Structural Studies on Heparitin Sulfate of Normal and Hurler Tissues*  

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SUMMARY

Heparitin sulfate isolated from human aorta is a polysaccharide of molecular weight 24,000 to 29,000, which is bound to protein by way of serine. The presence of serine, xylose, and galactose in a molar ratio of 1:1:2 suggests that the linkage region of this mucopolysaccharide is similar to that in chondroitin 4-sulfate and in heparin. Structural studies with nitrous acid indicate that the heparitin sulfate molecule contains a relatively large segment of N-acetylglucosaminuronic acid repeating units in the region of protein binding. Heparitin sulfate isolated from the livers and urine of patients with the Hurler's syndrome is heterogeneous and contains materials ranging in molecular weight from 2,700 to 5,500. Two distinct fractions were isolated, which differed in chemical analyses, amino acid composition, and structural characteristics. It is suggested that these may represent segments of a single parent molecule.

Following the original work of Muir (1), several investigators (2-5) have shown that a number of sulfated mucopolysaccharides are covalently bound to protein through the hydroxyl group of serine. A detailed study of the linkage region in both heparin (6) and chondroitin 4-sulfate (7, 8) has shown that mucopolysaccharide chains are bound to serine by way of a glucuronylgalactosylgalactosylxylosyl linkage region. The predominance of serine in a heparitin sulfate fraction obtained from human aorta after exhaustive proteolytic treatment (9) suggested a similar linkage pattern for this substance. Previous comparison of heparitin sulfate, isolated as a by-product of commercial heparin, with the polysaccharide obtained from the tissues and urine of patients with the Hurler's syndrome failed to indicate any significant chemical differences (10). Dorfman (11) showed that dermatan sulfate isolated from water extracts of Hurler's tissue or urine without the use of alkali or proteolytic enzymes contains only small amounts of amino acids (predominantly serine). In contrast, dermatan sulfate of normal human skin is only separated from protein by alkali treatment or exhaustive proteolytic digestion. Since dermatan sulfate is usually associated with heparitin sulfate in the Hurler syndrome, it seemed likely that a common metabolic defect involving both mucopolysaccharides was operative in this disease. Accordingly, a study was undertaken of the structure of heparitin sulfate in both the normal and the diseased state with particular emphasis on its protein-polysaccharide linkage. The results of such a study are presented in this paper. A preliminary report of these studies has appeared (12).

EXPERIMENTAL PROCEDURE

Materials

Liver specimens were obtained at autopsy of three patients with the Hurler's syndrome. Patients T. P. and G. I. were males aged 4 years and 10 years, respectively, whose clinical characteristics and family histories were consistent with the diagnosis of the autosomal recessive form of Hurler's syndrome (13). Patient G. C., a 12-year-old boy, lacked corneal opacities and had a maternal uncle who died from a similar disease. These facts suggest that the patient was suffering from the X-linked form of the disease, sometimes called Hunter's syndrome (13).

Urine samples were obtained from patient K. F., an 11-year-old male Oriental. In view of his clinical features, family history, and the large quantity of heparitin sulfate excreted, this patient might fit the classification of the Sanfilippo syndrome (13).

The specimens, as well as detailed case histories, were generously supplied by Drs. Ira M. Rosenthal, Russell Weller, and James A. Austin. Normal aortas were obtained through the courtesy of Drs. William E. Ehrich, Paul Szanto, James S. Kidd, and Robert W. Wissler.

Testicular hyaluronidase (20,000 i.u. per mg) was obtained from AB Leo (Hälsingborg, Sweden). D-Galactose dehydro-
membranes were prepared which were freely permeable to HCl and sufficient cetylpyridinium chloride was added to precipitate the mucopolysaccharide (approximately 3 mg of cetylpyridinium chloride per mg of polysaccharide). The precipitate which formed after standing several hours at 37° was removed by centrifugation and washed with 0.03 M NaCl-0.1% cetylpyridinium chloride. The cetylpyridinium chloride complex was dissolved with 2 M NaCl and the mucopolysaccharide was recovered by the addition of 3 volumes of absolute ethanol. The precipitate was washed twice with 80% ethanol, twice with absolute ethanol, once with ethyl ether, and air-dried.

The dry powder was dissolved in 0.05 M phosphate buffer pH 7.0, and the nucleic acid content was estimated on the basis of the absorbance at 260 μm. Digestion with pancreatic ribonuclease (Worthington, 2 times crystallized, enzyme to substrate ratio of 1:10) was carried out at 37° for 5 hours. After addition of MgCl 2 to 0.005 M, pancreatic deoxyribonuclease (Worthington, 1 time crystallized) in the same enzyme to substrate ratio was added. Digestion was continued overnight. Following dialysis against distilled water, the polysaccharide was recovered by precipitation with cetylpyridinium chloride, solution in 2.0 M NaCl, and precipitation with ethanol. The cetylpyridinium chloride and ethanol precipitations were repeated.

To determine the completeness of isolation of the mucopolysaccharide, the water-insoluble residue from the liver of patient T. F. was digested with papain as described below for the extraction of aortas. The cetylpyridinium chloride-precipitable material obtained from the papain digest was treated with nucleases and fractionated by gel filtration on Sephadex G-25. The uronic acid content of this material was less than 5% of that recovered by means of water extraction. It was not studied further.

Isolation of Mucopolysaccharide from Hurler Urine—Urine was collected in bottles containing thymol as a preservative. Three liters were dialyzed against distilled water at 4° and concentrated under reduced pressure to 100 ml. After centrifugation, polysaccharide was isolated from the supernatant solution by the same technique as that used for the water extract from Hurler liver.

Isolation of Mucopolysaccharides from Human Aorta—Aortas were obtained at autopsy from individuals between infancy and 30 years of age. Aortas from patients suffering from disturbances of carbohydrate metabolism and cardiac disease were excluded, and only those samples which were normal in gross appearance were used.

Immediately after autopsy, the aortas were placed in acetone. They were cleared of adventitia, but no attempt was made to separate the intima from the media. The combined tissues were minced and allowed to stand in several changes of acetone. Finally, they were washed with ether and air-dried. A mixture of dry ice and minced tissue was ground in a Wiley mill. The resultant fine powder was extracted with water and the mucopolysaccharides of the extract were isolated as described above.

Acetyl groups were measured as free acetic acid by gas chromatography performed by Mr. Gerald Meyer and Mr. William Lehnhardt in the laboratory of Dr. Richard Winzler, University of Illinois.

Acetylation of fractions obtained after HNO 3 treatment was performed by the procedure of Roseman and Daifner (18).

Amino acids were estimated by means of a Technicon amino acid analyzer after hydrolysis in 6 M HCl at 100° for 20 hours. The viscosity of the heparitin sulfate solutions was measured in a Cannon-Ubbelohde semi-microviscometer at 25°. The viscometer, when calibrated with 0.009 M sodium phosphate buffer (pH 6.8), 0.3 M NaCl, gave a flow time of 62.0 sec.

Number average molecular weights were calculated from osmotic pressure measurements made at 25° with a static osmometer (19). Heparitin sulfate samples from Hurler patients were found to dialyze slowly through regular Visking casing (20-mm diameter) as previously reported (20). Therefore, acetylated membranes (21) were prepared which were freely permeable to NaCl but retained tetrasaccharide prepared by hyaluronidase digestion of chondroitin 4-sulfate.

For electrophoresis of neutral sugars, hydrolysates were applied to strips (18 × 40 cm) of Whatman No. 3MM paper which had been soaked in 0.05 M sodium borate, pH 10.0. Electrophoresis was performed in an LKB apparatus at 300 volts for 4 hours. The paper was dried, neutralized by immersion in acidic methanol, dried, and stained with aniline-hydrogen phthalate.

Isolation of Mucopolysaccharide from Hurler Liver—After thawing at 4°, the tissues were minced and extracted with 5 volumes of distilled water in a Waring Blender for 10 min at 4°. The insoluble residue was removed by centrifugation, and the supernatant solution was dialyzed against distilled water in the cold and concentrated in a rotary evaporator. After clarification by centrifugation, the solution was made 0.03 M with respect to NaCl and sufficient cetylpyridinium chloride was added to precipitate the mucopolysaccharide (approximately 3 mg of cetylpyridinium chloride per mg of polysaccharide). The precipitate which formed after standing several hours at 37° was removed by centrifugation and washed with 0.03 M NaCl-0.1% cetylpyridinium chloride. The cetylpyridinium chloride complex was dissolved with 2 M NaCl and the mucopolysaccharide was recovered by the addition of 3 volumes of absolute ethanol. The precipitate was washed twice with 80% ethanol, twice with absolute ethanol, once with ethyl ether, and air-dried.

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The water-insoluble residue (53 g, dry weight) was suspended in 300 ml of a buffer containing 0.01 M EDTA, 0.005 M cysteine-HCl, and 0.1 M sodium acetate, pH 5.6. Crystalline papain (10 mg) was added and the suspension was incubated at 60°. Further additions of cysteine and papain were made over a 16-hour period until a total of 30 mg of papain was added. Following removal by centrifugation of the small amount of nondigested material, the supernatant solution was dialyzed against distilled...
The digestion of human aorta were fractionated on Dowex 1 as demucopolysaccharides obtained by water extraction and by papain revealed that the bulk of the hexosamine, 60%, was eluted with 0.02 M NaCl. Each fraction was dialyzed, concentrated, made 0.03 M with respect to NaCl, and precipitated first with cetylpyridinium chloride and then with ethanol. The results of this fractionation procedure are presented in Table I.

**Hurler Heparitin Sulfate**—During fractionation of the mucopolysaccharides from patient G. I., the material which was eluted from Dowex 1 with 1.0 M NaCl was only partially precipitated with cetylpyridinium chloride. This nonprecipitable polysaccharide was recovered from the cetylpyridinium chloride supernatant solution by precipitation with 3 volumes of ethanol. Upon rechromatography, it was consistently eluted with 1.0 M NaCl. It is identified as 1.0a M G. I. in Table III and in all subsequent tables.

After preliminary analysis, the principal fractions were digested with testicular hyaluronidase as follows. To 8 ml of a 0.5% solution of mucopolysaccharide in 0.1 M sodium acetate-0.15 M NaCl, pH 4.8, was added purified testicular hyaluronidase in 0.02 M Na2HPO4, pH 7.0, to a final concentration of 400 units per ml. Digestion was allowed to proceed for 5 days at 37°C under toluene, with daily additions of hyaluronidase. The digest was dialyzed against distilled water and concentrated. Gel filtration was performed on a column (2 X 40 cm) of Sephadex G-25, fine, equilibrated with 0.02 M NaCl. The uronic acid-containing material which emerged with the void volume was precipitated first with cetylpyridinium chloride and then with ethanol. The mucopolysaccharide was rechromatographed on Dowex 1 until constant chemical analyses, including amino acid content, were obtained. Such constancy was achieved with two or three repetitions of the procedure.

**Purification of Mucopolysaccharides from Hurler Tissues**—A 0.1% solution of the isolated mucopolysaccharide mixture was applied to a column (4 X 44 cm) of Dowex 1-X2, (Cl-) 200 to 400 mesh. Fractionation was achieved by stepwise elution with NaCl. Each fraction was dialyzed, concentrated, made 0.03 M with respect to NaCl, and precipitated first with cetylpyridinium chloride and then with ethanol. The results of this fractionation procedure are presented in Table I.

**Table I**

<table>
<thead>
<tr>
<th>NaCl concentration</th>
<th>T. P. (1.18 g)*</th>
<th>G. I. (1.24 g)*</th>
<th>G. C. (2.7 g)*</th>
<th>K. F. (0.4 g)* % dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>26</td>
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<td>52</td>
<td>61</td>
</tr>
<tr>
<td>1.3</td>
<td>23</td>
<td>18</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>1.5</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>1.7</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>1</td>
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<td>2.0</td>
<td>2</td>
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<td>2</td>
<td>0</td>
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<tr>
<td>Total recovery</td>
<td>67</td>
<td>83</td>
<td>82</td>
<td>80</td>
</tr>
</tbody>
</table>

* Dry weight of sample applied to the column.

**Table II**

<table>
<thead>
<tr>
<th>NaCl concentration</th>
<th>Mucopolysaccharide recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HzO extract (20 mg)*</td>
</tr>
<tr>
<td>1.0</td>
<td>50% dry weight</td>
</tr>
<tr>
<td>0.5</td>
<td>20% dry weight</td>
</tr>
<tr>
<td>1.0</td>
<td>5% dry weight</td>
</tr>
<tr>
<td>1.3</td>
<td>3% dry weight</td>
</tr>
<tr>
<td>1.5</td>
<td>0% dry weight</td>
</tr>
<tr>
<td>1.7</td>
<td>0% dry weight</td>
</tr>
<tr>
<td>2.0</td>
<td>0% dry weight</td>
</tr>
<tr>
<td>Total recovery</td>
<td>79% dry weight</td>
</tr>
</tbody>
</table>

* Dry weight of sample applied to the column.

**Isolation of Neutral Sugars from Heparitin Sulfate**—A 0.5% distilled water and 0.5 M NaCl. The results of uronic acid analyses of these fractions were difficult to evaluate, since a yellow color developed before the addition of carbazole. Nitrogen to hexosamine ratios were approximately 5, which suggests a high protein content. Analyses (carbazole to orcinol ratio and indole value) of the 1.0 M fraction suggested a mixture of mucopolysaccharides, containing a small amount of heparitin sulfate. However, insufficient material rendered further identification impossible.

Unlike mucopolysaccharides from Hurler tissues, the normal aorta mucopolysaccharides were only poorly extracted with water. Consequently, digestion with crystalline papain was necessary to isolate heparitin sulfate from this tissue. Under these conditions, 97.6% of the tissue was solubilized. The isolated mucopolysaccharides represented 0.9% of the dry weight of the aorta, in close agreement with previously reported data (25, 26).

Analyses (carbazole to orcinol ratios and indole values) of the fractions obtained by Dowex 1 chromatography of the papain-digested material indicated that the 1.0 M fraction was a rather pure preparation of heparitin sulfate, while the 1.3 M fraction appeared to be a mixture of heparitin sulfate and chondroitin sulfate-like polysaccharides. These two fractions were exhaustively digested with testicular hyaluronidase and the digests were applied to Sephadex G-25 columns. The mucopolysaccharides which emerged with the void volume were precipitated first with cetylpyridinium chloride and then with ethanol. Rechromatography on Dowex 1 followed and was repeated (twice) until constant chemical analyses, including amino acid content, were obtained. Hyluronidase digestion of the 1.3 M fraction resulted in a loss of 86% of the original material. The recovered polysaccharide, upon rechromatography on Dowex 1, was again eluted with 1.3 M NaCl. This fraction displayed an optical rotation of $-1^0$, a uronic acid to hexosamine ratio of 1:1, and an N-sulfate hexosamine content of 30%. It was not studied further.

In contrast to the 1.3 M fraction, 91% of the 1.0 M fraction was recovered after an identical purification procedure. Chromatography on Dowex 1, repeated twice, did not change the composition of this fraction. This preparation was utilized as the "normal" heparitin sulfate to which the Hurler heparitin sulfates were compared.

**Isolation of Neutral Sugars from Heparitin Sulfate**—A 0.5%
solution (1.0 ml) of heparitin sulfate in 1 m HCl was hydrolyzed
in a sealed tube at 100° for 12 hours. The hydrolysate was
diluted 25-fold, neutralized with Dowex 3 (CO3-) and applied
to a column (1 × 20 cm) of Dowex 1-X2 (Cl-),2 200 to 400
mesh. The column was washed with 2 bed volumes of water,
and the combined effluents were passed through a column (1 ×
20 cm) of Dowex 50W-X8 (H+), 200 to 400 mesh. The efflu-
ent was adjusted to pH 5 with Dowex 3 (CO3-) and con-
centrated. The residue, dissolved in water, was used for
qualitative (paper chromatography and electrophoresis) and
quantitative determination of galactose and xylose.

Nitrous Acid Degradation of Heparitin Sulfate—Based on
the method of Lagunoff and Warren (24) for the determination
of heparitin sulfate, Cifonelli (27, 28) has developed a method for
structural study, which utilizes the reaction of HNO3 with N-
sulfated groups. When the HNO3 reaction is carried out at −20°
in 60% glyme (1,2-dimethoxyethane), N-sulfate hexosamine
groups are converted to anhydromannose with the concomitant
rupture of adjacent glycoside bonds. Under these conditions,
free or acetylated amino groups of hexosamines, amino acids, or
dinitrophenyl-amin derivatives do not react with HNO3.

The nitrous acid was prepared at −20° by the method of
Scanley (29) and was approximately a 1% solution in 70% glyme.
Before reaction, 4 ml of a 1.0% solution of heparitin sulfate were
converted to the acid form by passage over a column (0.8 × 3.0
cm) of Dowex 50-X12 (H+), at 4°. The final reaction mixture
contained 1.5 mg of polysaccharide per ml and 0.06 N HNO3
in 70% glyme. Although the reaction appeared to be complete
in 8 to 10 hours, it was allowed to proceed for 24 hours. Excess
nitrous acid was removed by the addition of 12.5% ammonium
sulfamate at 4° until the solution gave a negative reaction
with starch iodide reagent. Finally, the solution was neutral-
ized with 0.5 N NaOH to pH 7 and concentrated in a rotary evap-
orator at room temperature.

The concentrated solution (0.5 ml) was applied to a column
(0.8 × 200 cm) of Sephadex G-25, fine, and eluted with 0.1 m
LiCl. Fractions of 1.4 to 1.6 ml were collected at a rate of 5 ml
per hour, and aliquots were analyzed for uranic acid, hexosamine,
and anhydromannose. On the basis of these analyses, contents
of tubco were pooled into several major fractions. The chemical
analyses and amino acid composition of each fraction were
determined.

The quantity of $N$-sulfate hexosamine, as measured by the
indole-HCl colorimetric method, did not correspond with values
based on the disappearance of hexosamine as measured by the
Elson-Morgan method (16). Therefore, this assay was used only
to follow the progress on HNO3 reaction and to provide some in-
dication of the final anhydromannose content of the fully reacted
preparation. The values so obtained are designated as −20°
anhydromannose in all tables dealing with the results of the
nitrous acid degradation studies. The fractions isolated after
complete reaction at −20° were treated with nitrous acid at room
temperature for 50 min. The anhydromannose value so
obtained is designated as +25° anhydromannose.

RESULTS

Analyses of Purified Heparitin Sulfate Preparations

Table III summarizes the results of analyses of the various
heparitin sulfate preparations.

The analytical data reveal striking similarities among the
heparitin sulfate fractions isolated from Hurler liver and urine.
In all cases, heparitin sulfate represented 80% or more of the
total mucopolysaccharide and emerged in two distinct fractions,
with one of which was eluted from Dowex 1 with 1.0 m and the other
with 1.3 m NaCl. The fractions eluted with higher salt concen-
trations contained chondroitin sulfate and were not investigated
further. The 1.0 m fraction consistently exhibited lower total
sulfate, lower N-sulfate (based on hexosamine determinations
after HNO3 treatment), and a higher positive optical rotation
than did that obtained with 1.3 m NaCl. The N-sulfate and
N-acetyl content were inversely related; the sum of the two is
approximately equal to the total hexosamine. Since the 1.3 m
fraction was similar to heparin in composition, these preparations
were tested for whole blood anticoagulant activity (30). No
anticoagulant activity was demonstrated.

The high nitrogen to hexosamine ratios are characteristic of
all heparitin sulfate fractions studied. These ratios cannot be

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Preparation & Fraction of & N\textsuperscript{a} & UA\textsuperscript{a} & SO\textsuperscript{a} & Acety\textsuperscript{b} & N−SO\textsuperscript{a} & M.W.\textsuperscript{c} & \textsuperscript{20} \beta \textsubscript{D} (\textdegree) \\
 & recovered & & & & & & & \\
% dry weight & mucopolysaccharide & & & & & & & \\
Aorta, 1.0 m & 20 & 1.30 & 1.31 & 0.68 & 0.29 & +67 & 24,000 & 0.98 \\
Liver, 1.0 m T. P. & 54 & 1.31 & 1.48 & 0.99 & 0.72 & 0.31 & +69 & 5,500 & 0.20 \\
Liver, 1.0 m G. I. & 31 & 1.51 & 1.35 & 1.08 & 0.46 & 0.50 & +65 & 3,200 & 0.10 \\
Liver, 1.0a m G. I. & 21 & 1.56 & 1.56 & 0.72 & 0.77 & 0.18 & +73 & 5,500 & 0.20 \\
Liver, 1.0 m C. & 64 & 1.50 & 1.60 & 1.20 & 0.40 & 0.48 & +70 & 3,600 & 0.11 \\
Urine, 1.0 m K. F. & 76 & 1.30 & 1.43 & 0.61 & 0.33 & +61 & 3,600 & 0.18 \\
Liver, 1.3 m T. P. & 35 & 1.60 & 1.35 & 2.02 & 0.26 & 0.80 & +49 & 4,000 & 0.13 \\
Liver, 1.3 m G. I. & 20 & 1.55 & 1.38 & 2.12 & 0.21 & 0.78 & +63 & 2,700 & 0.07 \\
Liver, 1.3 m G. C. & 17 & 1.20 & 1.30 & 3.00 & 0.24 & 0.83 & +56 & 2,800 & 0.08 \\
Urine, 1.3 m K. F. & 18 & 1.50 & 1.40 & 1.83 & 0.72 & 0.44 & +49 & 2,900 & 0.08 \\
\hline
\end{tabular}
\caption{Analyses of normal and abnormal heparitin sulfate preparations}
\end{table}

\textsuperscript{a} Molar ratios with hexosamine as 1.00.
\textsuperscript{b} Anhydrous concentrations were determined from the hexosamine values.
\textsuperscript{c} Calculated from osmometric data.
explained on the basis of amino acid content and were not significantly reduced by treatment with Dowex 50 (H+), acid-washed charcoal, or 0.1 N KOH at 4° for 24 hours. All heparitin sulfate preparations displayed an absorption peak at 254 mp; a 0.1% solution of the 1.0 m T. P. preparation exhibited an optical density of 0.14 per ml at this wavelength. No phosphate could be demonstrated in 10 mg of this preparation. Low molecular weight is a striking characteristic of the Hurler heparitin sulfate.

**Heparitin Sulfate of Normal and Hurler Tissues**

Aorta Heparitin Sulfate—The aorta preparation showed low sulfate content. In this respect, it is similar to the heparitin sulfate fraction isolated by Muir (31) from proteolytic digests of human aorta. Analytically, the aorta preparation most closely resembles 1.0 mM heparitin sulfate fractions obtained from the urine and livers of Hurler patients. The most dramatic difference between the "normal" and Hurler heparitin sulfate is in molecular weight. As judged both by osmotic pressure and viscosity measurements, the molecular weight of the polysaccharides of connective tissues, while that of preparations isolated from Hurler's tissue is markedly reduced. Notable was the absence in the aorta mucopolysaccharide of a fraction comparable to the 1.3 mM Hurler heparitin sulfate preparations.

**Amino Acid Composition**

Considerable care had been taken in the isolation and purification of Hurler mucopolysaccharide to avoid rupture not only of peptide bonds but also of any xylosylserine linkages. A study of the amino acid composition of these preparations was undertaken to obtain information as to the mode of binding of polysaccharides to protein in normal and Hurler heparitin sulfate.

Table IV summarizes the amino acid analyses of the heparitin sulfate preparations, Hurler and normal. In all the Hurler 1.0 mM fractions, serine is clearly the predominant amino acid. Considering the average molecular weights of these preparations, serine is present to the extent of approximately 1 in 4 to 10 heparitin sulfate molecules. The 1.3 mM fractions show a much lower total amino acid content with little evidence of predominance of serine. Approximately 1 serine residue is present for every 50 heparitin sulfate molecules. It is also to be noted that the 1.3 mM fraction contains some galactosamine, which might indicate that a portion of the amino acids present may be related to contaminating substances.

When one compares the amino acid analyses of the heparitin sulfate from aorta to those of the Hurler preparations, two important distinctions emerge. Unlike the Hurler polysaccharide, the heparitin sulfate from aorta contains approximately equimolar amounts of aspartic acid, serine, glutamic acid, and glycine. If it is assumed that serine is the amino acid involved at the binding site and that there is only 1 serine residue per polysaccharide chain, then a chain weight of 29,500 can be calculated from the amino acid data. This value agrees fairly well with the experimentally determined number average molecular weight of 24,000. This result strongly suggests that in normal aorta every molecule of heparitin sulfate is bound to protein.

**Identification of Neutral Sugars in Heparitin Sulfate**

The presence of neutral sugars in acid hydrolysates of the heparitin sulfate preparations was demonstrated by paper chromatography. Initial experiments employed Solvent c, but better resolution of pentoses was obtained with Solvents a and b, which were used for all subsequent chromatographs. Since the mobilities of xylose and xylose were similar in all three solvent systems, electrophoresis in borate buffer, pH 10, was used to distinguish these sugars. The results of these procedures applied to the hydrolysate of preparation 1.0 mM H. G. I. are illustrated in Figs. 1 through 5. Paper chromatography revealed one brown spot which migrated as does galactose and a faster moving pink spot with a mobility identical with that of xylose. Electrophoresis in borate buffer showed one major pink spot with a slightly trailing brownish area. The mobility of the pink spot was the same as that of xylose and arabinose and is clearly distinct from that of ribose and xylose. Identical results were obtained for all the Hurler 1.0 mM preparations, and for hydrolysates of heparitin sulfate obtained from aorta (Figs. 4 and 5). The chromatogram with Solvent a for the hydrolysate of the heparitin sulfate of normal aorta is not reproduced since it was identical with Fig. 1. It was not possible to demonstrate either galactose or xylose in hydrolysates of the Hurler 1.3 mM preparations.

**Estimation of Galactose and Xylose**

The galactose and xylose contents of the heparitin sulfate preparations, both Hurler and normal, are listed in Table V. Enzy-

**Table IV**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Galactosamine</th>
<th>Aspartic acid</th>
<th>Threonine</th>
<th>Serine</th>
<th>Glutamine</th>
<th>Glycine</th>
<th>Alanine</th>
<th>Isoleucine</th>
<th>Leucine</th>
<th>Lysine</th>
<th>Histidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta, 1.0 m</td>
<td>0.9</td>
<td>2.2</td>
<td>0.2</td>
<td>1.7</td>
<td>1.6</td>
<td>1.9</td>
<td>0.7</td>
<td>0.3</td>
<td>0.5</td>
<td>0.2</td>
<td>0.3</td>
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<tr>
<td>Liver, 1.0 m T. P.</td>
<td>0</td>
<td>0.3</td>
<td>0.1</td>
<td>1.8</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Liver, 1.0 m G. I.</td>
<td>Tr.</td>
<td>0.4</td>
<td>0</td>
<td>0.9</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Liver, 1.0 m G. I.</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>1.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Liver, 1.0 m G. I.</td>
<td>0.9</td>
<td>0.8</td>
<td>0.2</td>
<td>1.3</td>
<td>0.5</td>
<td>0.3</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Liver, 1.3 m T. P.</td>
<td>2.0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Liver, 1.3 m G. I.</td>
<td>3.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Liver, 1.3 m G. C.</td>
<td>5.0</td>
<td>0.2</td>
<td>0.3</td>
<td>0.6</td>
<td>0.6</td>
<td>0.7</td>
<td>0</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urine, 1.3 m K. F.</td>
<td>5.0</td>
<td>0.5</td>
<td>0.3</td>
<td>0.7</td>
<td>0.6</td>
<td>0.6</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*All results are expressed as micromoles of amino acid per 100 μmoles of glucosamine as determined by the Boas method (16).*
A striking finding was that the molar ratios of serine to xylose to galactose in the Hurler heparitin sulfate preparations approach 1:1:2. Since only 1 in 4 to 10 molecules in the Hurler 1.0 M fractions contained serine, it seemed possible that serine-free polysaccharide chains were present which contained variable parts of the linkage region. Such does not seem to be the case. If serine is present, the other two components of the linkage region are present. If it is missing, galactose and xylose are also absent. In the Hurler 1.3 M preparations, neither galactose nor xylose was detectable in hydrolysates of 10 mg of these fractions.

Heparitin sulfate from aorta was also found to contain serine-xylose-galactose in a ratio of approximately 1:1:2. This result tends to confirm the conclusion, deduced from the amino acid and molecular weight data, that in normal heparitin sulfate there is one serine per carbohydrate chain.

**Nitrous Acid Degradation**

The absence of the neutral sugars, together with their unusual chemical composition, led to the hypothesis that the 1.3 M Hurler preparations represent molecular fragments not involved in protein binding. This possibility was further examined by structural studies with nitrous acid. The method also afforded the opportunity of localizing in the 1.0 M fraction the area of binding to protein.
TABLE V
Neutral sugar content of heparilin sulfate preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Serine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Xylose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Galactose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Serine-xylose-galactose&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta, 1.0 m</td>
<td>1.7</td>
<td>1.5</td>
<td>3.1</td>
<td>1.00:0.88:1.80</td>
</tr>
<tr>
<td>Liver, 1.0 m T. P.</td>
<td>1.8</td>
<td>2.0</td>
<td>4.0</td>
<td>1.00:1.11:2.22</td>
</tr>
<tr>
<td>Liver, 1.0 m G. I.</td>
<td>0.9</td>
<td>0.8</td>
<td>2.1</td>
<td>1.00:0.80:2.33</td>
</tr>
<tr>
<td>Liver, 1.0a m G. I.</td>
<td>2.9</td>
<td>3.2</td>
<td>6.4</td>
<td>1.00:1.10:2.20</td>
</tr>
<tr>
<td>Liver, 1.0 m G. C.</td>
<td>1.0</td>
<td>0.9</td>
<td>2.1</td>
<td>1.00:0.90:2.10</td>
</tr>
<tr>
<td>Urine, 1.0 m K. F.</td>
<td>1.3</td>
<td>1.1</td>
<td>2.5</td>
<td>1.00:0.85:1.92</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values expressed as micromoles per 100 μmoles of glucosamine.

<sup>b</sup> Molar ratios with serine as 1.00.

HNO<sub>2</sub> Degradation of Hurler 1.0 m Heparitin Sulfate Preparations—The reaction products resulting from nitrous acid treatment of preparation 1.0 m T. P. were fractionated by gel filtration on Sephadex G-25. The results are presented in Fig. 6. One major peak was obtained, which emerged with the void volume. This was divided into two fractions. Fractions 3 and 4 represented minor subsequent peaks. These appeared as a result of the nitrous acid treatment, since the untreated heparitin sulfate emerged as a single peak with the void volume. Analyses of the material from each of the four fractions are shown in Table VI.

The total hexosamine recovered from the combined fractions represented 69% of the amino sugar in the original preparation. By difference, the remaining 31% was N-sulfate hexosamine. This value is supported by the N-acetyl analyses which indicated that 72% of the hexosamine of the starting material was N-acetylated. Theoretical nitrogen to hexosamine ratios were found in Fractions 1, 2, and 3. Contamination with ammonium sulfamate and inorganic sulfate precluded accurate determinations of nitrogen and sulfate in Fraction 4. In Fraction 1, 87% of the hexosamine is N-acetylated, an indication of a minimal amount of N-sulfate. Since the total SO₄ was 0.25, the amount of O—SO₄ was also low.

An unusual finding was the discrepancy between the −20° and +25° anhydromannose determinations in Fractions 1 and 2. The production of additional anhydromannose groups by nitrous acid at room temperature indicated either incomplete reaction of the N-sulfate hexosamine groups in the cold or the presence of reactive groups, such as free amino groups, in these fractions. The former possibility was tested by repetition of the nitrous acid procedure. After 8 hours, the anhydromannose content remained unchanged. The possibility of the occurrence of free amino groups was tested by determining the effect of acetylation on the 25° anhydromannose reaction. While acetylation reduced the 25° anhydromannose reaction of N-desulfated heparin to the extent of 96%, this procedure resulted in only a 50% decrease of the 25° anhydromannose reaction of Fractions 1 and 2 of the 1.0 m preparations. The significance of these findings is not clear. They suggest the presence of some free amino groups on glucosa-

TABLE VI
Analyses of fractions obtained after HNO<sub>2</sub> treatment of liver preparation 1.0 m T. P.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hexosamine recovered</th>
<th>Serine recovered</th>
<th>N&lt;sup&gt;a&lt;/sup&gt;</th>
<th>VA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SO₄&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Acetyl&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Anhydromannose&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>%</td>
<td>μM</td>
<td>%</td>
<td></td>
<td></td>
<td>−20°</td>
</tr>
<tr>
<td>Starting material</td>
<td>53</td>
<td>100</td>
<td>1.00</td>
<td>100</td>
<td>1.31</td>
<td>1.48</td>
<td>0.49</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>38.0</td>
<td>0.68</td>
<td>68</td>
<td>0.97</td>
<td>1.34</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>10.4</td>
<td>19.6</td>
<td>0.14</td>
<td>14</td>
<td>0.99</td>
<td>1.23</td>
<td>0.40</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>7.1</td>
<td>Trace</td>
<td></td>
<td>1.05</td>
<td>1.70</td>
<td>1.70</td>
</tr>
<tr>
<td>4</td>
<td>2.3</td>
<td>4.4</td>
<td>0.03</td>
<td>3</td>
<td>1.73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Values expressed as molar ratios with hexosamine as 1.00.
Table VII

Analyses of fractions obtained after HNO₂ treatment of liver preparation 1.3 M T. P.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hexosamine recovered</th>
<th>Serine recovered</th>
<th>N⁺</th>
<th>UA⁺</th>
<th>SO₄⁻</th>
<th>Anhydromannose*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>%</td>
<td>µM</td>
<td>%</td>
<td></td>
<td>−20°</td>
</tr>
<tr>
<td>Starting material</td>
<td>45</td>
<td>100</td>
<td>0.09</td>
<td>100</td>
<td>1.60</td>
<td>1.35</td>
</tr>
<tr>
<td>1</td>
<td>1.1 Galactosamine</td>
<td>9.0</td>
<td>0.04</td>
<td>45</td>
<td>1.16</td>
<td>1.12</td>
</tr>
<tr>
<td>2</td>
<td>2.8 Glucosamine</td>
<td>7.0</td>
<td>0</td>
<td>1.22</td>
<td>1.80</td>
<td>3.30</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>5.0</td>
<td>0</td>
<td>1.45</td>
<td>3.25</td>
<td>11.8</td>
</tr>
<tr>
<td>4</td>
<td>1.1</td>
<td>2.5</td>
<td>0</td>
<td>3.70</td>
<td>12.0</td>
<td></td>
</tr>
</tbody>
</table>

a All values expressed as molar ratios with hexosamine as 1.00.

mine residues as well as glucosamine residues substituted with unidentified groups which react with HNO₂ at 25° but not at −20°. A recent report provides some evidence for the presence of an acid-labile N-substituent other than sulfate in a heparitin sulfate fraction from human aorta (31). However, the presence of N-sulfate glucosamine residues which, for steric or other reasons, do not react with HNO₂ in the cold must be considered as a possible explanation.

HNO₂ Degradation of Hurler 1.3 M Heparitin Sulfate Fractions—The elution profile of the HNO₂ reaction products from preparation 1.3 M T. P. (Fig. 7) is clearly different from that obtained with the 1.0 M Hurler heparitin sulfate preparations. Although the 1.3 M starting material emerged from this column with the void volume as a sharp peak, all reaction products were retarded by Sephadex G-25. Analyses of the four fractions are given in Table VII. Similar results were obtained for all Hurler 1.3 M heparitin sulfate preparations.

Approximately 25% of the hexosamine of the starting material was recovered after HNO₂ treatment, a significant portion of which was galactosamine. In contrast to the 1.0 M Hurler preparations, there is a close correlation between the −20° and +25° anhydromannose values and retention of a high nitrogen to hexosamine ratio in the reaction products. This finding strongly suggests that the group containing the excess nitrogen is associated with the N-sulfate hexosamine residues of the Hurler 1.0 and 1.3 M heparitin sulfate preparations.

It is apparent that the Hurler 1.3 M preparation contains few, if any, molecules with large segments of N-acetylhexosamine-uronic acid repeating units. The failure to obtain sharp peaks and the persistence of hexosamine in Fractions 2, 3, and 4 may reflect either heterogeneity of the starting material or the occurrence of N-acetylhexosamine-uronic acid units between large segments of N-sulfate hexosamine-uronic acid.

The significance of the preponderance of serine in Fraction 1 is uncertain because of the low recovery of this amino acid and the high galactosamine content of this material. It is nonetheless clear that serine is not associated with fractions of high anhydromannose and sulfate content.

HNO₂ Degradation of Aorta Heparitin Sulfate—The results of the fractionation resulting from HNO₂ treatment of aorta heparitin sulfate are illustrated in Fig. 8. The elution pattern is

Table VIII

Analyses of fractions obtained after HNO₂ treatment of heparitin sulfate from human aorta

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hexosamine recovered</th>
<th>Serine recovered</th>
<th>N⁺</th>
<th>UA⁺</th>
<th>SO₄⁻</th>
<th>Anhydromannose*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>%</td>
<td>µM</td>
<td>%</td>
<td></td>
<td>−20°</td>
</tr>
<tr>
<td>Starting material</td>
<td>9.6</td>
<td>100</td>
<td>0.16</td>
<td>100</td>
<td>1.3</td>
<td>1.31</td>
</tr>
<tr>
<td>1</td>
<td>4.9</td>
<td>51.0</td>
<td>0.10</td>
<td>63</td>
<td>0.93</td>
<td>1.12</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>10.5</td>
<td>0.03</td>
<td>19</td>
<td>1.14</td>
<td>1.40</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>3.0</td>
<td>Trace</td>
<td>1.60</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>2.0</td>
<td>Trace</td>
<td>1.45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a All values expressed as molar ratios with hexosamine as 1.00.
remarkably similar to that obtained with the 1.0 M Hurler heparitin sulfate preparations. Analyses of the fractions obtained from these peaks are given in Table VIII. As in the Hurler 1.0 M samples, serine is strikingly concentrated in the first fraction. The elution position of this fraction, as well as its analyses, indicates that heparitin sulfate from normal aorta contains a relatively large N-acetylgalactosamine-rich region in which the protein binding site is localized. Regions of high N-sulfate hexosamine content do not appear to be involved in protein linkage of the polysaccharide as isolated from normal tissue.

Analyses of the material in the second fraction suggest that a small percentage of N-acetylgalactosamine-uronic acid chains of moderate size and little or no amino acids are present in the aorta preparation.

**Discussion**

Heparitin sulfate, as isolated after proteolytic digestion of combined, normal, human aortas, appears to be a carbohydrate-protein complex of molecular weight 24,000 to 29,000. The polysaccharide moiety is characterized by a high N-acetyl and a low N-sulfate glucosamine content. After digestion with papain, the residual peptide contains, predominantly, asparagine, glycine, serine, glutamic acid, and alanine. Demonstration of an approximately 1:1:2 molar ratio of serine to xylose to galactose indicates that the linkage region of heparitin sulfate is similar to that found in chondroitin 4-sulfate and heparin.

Nitrous acid degradation studies show that the mucopolysaccharide is not a linear hybrid polymer of alternating N-acetylgalactosamine-uronic acid and N-sulfate glucosamine-uronic acid disaccharides. A similar conclusion has been reported for heparitin sulfate isolated as a by-product of commercial heparin (27, 32). The data are inadequate to distinguish between heterogeneity of the original material or random distribution of N-sulfate and N-acetyltetrasulfonates in a single parent molecule. However, the data do clearly indicate that a majority of the heparitin sulfate molecules of aorta consists predominantly of a rather large segment composed of repeating units of N-acetylgalactosamine and uronic acid. Very small quantities of sulfate are found in this segment, in conformation of previous reports that the bulk of the N-acetylgalactosamine residues are free of ester sulfate (27, 32). A similar conclusion regarding the structure of heparin has been reached by Lindahl (6). The association of serine with this structural unit suggests that protein binding is localized to this portion of the heparitin sulfate molecule. Fractions rich in N-sulfate hexosamine do not appear to contain the region involved in linkage to protein.

Heparitin sulfate isolated from the livers and urine of patients with Hurler syndrome differs markedly from that obtained from human aorta. Two fractions are found which are chemically and structurally distinct. The 1.0 M fraction is closely related chemically to the normal aorta heparitin sulfate preparation. However, it is distinguished from the normal material by its low molecular weight (approximately one-fifth that of the aorta polysaccharide) and its markedly reduced amino acid content (1 serine per 4 to 10 polysaccharide chains). The predominance of serine in this fraction supports the original suggestion by Jacobs and Muir (9) that binding of heparitin sulfate to protein is mediated by this amino acid. An approximately 1:1:2 serine to xylose to galactose molar ratio indicates that this binding is normal, albeit deficient, in the heparitin sulfate from abnormal tissue. Structurally, the preparation appears to consist mainly of molecules with repeating N-acetylgalactosamine-uronic acid disaccharide units. The association of serine with this unit indicates that the location of complex formation, when it occurs, is normal. The 1.3 M Hurler heparitin sulfate fraction bears a closer chemical relationship to heparin. It is characterized by the absence of a significant percentage of molecules with N-acetyltetrasulfonate-uronic acid repeating units. Associated with this absence is the lack of both serine and the neutral sugar components of the protein-polysaccharide linkage region. These findings suggest that Hurler heparitin sulfate may arise from partial hydrolysis of a parent molecule similar in structure to heparitin sulfate from normal aorta. These findings seem relevant to the genetic defect in the Hurler's syndrome, but the exact mechanisms are not clear. Comparative studies by Matalon and Dorfman (33) on fibroblasts cultured from the skin of Hurler and normal patients have shown that the rates of synthesis and storage of dermatan sulfate and hyaluronic acid are increased within the disease cell. Puromycin inhibits the synthesis of the mucopolysaccharides, which suggests that a protein acceptor is necessary for synthesis of polysaccharides in Hurler fibroblasts as it is in normal chick chondrocytes (34). This finding eliminates the possibility that in Hurler fibroblasts sulfated mucopolysaccharide chains are synthesized independently of a protein acceptor. If one assumes that a similar mechanism is obligatory for heparitin sulfate synthesis, then the 1.3 M Hurler heparitin sulfate fractions could only arise as a result of a degradative process. A report by Hutterer (35) of an enzyme from rat liver lysosomes capable of hydrolyzing heparitin sulfate indicates that enzymatic mechanisms for degradation of this mucopolysaccharide are present in the normal animal. (The absence of intact heparitin sulfate-protein complex in Hurler liver is significant.)

Separate studies of the dermatan sulfate of Hurler's tissues similarly indicate that the stored material is partially degraded.

In the case of dermatan sulfate, the mechanism of degradation has become more apparent as a result of the studies of Fransson and Rodén (36). The studies indicate that dermatan sulfate is a hybrid molecule containing both N-glucuronic and L-iduronic acid. Since hyaluronidase has recently been found in lysosomes (37), it seems reasonable to postulate that this enzyme may be responsible for the partial degradation of dermatan sulfate in Hurler's disease. If it is assumed that N-glucuronic acid is randomly distributed in the dermatan sulfate molecule and that hyaluronidase acts on some or all of the linkages of N-acetyltetrasulfonate to glucuronic acid, it may be predicted that the resultant dermatan sulfate will have a decreased molecular weight and a decrease of linkage region.

On the basis of the evidence of excessive synthesis of mucopolysaccharides in Hurler's syndrome, it may then be postulated that both heparitin sulfate and dermatan sulfate are partially degraded by intracellular (lysosomal) enzymes. Storage may result from the limited capacity to degrade these two polysaccharides.

**Acknowledgments**—We wish to thank Dr. Lennart Rodén for many helpful discussions. We would also like to express our gratitude to Dr. Robert Austrian and Dr. Gerald Schiffman for their generosity in providing laboratory facilities for a portion of this work. The expert technical assistance of Miss Pei-Lee Ho and Mrs. Louise K. Roth is gratefully acknowledged.

\(^1\) J. Knecht, P. Ho, and A. Dorfman, unpublished results.
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Structural Studies on Heparitin Sulfate of Normal and Hurler Tissues
Judith Knecht, J. Anthony Cifonelli and Albert Dorfman


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