded.

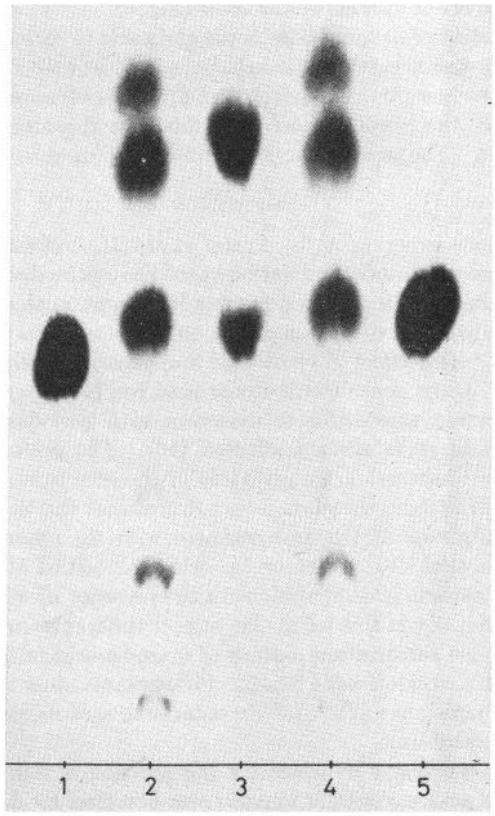

FIG. 6. Paper chromatography of uronic acid monosaccharides derived from Fraction 4a. *1* and *5*, D-glucuronic acid; *2*, uronic acids liberated from the *N*-acetylated Fraction 4a by acid hydrolysis; *3*, D-glucuronic acid and L-iduronic acid; *4*, uronic acids liberated from Fraction 4a by treatment with nitrous acid.

Effect of Nitrous Acid on Oligosaccharides Obtained by Acid Hydrolysis of Heparin—Uronic acid monosaccharides were liberated from the reducing terminals of heparin oligosaccharides by treatment with nitrous acid and were then identified by paper chromatography. The uronic acid patterns thus obtained were related to the uronic acid composition of the corresponding oligosaccharide fractions (Table II). Although the results obtained were only semiquantitative the following observations would seem to be valid. (*a*) None of the oligosaccharide fractions contained glucuronic acid as the only uronic acid, nor did nitrous acid deamination of any of the fractions yield glucuronic acid exclusively. (*b*) Iduronic acid was the only significant uronic acid constituent of two of the oligosaccharide fractions (Fractions 1 and 5b) and was the only uronic acid monosaccharide produced by these fractions on treatment with nitrous acid. Hence, the possibility of nitrous acid-induced epimerization of iduronic acid to glucuronic acid appears to be excluded. (*c*) All uronic acids liberated with nitrous acid invariably occurred as major components of the corresponding nondeaminated oligosaccharide fractions. (*d*) Fraction 3a contained significant amounts of both iduronic acid and glucuronic acid and yielded only iduronic acid on deamination. Similarly, the uronic acids obtained from Fractions 2, 7b, and 8 by treatment with nitrous acid showed higher iduronic acid to glucuronic acid ratios than did hydrolysates of the corresponding *N*-acetylated oligosaccharides. (*e*) Deamination of Fraction 4a, essentially consisting of three hexosaminyluronic acid disaccharides with different uronic acid constituents, did not appreciably affect the relative amounts of the various uronic acids (Fig. 6).

The effect of deamination on the carbazole to carbazole-borate ratios of the heparin oligosaccharides is illustrated in Table II. With one exception (Fraction 5a) these ratios were invariably lower for the nitrous acid-treated than for the intact oligosaccharides. The significance of this finding will be discussed below.

DISCUSSION

Previous experiments by Foster *et al.* (16) indicated the potential use of periodate oxidation in studies on the distribution of sulfate in heparin. In the present investigation this technique has been applied to the identification of the sulfated uronic acid residue. The effect of periodate on heparin is illustrated in Fig. 7. Only nonsulfated uronic acid residues contain the α-glycol group susceptible to oxidation with periodate; sulfated uronic acid units are not affected (16). The preferential and extensive destruction by periodate of the glucuronic acid component of heparin therefore demonstrates that this uronic acid is essentially nonsulfated, in agreement with the observations by Wolfrom *et al.* (14). The uronic acid composition of periodate-treated heparin is compatible with the presence of sulfated iduronic acid, but in this particular respect the results are inconclusive, as the substitution pattern of iduronic acid in heparin has not been characterized in detail. Substituents other than sulfate ester groups thus might confer resistance toward periodate on the iduronic acid unit.

More conclusive evidence for the location of sulfate on the iduronic acid moieties of heparin was obtained by deaminative degradation of the polysaccharide with nitrous acid. By this procedure *N*-sulfated hexosamine units are converted to anhydromannose residues with concomitant cleavage of the corresponding glycosidic bonds (Fig. 7), whereas *N*-acetylated hexosamine residues are not affected (24). Since approximately half of the uronic acid units of heparin are sulfated (16), the major expected degradation products of heparin would be mono- and disulfated uronosylanhydromannose disaccharides (Fig. 7). Such disaccharides were recovered from Fractions C and D, respectively; in addition substantial amounts of nonsulfated (Fraction B) and monosulfated (Fraction E) uronic acid monosaccharides were isolated. The yield of mono- and disaccharides amounted to about 30% of the uronic acid content of the starting material, a considerable portion of the losses representing larger oligosaccharides and carbohydrate-serine compounds (7) removed by gel chromatography (Fig. 1*A*). The liberation by nitrous acid of uronic acid monosaccharides was unexpected in view of the current concept of heparin structure. However, it seems possible that at least part of these monosaccharides was derived from the reducing ends of polysaccharide chains lacking the polysaccharide-protein linkage region, since we have shown previously that such molecules constitute a major portion of heparin preparations of the type used in this study (17). Owing to the preferential recovery of low molecular weight components during the fractionation procedure (Fig. 1*A*) the yields given in Table I do not indicate the actual monosaccharide to oligosaccharide ratio of the initial heparin deamination mixture.

The isolated mono- and disaccharides were found to differ drastically with regard to uronic acid composition. The uronic acid component of the sulfated uronic acid monosaccharide (Fraction E) and of the disulfated uronosylanhydromannose disaccharide (Fraction D) was identified as iduronic acid, glucuronic acid being present in trace amounts only. In contrast, the nonsulfated uronic acid monosaccharide (Fraction B) and the

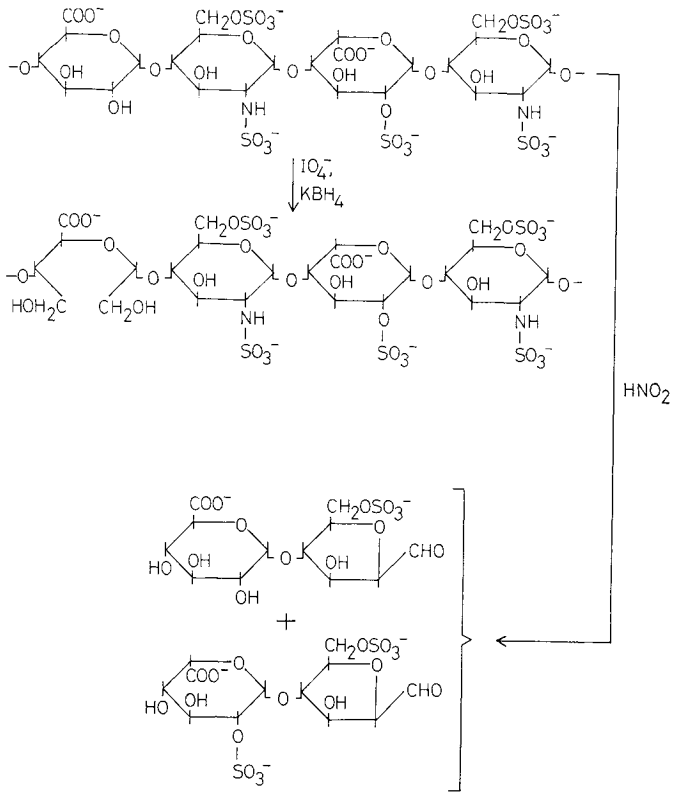

FIG. 7. Scheme illustrating the reactions of heparin with periodate and with nitrous acid. In accordance with the results of the present study the uronic acid-bound sulfate group is located on an iduronic acid residue of the heparin tetrasaccharide fragment.

monosulfated uronosylanhydromannose disaccharide (Fraction C) both contained considerable amounts of glucuronic acid in addition to iduronic acid. Since the uronic acid moiety of Fraction D is presumably sulfated (Fig. 7), whereas that of Fraction C is nonsulfated (16) it is concluded that iduronic acid is the major sulfated uronic acid of the heparin preparation investigated. This conclusion is supported by the composition of Fraction E.

The interpretation of the results obtained by the deamination technique depends on the assumption that the configuration at C-5 of the glucuronic acid residues of heparin is unaffected by the treatment with nitrous acid. The generally accepted mechanism of the deamination reaction (27) would not seem to promote a configurational change at this site. Nevertheless, Yamauchi, Kosakai, and Yosizawa (28) suggested that the deaminative cleavage of hexosaminidic linkages in heparin results in concomitant epimerization at C-5 of the D-glucuronic acid residues involved in these linkages, thus yielding L-iduronic acid (28). According to these authors any glucuronic acid present in the deamination product would be linked to a nitrous acid-resistant glucosamine residue having an acetylated rather than a sulfated amino group. This notion is clearly at odds with the results of the present study, wherein the disaccharide glucuronosylanhydromannose (Fraction C) is shown to be a deamination product of heparin. The glucuronic acid component of this disaccharide must have been derived from a glucuronic acid unit of the intact polysaccharide, as iduronic acid was liberated by nitrous acid from heparin oligosaccharides (Fractions 1 and 5b, isolated after acid hydrolysis of heparin; Table II) without converting into glucuronic acid. These results indicate that nitrous acid is capable of liberating glucuronosylanhydromannose disaccharides from heparin without concomitant epimerization at C-5 of the uronic acid unit. However, the possibility of epimerization affecting only some of the glucuronic acid residues could not be excluded, and therefore, the effect of nitrous acid on oligosaccharides obtained by acid hydrolysis of heparin was investigated in more detail.

The ideal model compound for the demonstration of nitrous acid-induced conversion of glucuronic acid to iduronic acid would be a heparin oligosaccharide lacking iduronic acid and with glucuronic acid in the reducing terminal position. Attempts to isolate such a compound were unsuccessful. Although the heparin hydrolysate was extensively fractionated all glucuronic acid-containing fractions also contained iduronic acid as a major constituent (Table II). Unfortunately, the glucuronic acid to iduronic acid ratios of these oligosaccharide fractions could not be accurately determined, as the techniques currently available for this purpose depend on the quantitative conversion of oligosaccharides to monosaccharide units. Extensive acid hydrolysis of the *N*-acetylated oligosaccharides results in appreciable destruction of uronic acid residues; hence, a semiquantitative estimation of these ratios following partial hydrolysis of the oligosaccharides was considered more adequate. Whereas most of the fractions containing iduronic as well as glucuronic acid yielded both sugars as monosaccharides on treatment with nitrous acid (Table II), one fraction (Fraction 3a) produced iduronic acid only (in addition to Fractions 1 and 5b, which lacked glucuronic acid even prior to nitrous acid treatment). Also, the uronic acid monosaccharides liberated by nitrous acid from some of the fractions (Fractions 2, 7b, and 8) appeared to show higher iduronic acid to glucuronic acid ratios than did the parent oligosaccharides. Although these latter findings are compatible with the C-5 epimerization mechanism postulated by Yamauchi *et al.* (28), they might equally well reflect the predominant location, in these fractions, of iduronic rather than glucuronic acid in the reducing terminal position.

The effects of nitrous acid on glucosaminyluronic acid disaccharides are more amenable to evaluation, as the glucuronic acid to iduronic acid ratios observed before and after deamination refer to a substituent at one single position. These ratios are thus directly comparable. Yamauchi *et al.* (28) isolated a disaccharide, claimed to be identical with 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-D-glucuronic acid, from an acid hydrolysate of porcine intestinal heparin. On treatment with nitrous acid this disaccharide, the isolation and characterization of which was not reported in detail, yielded iduronic acid as the only detectable uronic acid monosaccharide. In the present investigation the major hexosaminyluronic acid disaccharide, also isolated from porcine heparin, was identified as glucosaminyliduronic acid (Fraction 5b). Hexosaminylglucuronic acid was detected, although in much smaller quantities, as a component of a disaccharide fraction (Fraction 4a) containing in addition two other hexosaminyluronic acid disaccharides. The uronic acid patterns obtained by paper chromatography before and after deamination of this fraction did not conform to the concept of nitrous acid-induced epimerization of glucuronic acid to iduronic acid (Fig. 6). This finding, as well as the results provided by nitrous acid treatment of the larger heparin oligosaccharides (Table II), supports the view that the iduronic acid constituents of Fractions B to E did not originate from glucuronic acid units of the heparin preparation subjected to deamination. Further evidence in favor of this conclusion relates to the apparent lack of sulfated glucuronic acid, as demonstrated by periodate oxidation. The amount of sulfated glucuronic acid present, if any, in the intact polymer is thus too low to account for the sulfated iduronic acid recovered as a deamination product in Fractions D and E.

The conception of sulfate distribution in heparin inferred from the above results may be compared with that presented recently by Danishefsky *et al.* (13), who suggested on the basis of methylation studies that about one-third of the glucuronic acid moieties in heparin are sulfated. Acetamino heparin was permethylated, reduced, and hydrolyzed, yielding 3-*O*-methyl-D-glucose and 2,3-dimethyl-D-glucose representing 4% and 10% (minimal values), respectively, of the modified polysaccharide. No methylated idose was found, possibly due to the marked tendency of idose to convert, under acidic conditions, into 1,6-anhydroidopyranose (29). Since the procedure employed did not involve methylation at C-6 of the hexoses formed by reduction of uronic acids, the possibility remains that any methylated idose formed may have escaped detection by conversion into the corresponding anhydrosugar. It should also be observed in this context that our results do not exclude the occurrence in heparin of small amounts of sulfated glucuronic acid.

Although the occurrence of L-iduronic acid in heparin has been recognized for several years the relative amounts of glucuronic acid and iduronic acid in this polysaccharide are unknown. The apparent preponderance of iduronic acid over glucuronic acid in acid hydrolysates of heparin has been ascribed to the greater susceptibility of the L-idosyluronic linkage to hydrolysis with acid (8). The detection of D-glucose as the only hexose present in acid hydrolysates of carboxyl-reduced heparin has been taken as evidence for the absence of significant amounts of uronic acids

other than D-glucuronic acid (2). However, subsequent investigations have demonstrated that L-idose can be readily detected by the use of less drastic hydrolysis procedures (8). The present experiments show that about 30% of the total uronic acid and almost all of the glucuronic acid of heparin were destroyed during oxidation with periodate. Also, a large proportion of iduronic acid was indicated by the relative yields of the various heparin deamination products (Table I); all of the fractions characterized contained iduronic acid, whereas only Fractions B and C contained significant amounts of glucuronic acid. Although these observations do not permit any definite conclusions as to the relative amounts of the two uronic acids in heparin, it seems reasonable to infer that iduronic acid might account for more than half of the total uronic acid content. This conclusion is in accordance with recent observations of Perlin and Sanderson (9).

Various colorimetric procedures have been employed for determination of the relative amounts of glucuronic acid and iduronic acid in polysaccharide preparations. In the original carbazole reaction of Dische (30) iduronic acid as well as the iduronic acid-containing polysaccharide dermatan sulfate show low color yields compared to glucuronic acid, whereas the uronic acid values obtained for heparin and heparan sulfate are anomalously high (2). The modified carbazole method of Bitter and Muir (denoted carbazole-borate method in the present paper) shows higher color yields of iduronic acid relative to glucuronic acid, and lower color yields of heparin and heparan sulfate relative to other glycosaminoglycuronans, than does the original carbazole procedure (18). Yamauchi *et al.* (28) observed that the carbazole to carbazole-borate ratio of heparin was drastically decreased upon treatment of the polysaccharide with nitrous acid. This decrease was interpreted as evidence for C-5 epimerization of glucuronic acid. Similar results were obtained in the present study on deaminating oligosaccharides isolated from an acid hydrolysate of heparin (Table II). However, this decrease in carbazole to carbazole-borate ratio should apparently not be attributed to a change in uronic acid composition, as the high ratios of the nondeaminated fractions showed no correlation to the glucuronic acid to iduronic acid ratios. Similar high ratios were thus displayed by Fractions 1 and 5b, which contain iduronic acid only, and by Fractions 2 and 8, which apparently contain more glucuronic than iduronic acid (Table II). It therefore seems reasonable to infer that the carbazole color yields of heparin and of heparin oligosaccharides are to a large extent influenced by the hexosamine substituents at the constituent uronic acid residues. In contrast, the carbazole to carbazole-borate ratios recorded for the corresponding deamination products, which lack these substituents, appear to essentially reflect the uronic acid composition of the various fractions (Table II). Of particular interest in this context is the high ratio of the nitrous acid-treated Fraction 5a, which provides additional evidence for the presence in this fraction, as well as in Fraction 4a (Fig. 6), of a uronic acid or uronic acid derivative distinctly different from glucuronic acid or iduronic acid. Although the possibility remains that this component is an artifact of the hydrolysis procedure, it is noteworthy that the occurrence in heparin of uronic acids other than iduronic or glucuronic has been indicated by other studies (31–33).

Note Added in Proof—A recent study on the proton magnetic resonance of heparin (34) indicated, in accord with the results of the present investigation, that iduronic acid is the major uronic acid constituent of this polysaccharide.

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