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THE HEPARITIN SULFATES (HEPARAN SULFATES)

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ABSTRACT

Heparitin sulfate has been isolated from several sources, namely: a commercial lung polysaccharide preparation, beef lung, beef aorta, human amyloid liver, human intestine, and urine of a patient with mucopolysaccharidosis. The polysaccharides isolated were extensively purified, fractionated, and characterized. The data obtained show that heparitin sulfate is not a single compound but constitutes a family of related polymers which differ in sulfate content and in the arrangement of charged groups. These compounds are readily distinguished from most other glycosaminoglycuronans (mucopolysaccharides) by composition and optical rotation. They can be differentiated from heparin by their content of sulfate and *N*-acetyl groups, and D-glucuronic acid residues and by the ratio of the carbazole to orcinol uronic acid values. Due to the variations of charge distribution and molecular size, the heparitin sulfates are found to a considerable extent in admixture with other acidic polysaccharides during isolation and fractionation procedures. The particular type of heparitin sulfate obtained varies considerably with the tissue of origin. The lung-derived material was found to contain the largest range of subfractions. The heparitins isolated from aorta and amyloid liver were fairly homogenous in themselves, but differed from each other.

INTRODUCTION

Glycosaminoglycuronan sulfates (acid mucopolysaccharides) occur in a large variety of organisms and seem of particular importance in mammalian connective tissue where six different glycosaminoglycuronan sulfates (GGS)* have been recognized. Originally thought to be composed of carbohydrate only and to contain fairly simple and uniform repeating units, they are now recognized to be proteoglycans and they share with other glycoproteins the property of considerable heterogeneity. This manifests itself in variations in molecular size, in monosaccharide residue composition, and in degree of sulfation. Dermatan sulfates, for instance, show

*Abbreviations used are: GGS glycosaminoglycan sulfates (acid mucopolysaccharides); CPC, cetylpyridinium chloride.

significant variation in the relative proportion of D-glucuronic and L-iduronic acid residues and in degree of sulfation depending on their source[1–3]. Keratan sulfates show differences in their attachment to protein, neutral sugar content, and distribution of sulfate groups[4]. Heparatin sulfate (or heparan sulfate) appears to be perhaps the most heterogeneous of all. This GGS contains D-glucosamine, D-glucuronic acid, L-iduronic acid, *N*-acetyl, and *N*-sulfate, and *O*-sulfate groups. The distribution of charges in particular seems extremely variable[5,6] and the polysaccharide shows resemblance to, as well as differences from heparin[7].

No direct evidence for the biological role of heparitin sulfate is available, but this compound is of obvious interest owing to its presence in blood-vessel walls and cell membranes[8] and its involvement in such disease processes as the mucopolysaccharidoses[9], amyloidosis[10–12], and perhaps atherosclerosis[13]. With the exception of some careful and detailed studies[14–16], most of the data reported are concerned with either the presence or the relative amounts of this polysaccharide in various tissues or organs. In general, no detailed characterization or extensive fractionations have been carried out, except as noted above.

One of the questions arising is whether heterogeneity is apparent rather than real, namely, how great is the variation within a given polysaccharide chain rather than in a family of chains isolated from a particular source and how closely is this heterogeneity related to a particular tissue or origin. An additional problem is the presence and the quantitation of heparitin sulfate in a particular source, as this is often based on rather arbitrary criteria, such as chromatographic elution peaks, electrophoretic mobility, and liberation of amino groups during dilute acid hydrolysis. The heterogeneity of the heparitin sulfates with regard to physical and chemical properties complicates isolation and identification procedures and necessitates more complete characterization of polysaccharide fractions than usual. When the biological role of these polymers is investigated, it is certainly not sufficient to be aware that one is dealing with "heparitin sulfate", but it becomes essential to know which heparitin sulfate is involved in a particular instance.

This paper attempts to identify and characterize in greater detail the individual components of the family of heparitin sulfates from a variety of sources, in order to better ascertain the structure, heterogeneity, and biological function.

EXPERIMENTAL AND RESULTS

Analytical methods. — Uronic acid contents were determined by the carbazole[17] and orcinol[18] procedures, total hexosamine contents by a modified Elson–Morgan method[19], and *N*-sulfated hexosamine contents by a nitrous acid procedure[20]. *N*-Acetyl groups were determined colorimetrically[21] and sulfate groups by titration[22] or a modification of the titration method[23]. Free amino groups were measured by the 2,4-dinitrofluorobenzene procedure[24]. The proportion of D-glucosamine to D-galactosamine was determined by ninhydrin degradation, followed by paper chromatography[25]. The proportion of D-glucuronic to L-iduronic acid was determined by

hydrolysis followed by paper chromatography, as described by Lindahl *et al.*[26]. The intensity of spots was measured by a densitometer (Photovolt Corp., New York, N.Y. 10010).

Electrophoresis. — Strips of Sepraphore III (Gelman Instr. Co., Ann Arbor, Mich. 48106) were used in a high-voltage electrophoresis apparatus (Savant Instr., Inc., Hicksville, N.Y. 11801, flat plate) with pyridine–formic acid buffer[27] at 35 V/cm for 1 h. A Brinkman Microphor system with cellulose acetate strips was also used. Electrophoresis was carried out in 0.2M calcium acetate[28] or in the same pyridine–formic buffer as just described at 5 mA per strip for 2 h. Very good separation between heparitin sulfate with a low sulfate content, dermatan sulfate, and chondroitin 4- or 6-sulfate was obtained in the calcium acetate solution. However, charge differences are minimized in this system and heparin migrates barely faster than chondroitin 4-sulfate.

Column chromatography. — Polysaccharides were fractionated on columns of AG (1 × 8, 200–400 mesh, Bio-Rad Lab., Richmond, Cal. 94804) by a modification of the method of Schiller *et al.*[29]. At least 15 g of resin were used per 100 mg of GGS. Columns were eluted with 0.1, 0.5, 0.9, 1.2, 1.4, and 2.0M solutions of sodium chloride in stepwise increment. Approximately 5 ml per tube were collected and analyzed by the orcinol reaction[18]. The contents of peak tubes were combined, dialyzed for at least 48 h against distilled water, evaporated to a small volume on a flash evaporator, and lyophilized. The material obtained was characterized by analysis and electrophoresis and further purified if necessary, as will be indicated.

Isolation from tissues. — No method of isolation appears to exist which is generally applicable to all tissues. Therefore, some variations in isolation procedures were used as will be indicated. In general, the following procedure based on a method developed by Pearce *et al.*[30] was used at least for the initial stages of isolation. Fresh or frozen tissues were ground in a meat grinder and suspended in acetone (4 vol.), which was changed at least twice by decantation. The tissue was finally spread in evaporating dishes, dried at room temperature, ground in a Wiley mill and the resulting fine powder dried in a desiccator and weighed. The acetone powder (100 g) was suspended in 0.1M acetate buffer (500 ml, pH 5.5) containing 5mM ethylenediaminetetraacetate (EDTA) and cysteine. A suspension of crystalline papain (50 mg in 1.66 ml, 14.8 units/mg, Worthington Co.) was added, and the mixture was incubated for 24 h at 60°; additional enzyme (12.5 mg) was added at this time and incubation continued for 24 h. The digest was centrifuged and the precipitate, if small, was discarded. Material in the supernatant was either precipitated with ethanol (2 vol.) or 10% CPC (10 ml/l) was added as precipitant. In either case, the suspension was kept in the refrigerator for 24 h and then centrifuged. Pronase digestion was performed by incubation of crude fractions with Pronase (0.2 g/l, Calbiochem, A grade, 120,000 PUK/g) in 0.1M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.3) for 2 days at 37°.

Unless indicated otherwise, GGS were recovered from enzyme digests or other solutions by precipitation with ethanol (2 vol.). The precipitates were collected by

centrifugation, washed with 80%, 95%, and abs. ethanol and dried in a desiccator over phosphorus pentaoxide. The precipitates of the crude GGS were further treated as will be described. The heparitin sulfate fractions to be described were characterized by the analytical data, the presence of D-glucosamine as the only amino sugar, optical rotation, electrophoresis on cellulose acetate, and, when necessary, behavior with specific enzymes. As the emphasis in this report is on heparitin sulfate, other GGS present were characterized only sufficiently for identification. This was done mainly to obtain values for the relative amounts present in various sources, but no detailed analysis was carried out. A variety of biological sources and one commercial sample were used for GGS isolation. Isolation will be described separately for each source.

Byproducts of heparin preparation from beef lung. — This material, obtained from The Upjohn Co., Kalamazoo, Mich. 49001 and also used by other laboratories[14,31] as a starting material for heparitin sulfate fractionation, contains about 18% uronic acid (carbazole reaction) which corresponds to a total GGS content of about 50%. At first, purification by a CPC-fractionation method[32] was attempted. Some purification was achieved, but considerable overlap between different sodium chloride eluates could be detected by electrophoresis. Therefore, in earlier experiments, material obtained from the CPC procedure was chromatographed on columns of Bio-Rad AG (1 × 8), and in later experiments, the crude GGS was fractionated directly on the resin columns.

The crude GGS (2 g) was dissolved in water (2 liters) and the solution was added to a 4 × 50 cm column containing 400 g of AG (1 × 8, 200–400 mesh) which was then eluted with a total of 500–1000 ml of each sodium chloride solution mentioned earlier. The peak fractions were isolated, analyzed, and monitored by electrophoresis. Most of these fractions were heterogeneous either as heparitin sulfate subfractions or because they contained nucleic acid, chondroitin sulfates, or protein. Therefore, they were further purified by ethanol extraction[33]; 65% ethanol (5 ml per 100 mg of GGS) was used first and it appeared to remove mainly nucleic acids and proteins. This was followed by extraction with 50% and 35% ethanol which solubilized mainly heparitin sulfate; the composition of the two ethanol extracts were somewhat different.

Any fractions found to contain D-galactosamine and, therefore, chondroitin sulfate were first treated with testicular hyaluronidase* (Sigma Chem. Co., St. Louis, MO. 63178, 750 units per mg) as described previously[34]. The hydrolyzed samples were dialyzed for 3 days against distilled water and concentrated on a flash evaporator. Sodium acetate was added to a 5% concentration, and acetic acid to a 0.1M concentration, and ethanol (2 vol.) was added for precipitation. Fractions containing dermatan sulfate, which is not hydrolyzed adequately by the testicular enzyme, were further fractionated by precipitation of this GGS with Benedict reagent[35]. Approximately 350 mg of the total GGS were obtained from 1 g of starting material, 180 mg being heparitin sulfate fractions, 140 mg heparin, and 30 mg chondroitin sulfates.

*We could not detect any action of this enzyme on purified heparitin sulfate fractions obtained from this source or from aorta which had been prepared without the use of testicular hyaluronidase during the isolation procedure.

Analyses of the major purified heparitin sulfate fractions are shown in Table I and electrophoresis patterns in Fig. 1. Fractions are designated by the molarity of sodium chloride used to elute them from the resin column. Ethanol extraction used for further purification, as mentioned in the preceding paragraphs, will be indicated in the text where applicable.

TABLE I

HEPARITIN SULFATES ISOLATED FROM HEPARIN BYPRODUCTS

Properties	*Fractions eluted with sodium chloride of molarity*				
	0.5	*0.9*	*1.2*	*1.4*	*2.0*[a]
Yield[b] (%)	2.5	32 0	36.0	29 5	
Uronic acid residues (%)					
Carbazole test	42	47	48	46	36
Orcinol test	29	25	21	18	8
Sulfate groups[c] (%)	1.5 (0 1)	9 0 (0.7)	13 0 (0 9)	17 0 (1.0)	28 (2 3)
Acetyl groups[c] (%)	10 0 (1 4)	4 9 (0.8)	3 9 (0.6)	3 7 (0.5)	0.72
Hexosamine residues[c] (%)					
Total	28	24	28	30	21
Indole test	5 (0.2)	17 (0.7)	24 (0.8)	27 (0.9)	27 (1.2)
Free amino groups[d] (%)	12	8	7	8	2
Optical rotation $[\alpha]_D^{25}$ (c 1.0, water) (°)	+80	+82	+75	+65	+42
D-Glucuronic acid[e] residues (%)	90	80	80	70	30

[a]Ref 5. [b]Percent of total heparitin sulfate isolated. [c]In parentheses, molar ratio to total hexosamine residues. [d]Percent of total amino groups which are unsubstituted. This would include amino groups of peptides, if present. [e]Percent of total uronic acid residues The difference from 100% is L-iduronic acid. Not corrected for variable loss during hydrolysis.

Heparitin 0 5. This fraction was extracted with 50% ethanol from the 0.5M-sodium chloride eluate fraction. Very little GGS was present in the other ethanol extracts. As can be seen in Table I, the yield of this fraction is very low. The compound is, however, of considerable interest as it is almost completely *N*-acetylated and nonsulfated, and contains very little L-iduronic acid A small amount of free amino groups appears to be present, but could be contributed in part by small amounts of peptides

Heparitin 0.9. The two fractions extracted with 50% and 35% ethanol from the original column eluate were combined, since little difference between both materials could be observed, to give one of the major fractions (Table I). It contains about equivalent amounts of *N*-sulfate and *N*-acetyl groups and the relation between the analyses for total sulfate groups, indole hexosamine residues*, and acetyl groups indicates

*The indole reaction measures hexosamine residues having *N*-sulfated or free amino groups, *N*-acetylhexosamines do not react

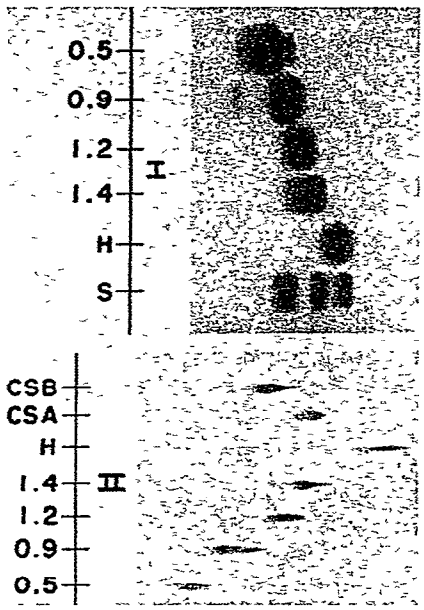

Fig 1. Electrophoresis patterns of heparitin sulfate fractions from heparin byproduct material. I, In 0 2M calcium acetate II, In pyridine–formic acid buffer at high voltage; for the Heparitin 0.5, 0.9, 1.2, 1.4 fractions, see text and Table I. H, heparin standard, S, standards of the Heparitin 0.9 fraction, dermatan sulfate, and chondroitin 4-sulfate; CSB, dermatan sulfate; CSA, chondroitin 4-sulfate.

that about 28% of the total sulfate groups are present as *O*-sulfate*, the rest as *N*-sulfate groups.

Heparitin 1 2. This fraction was extracted from the 1 2M sodium chloride eluate fraction almost entirely by 50% ethanol and it is the second major component obtained. It contains (Table I) somewhat more total sulfate and more *N*-sulfate groups (as indicated by the indole hexosamine reaction), and less *N*-acetyl groups than the Heparitin 0.9 fraction. About 30% of the total sulfate groups are *O*-sulfate*.

Heparitin 1.4. This fraction was completely extracted by 50% ethanol from the column-eluate fraction. This again is one of the major fractions and its properties (Table I) continue to resemble increasingly those of heparin. The content of total sulfate and *N*-sulfate groups is relatively high and that of *N*-acetyl groups low. About 40% of the total sulfate groups are *O*-sulfate*. The D-glucuronic acid content is lower than that of the previously described fractions, and therefore the L-iduronic acid content is higher. The yield of this fraction was much lower when the CPC method was used.

In general, it should be noted that as the content of sulfate groups increases, the orcinol value, optical rotation, and the proportion of D-glucuronic *vs.* L-iduronic acid decrease. As the content of *N*-sulfate groups increases, as indicated by the indole hexosamine reaction, that of the *N*-acetyl groups naturally decreases. The 2.0M-

*This value is only approximate as it is based on the difference between the indole and total sulfate groups analyses

sodium chloride fraction is evidently heparin, as shown by the analytical results and electrophoretic behavior. It constitutes about 40% of the total GGS present in the crude byproduct material. It should be emphasized very strongly that the heparitin fractions, though their properties show a drift toward heparin-like properties, are quite distinct. There is no gradual transition of the properties from the nonsulfated fraction to heparin. This is particularly apparent for the sulfate groups content (17% for the heparitin fraction containing the highest proportion and 28% for heparin), the orcinol values (which are 18% and 8%, respectively), the very low *N*-acetyl groups content of heparin and the much lower percentage of D-glucuronic acid in heparin as compared to that of the nearest heparitin fraction.

A comparison of the results of Table I with those of Fig. 1 shows a good correlation between the sulfate group content of fractions and the electrophoretic behavior. It is also obvious that considerable overlap between some of the heparitin fractions and the chondroitin sulfates occurs in the pyridine–formic acid buffer and somewhat less so in calcium acetate.

Direct isolation from beef lung. — The heterogeneity of the heparitin sulfate preparations described in the preceding paragraphs raised the obvious question whether this was a characteristic of the GGS obtained from this particular tissue source or whether some of the variations could be artifacts of the commercial preparative method. Therefore, the GGS was isolated directly from fresh beef lung as follows: The major cartilaginous portions of fresh lungs, obtained from a slaughter house, were removed, and the rest ground and dried. The acetone powder (1053 g) obtained was digested with papain, and then with Pronase. The mixture was centrifuged and the small residue discarded. The supernatant was dialyzed for one day and 10% CPC (100 ml per 3 liters) was added. The suspension was kept at room temperature for one day, centrifuged, the precipitate washed twice with 95% ethanol saturated with sodium chloride, and then dried in a desiccator over phosphorus pentaoxide to give a material (7.6 g) having a uronic acid content of 17% (carbazole reaction). The crude GGS was adsorbed on a column of AG 1 × 8 and eluted into fractions with 0.5M (65 mg), 0.9M (315 mg), 1.2M (535 mg), 1.4M (465 mg), 1.6M (685 mg), and 2.0M sodium chloride (573 mg). Dermatan sulfate, in addition to heparitin sulfate, was present in the 1.4, 1.6, and 2.0M-sodium chloride eluates, as shown by electrophoresis and by the presence of D-galactosamine. These fractions were therefore individually treated with Benedicts reagent[35] to precipitate the dermatan sulfate, which was isolated (732 mg consisting mainly of dermatan sulfate). The material in the supernatants was isolated as described previously[16].

Fractions derived from the 1.6M and 2.0M-sodium chloride eluates contained mainly heparin, as shown by electrophoresis and analysis. From 1 kg of dried lung, approximately 534 mg of total heparitin sulfate, 410 mg of heparin, and 730 mg of dermatan sulfate were obtained Relatively small amounts of other chondroitin sulfates were also present.

Analysis of the heparitin sulfate fractions (Table II) showed that the material derived from the 0.5M-sodium chloride eluate was still fairly impure and electro-

TABLE II

HEPARITIN SULFATES ISOLATED FROM BOVINE LUNG

Properties	*Fractions eluted with sodium chloride of molarity*			
	0.5	*0.9*	*1.2*	*1.4*
Yield[a] (mg)	11	112	198	218
(%)	1	21	37	41
Uronic acid residues (%)				
Carbazole test	12	42	46	41
Orcinol test	14	27	26	18
Sulfate groups[b] (%)		9.5 (0.7)	14.0 (1.0)	19.1 (1.3)
Acetyl groups[b] (%)		4.8 (0.80)	3.9 (0.65)	3.7 (0.56)
Hexosamine residues[b] (%)				
Total		25	25	26
Indole test		10 (0.40)	20 (0.80)	24 (0.94)
Free amino groups[c]		5.0	3.8	6.4
Optical rotation $[\alpha]_D^{25}$				
(*c* 1.0, water) (°)	+13	+68	+71	+73
D-Glucuronic acid residues[d] (%)		75		70

[a]Percent of total heparitin sulfate isolated [b]In parentheses, molar ratio to total hexosamine residues [c]Percent of total amino groups which are unsubstituted. [d]Percent of total uronic acid residues The difference from 100% is L-iduronic acid. Not corrected for variable loss during hydrolysis.

phoresis in calcium acetate revealed 3 bands; however, the major band moved at the same rate as that of the 0.5M sodium chloride fraction of Table I and disappeared after treatment of the mixture with a specific heparitinase[36]. The other bands were not affected by this enzyme and insufficient material was available for further purification. The electrophoretic mobility of the other eluate fractions was identical to that of the corresponding fraction from the heparin byproduct preparation.

Comparison of the results of Table II with those of Table I shows that the distribution of heparitin sulfate fractions obtained when using fresh lung and a mild isolation procedure was very similar to that obtained when using the commercial heparin byproduct material.

Isolation from beef aorta. — Owing to the variety of proteoglycans and polysaccharides present in this tissue, such as hyaluronic acid, chondroitin 4-sulfate, dermatan sulfate, and heparitin sulfate[37,38], this tissue is a rather poor source for the preparation of heparitin sulfate, which is only a minor component. Fresh aorta, obtained from a slaughterhouse, was cleaned of fat and adventitia, ground, and dried. The acetone powder (463 g) was digested with papain, the insoluble material removed by centrifugation, and the total GGS precipitated with CPC (ppt. I). In view of the problems found in the precipitation of low-sulfated heparitin from urine[39], the supernatant from the CPC precipitation (3.5 l) was dialyzed for one day. A 10% solution of CPC (50 ml) was added to the nondialyzable portion, the suspension kept for 24 h at room temperature, and the precipitate collected by centrifugation (ppt. II) Each precipitate (I and II) was dissolved in 2M potassium chloride and reprecipitated

with ethanol–acetic acid[30]. The material obtained was treated with Pronase, ethanol (3 vol.) was added, and the suspensions kept for 24 h at 5°, and then centrifuged. The precipitates were washed with ethanol and dried. The material derived from CPC-ppt. I weighed 763 mg and that from CPC-ppt. II 3.73 g; thus the larger amount of material had not precipitated directly with CPC. Both fractions contained about 25% of uronic acid (carbazole method). The CPC-ppt. I was fractionated with ethanol as previously described[40], and all fractions were found to contain D-galactosamine as the only amino sugar. The material precipitated by 20% ethanol (165 mg) appeared to be dermatan sulfate as indicated by electrophoresis, analysis, and relative resistance to testicular hyaluronidase. The material precipitated by 35% ethanol (526 mg) appeared to be a mixture of dermatan sulfate and chondroitin 4-sulfate. The 60%-ethanol fraction was mainly chondroitin 6-sulfate. No heparitin sulfate could be detected in any of these fractions.

Material derived from the dialyzed supernatant (CPC-ppt. II) was also fractionated with ethanol. Major amounts of material were obtained at 20%, 35%, and 65% ethanol. The 20%-ethanol fraction (1.42 g) contained D-glucosamine and D-galactosamine and had a fairly low uronic acid content. This material was therefore further purified on a column of AG 1 × 8 as described in previous paragraphs. The 0.5M-sodium chloride eluate contained only hyaluronic acid (10 mg) as shown by analysis, electrophoresis, and digestion with testicular hyaluronidase. The 0.9M-sodium chloride eluate contained, D-glucosamine and D-galactosamine. Electrophoresis in calcium acetate (Fig. 2) showed the presence of heparitin sulfate*, dermatan sulfate, and chondroitin 4- or 6-sulfate or both. The same results were obtained with the 1.2M-sodium chloride eluate, whereas the 1.4M- and 2.0M-sodium chloride eluates contained dermatan sulfate and chondroitin 4- or 6-sulfate, or both (Fig 2). In order to remove the dermatan sulfate component from the fractions containing heparitin sulfate, the 0.9M- and the 1 2M-sodium chloride eluates were combined and treated with Benedict reagent[35] Dermatan sulfate was obtained in a yield of 115 mg. The supernatant of the Benedict reagent treatment contained mainly heparitin sulfate and some chondroitin 4- or 6-sulfate, or both as shown by electrophoresis (Fig. 2). This material was therefore digested with testicular hyaluronidase[34] and the resistant heparitin sulfate isolated by precipitation with ethanol (2 vol.) The material obtained (35 mg, A-1, Table III) contained D-glucosamine as the only amino sugar, and moved on electrophoresis in calcium acetate at the same rate as that of the low-sulfated heparitin standard (Fig. 2). As some of the chondroitin sulfate was removed by hydrolysis, exact amounts of each GGS were difficult to calculate. However, the 20%-ethanol fraction of the CPC-ppt II contained approximately 20% of heparitin sulfate, 50% of dermatan sulfate, 25% of chondroitin 4- or 6-sulfate, and a small proportion of hyaluronic acid. It should also be pointed out that in our

*The 0.9M-sodium chloride fraction of the heparin byproduct material (Fig. 1 and Table I) was used as standard as it is the best to separate from the chondroitin sulfates. It must be kept in mind that more highly sulfated heparitin, if present, would migrate somewhat faster (Figs 1 and 4)

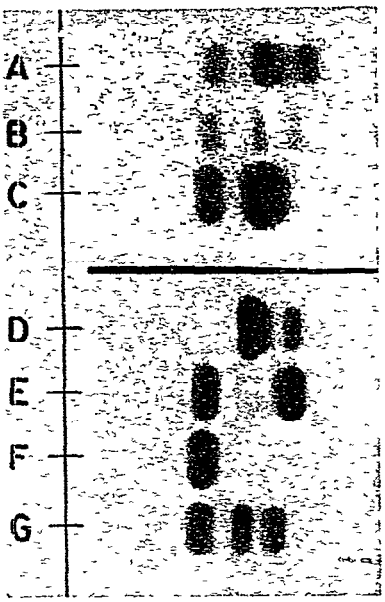

Fig 2 Electrophoresis patterns of GGS isolated from beef aorta. in 0.2M calcium acetate. A, 0.9M- and 1.2M-sodium chloride eluate fractions B, Standards of. heparitin sulfate (0.9M fraction of the heparin byproduct material), dermatan sulfate and chondroitin 4-sulfate in order of increasing migration rate. C, 0.9M-sodium chloride eluate fraction treated with testicular hyaluronidase. D, 1.4- and 2.0M sodium chloride fractions. E, Supernatant of Benedict solution treatment of the 0.9M-sodium chloride fraction. F, Fraction A-1. G, Standards as in B.

experience, at least, some material was invariably lost on ion-exchange chromatography. This was quite variable, but as much as a 25% loss did occur at times.

The 35%-ethanol fraction (1.89 g) of CPC-ppt. II contained about equal amounts of D-glucosamine and D-galactosamine. It was further fractionated on a column of AG 1 × 8. Significant amounts of material were obtained in the 0.5M, 0.9M, 1.2M, 1.4M, and 2.0M-sodium chloride eluates. The 0.5M-sodium chloride eluate contained 110 mg of material consisting entirely of hyaluronic acid as shown by electrophoresis, analysis, and digestion with testicular hyaluronidase. The 0.9M-sodium chloride fraction (203 mg) contained D-glucosamine as the only amino sugar. Electrophoresis indicated the presence of hyaluronic acid (or a nonsulfated heparitin) and of heparitin sulfate. This fraction was therefore treated with testicular hyaluronidase (Sigma, 750 units/mg) as described[34]. An enzyme-resistant GGS was isolated by precipitation with ethanol (2 vol.). Analyses of this material (A-2) obtained in a yield of 142 mg are shown in Table III and electrophoresis in Fig. 3. The 1.2M-sodium chloride eluate fraction (105 mg) contained D-glucosamine and D-galactosamine in a ratio of 2:1. Electrophoresis showed the presence of heparitin sulfate and chondroitin 4- or 6-sulfate. This mixture of GGS was therefore hydrolyzed with testicular hyaluronidase, as just described and the resistant material isolated. Analyses of this compound (A-3) obtained in a yield of 77 mg are shown in Table III and electro-

TABLE III

HEPARITIN SULFATES ISOLATED FROM BEEF AORTA

Properties	*Fractions*		
	A-1	*A-2*	*A-3*
Yield[a] (%)	14	56	30
Uronic acid residues (%)			
Carbazole test	34	41	40
Orcinol test	22	24	23
Sulfate groups[b] (%)	5.5	6.6 (0.5)	8.0 (0.6)
Acetyl groups[b] (%)		5 2 (0 83)	5.8 (0.89)
Hexosamine residues[b] (%)			
Total		26	27
Indole test	10	12 (0.46)	12 (0.45)
Free amino groups[c]		3.5	
Optical rotation $[\alpha]_D^{25}$ (*c.* 1 0, water) (°)	+68	+60	+72
Glucuronic acid residues[d] (%)		80	85

[a]Percent of total heparitin sulfate isolated [b]In parentheses, molar ratio to total hexosamine residues. [c]Percent of total amino groups which are unsubstituted. [d]Percent of total uronic acid residues. The difference from 100% is L-iduronic acid. Not corrected for variable loss during hydrolysis.

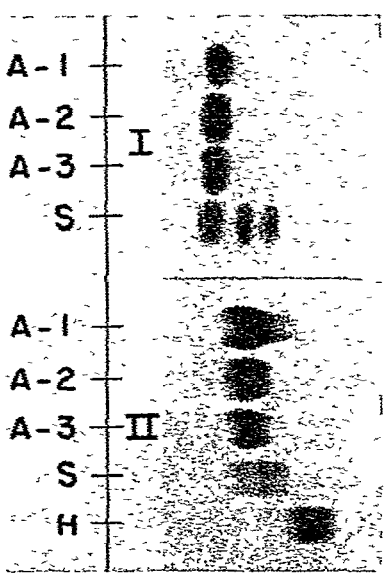

Fig. 3. Electrophoresis patterns of heparitin sulfate fractions isolated from beef aorta. I, In 0 2M calcium acetate, II, In pyridine–formic acid buffer, Microphor apparatus. Fractions A-1, A-2, A-3, see text. S, Standards of heparitin sulfate (see Fig. 2), dermatan sulfate, and chondroitin 4-sulfate in order of increasing migration rate. H, Standard of heparin (N. B Co)

phoresis in Fig. 3. The analytical data (Table III) quite clearly show that the aorta heparitin sulfate is much less heterogenous than that obtained from lung (commercial or fresh). The fractions differ little from each other, particularly in the content of sulfate groups and in the indole hexosamine test, both of which are relatively low. The D-glucuronic acid content is high as compared to that of L-iduronic acid. All these results resemble those of the 0.9M-sodium chloride eluate of the heparin byproduct material. The difference between fractions which permit fractionation resides probably in the molecular size, as will be described later. Despite considerable effort to find very low or high sulfated fractions which may be hidden by the other GGS present, none could be detected. Electrophoresis of the 1.4M-sodium chloride eluate fraction (465 mg) showed the presence of a small proportion of heparitin sulfate migrating like the "standard" and a large proportion of dermatan sulfate and chondroitin 4- or 6-sulfate. We were unable to isolate the small fraction of heparitin by either chromatography, hyaluronidase treatment, or ethanol fractionation. The 2.0M-sodium chloride eluate fraction (265 mg) contained D-galactosamine as the only amino sugar, and electrophoresis showed the presence of dermatan sulfate and chondroitin 4- or 6-sulfate in a ratio of 1:2. The approximate overall composition of the 35%-ethanol fraction of CPC-ppt. II was 22% of heparitin sulfate. 10% of dermatan sulfate, 50% of chondroitin 4- or 6-sulfate, or both and 18% of hyaluronic acid.

The 60%-ethanol fraction of CPC-ppt. II contained D-galactosamine as the only amino sugar and was completely digested by testicular hyaluronidase. The i.r. spectrum indicated the presence of both chondroitin 4- and 6-sulfate.

Isolation of heparitin sulfate from amyloid liver. — A portion of liver was obtained at autopsy from a patient with amyloidosis. The clinical diagnosis was verified by histological sections of liver and spleen which amyloid deposits stained characteristically.

An acetone powder of the liver was prepared as previously described, dried (360 g), and then digested with papain The solid residue was removed by centrifugation and saved. The GGS was precipitated from the supernatant with ethanol (2 vol.), the suspension was kept in the refrigerator for one day and centrifuged. This precipitate was redissolved in 5% sodium acetate (300 ml, 0.5M in acetic acid) and stirred with an equal volume of 4.1 chloroform–pentanol. The suspension was centrifuged, the precipitate discarded, and ethanol (2 vol.) added to the supernatant which was kept in the refrigerator for one day. The precipitate was centrifuged off, washed with ethanol, and dried (12.33 g).

The residue not digested by papain was suspended in 0 1M hydrochloric acid (500 ml), pepsin (400 mg, Difco 1:10,000) added, and the suspension kept for 24 h at 37°. The pH was readjusted to 1.0 and incubation continued for 6 h. The suspension was brought to a pH of 7.0, trypsin (200 mg, Difco 1:250) added, and incubation continued for 24 h at 37°. A small amount of insoluble material was removed by centrifugation and ethanol (2 vol.) added to the supernatant. The precipitate was isolated as just described (1.1 g). As it is possible that the papain-resistant residue contains GGS different from those of the papain digest, it was processed separately.

Each of the crude GGS fractions were treated with Pronase, a small amount of Lloyd's reagent was added to each digest, the suspension stirred and centrifuged, and the GGS in the supernatants precipitated with ethanol. The yield of GGS from the papain digest (L-1) was 4.2 g and from the papain residue (L-2) 0.6 g. These fractions still contained only about 13% uronic acid (carbazole reaction). Therefore, the crude GGS were further purified on columns of AG 1 × 8. The following fractions were obtained from the papain digest (L-1): The 0.5M-sodium chloride eluate in this case contained only insignificant amounts of material. The 0.9M-sodium chloride eluate appeared contaminated with protein and was therefore treated with 10% trichloroacetic acid to give a GGS (65 mg; L-1-0.9) containing D-glucosamine as the only amino sugar (Table IV, Fig. 4). The 1.2M-sodium chloride eluate was also treated with trichloroacetic acid and the purified GGS (105 mg; L-1-1.2) again contained only D-glucosamine as amino sugar (Table IV); the electrophoretic migration in calcium

TABLE IV

HEPARITIN SULFATES ISOLATED FROM AMYLOID LIVER

Properties	*Fractions*				
	L-1-0.9	*L-1-1.2*	*L-1-1.4*	*L-2-0.5*	*L-2-0.9*
Yield[a] (%)	18	28	32	15	7
Uronic acid residues (%)					
Carbazole test	33	42	40	42	39
Orcinol test	19	18	20	25	26
Sulfate groups[b] (%)	12.0 (1.0)	14.0 (1.0)	13.0 (1.0)	4.0 (0.3)	10
Acetyl groups[b] (%)		4.0 (0.64)	3.8 (0.65)	4.8 (0.82)	
Hexosamine residues[b] (%)					
Total	22	26	23	23	
Indole test	12 (0.55)	18 (0.70)	12 (0.52)	20 (0.87)	16
Free amino groups[c] (%)		6.0	2.0	42.0	40.0
Optical rotation $[\alpha]_D^{25}$					
(*c.* 1.0, water) (°)		+70	+60	+75	+60
Glucuronic acid residues[d] (%)		70	80	80	

[a]Percent of total heparitin sulfate isolated. [b]In parentheses, molar ratio to total hexosamine residues. [c]Percent of total amino groups which are unsubstituted. [d]Percent of total uronic acid residues. The difference from 100% is L-iduronic acid. Not corrected for variable loss during hydrolysis.

acetate was identical to that of L-1-0.9. The 1.4M-sodium chloride eluate contained about 10–15% of chondroitin 4- or 6-sulfate as indicated by electrophoresis, and in agreement with this, 10–20% of the total hexosamine was D-galactosamine. Digestion with testicular hyaluronidase removed all the chondroitin sulfate, and the reisolated GGS (L-1-1.4; 120 mg) contained D-glucosamine as the only amino sugar (Table IV, Fig. 4). The 2.0M-sodium chloride eluate contained 60 mg of material consisting mostly of dermatan sulfate; only a small amount of heparitin sulfate was present.

The following fractions were obtained from column chromatography of the papain residue (L-2): The 0.5M-sodium chloride eluate contained 57 mg of material

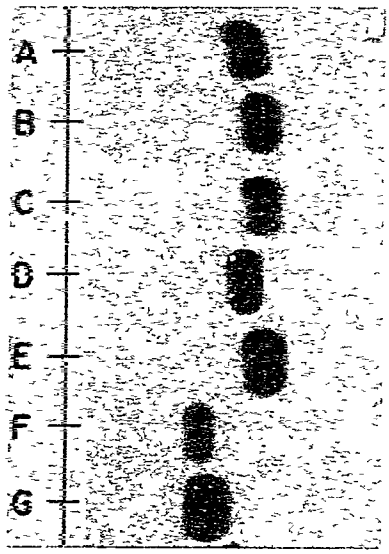

Fig. 4. Electrophoresis patterns of heparitin sulfate fractions isolated from amyloid liver in 0 2M calcium acetate. A, Fraction L-1-0.9; B, fraction L-1-1.2; C, fraction L-1-1.4; D, heparitin sulfate standard, (0.9M fraction of the heparin byproduct material); E, heparitin sulfate standard (1.2M fraction of the heparin byproduct material), F, fraction L-2-0 5; G, fraction L-2-0 9.

(L-2-0.5); D-glucosamine was the only amino sugar component (Table IV); half of it having a free amino group and, consequently, the sulfate group content being quite low and representing mostly *O*-sulfate groups. The remaining hexosamine residues appear to be *N*-acetylated. On electrophoresis in calcium acetate L-2-0.5 migrated slower than the heparitin sulfate standard (Fig. 4). The 0.9M-sodium chloride eluate contained 25 mg of material (L-2-0.9); D-glucosamine was the only amino sugar component (Table IV), a substantial amount of it having a free amino group but more being *N*-sulfated than in the 0.5M-sodium chloride eluate (Fig. 4). The 1.2M-sodium chloride eluate contained 10 mg of material consisting mostly of chondroitin 4- or 6-sulfate. No material was obtained at higher sodium chloride concentrations.

Therefore, the following approximate distribution of GGS occurs in amyloid liver: 80% of heparitin sulfate, 12% of dermatan sulfate, and 8% of chondroitin 4- or 6-sulfate. With the exception of the fractions containing free amino groups, the others show fairly little heterogeneity and are similar to the 1.2M-sodium chloride eluate of the heparin byproduct material (Table I). The presence of a substantial proportion of free amino groups in the L-2 fractions is unusual and may either bear a relation to amyloidosis or be a result of the isolation procedure, which involved in this case a pepsin digestion at pH 1.0 for 48 h; however, these conditions did not lead to extensive *N*-desulfation when tested on standard heparin samples.

Heparitin sulfate from the urine of a patient suffering from Sanfilippo's syndrome. — As these patients excrete heparitin sulfate in large amounts and other GGS in only small quantities[41], purification is relatively simple. The patient showed all the clinical

symptoms of Sanfilippo's syndrome and excreted from 100–120 mg of uronic acid per gram of creatinine[39]. Most of this was heparitin sulfate which was isolated from urine as described previously[41]. The GGS (100 mg) was fractionated on a column of AG 1 × 8, material being eluted only with 0.9M, 1.2M, and 1.4M sodium chloride. The overall yield was rather low, owing to loss partly on the column and partly on dialysis. In contrast to the tissue GGS described in the preceding paragraphs, these urine fractions gave rather unsharp, broad peaks on elution from the resin. Considerable tailing occurred in the eluate with a given salt concentration to the next. On electrophoresis in calcium acetate or pyridine–formic acid buffer very little difference in migration rate between the 3 eluate fractions was observed; all fractions moved somewhat faster than the "heparitin sulfate standard". The properties of the fractions differed little (Table V), all fractions being fairly highly sulfated. The molecular weight was reported to be quite low[15] (see Discussion).

TABLE V

HEPARITIN SULFATES ISOLATED FROM THE URINE OF A PATIENT SUFFERING FROM SANFILIPPO'S SYNDROME

Properties	*Heparitin sulfate before fractionation*	*Fractions eluted with sodium chloride of molarity*		
		0.9	*1.2*	*1.4*
Yield (mg)	100	25	15	20
Uronic acid residues (%)				
Carbazole test	42	40	42	43
Orcinol test	19	17	16	17
Sulfate groups (%)	14.0	13.0	15.0	14.0
Acetyl groups (%)	5.7			3.8
Hexosamine residues (%)				
Total	21			
Indole test	15			
Optical rotation $[\alpha]_D^{25}$				
(*c* 1.0, water) (°)	+58			
D-Glucuronic acid residues[a] (%)	75			

[a]Percent of total uronic acid residues. The difference from 100% is L-iduronic acid.

Heparitin sulfate from human intestine. — This procedure was originally intended to isolate and characterize intestinal heparin for comparison purposes. Small intestine obtained at autopsy was ground and dried. The acetone powder (170 g) was carried through the same procedure for the isolation of GGS as just described for beef aorta. The CPC precipitate was fractionated with ethanol and 3 major fractions were obtained: The 20%-ethanol fraction contained 38 mg of dermatan sulfate as determined by analyses and electrophoresis; the 50%-ethanol fraction contained 7 mg of chondroitin 4- or 6-sulfate; and the 30%-ethanol fraction (15 mg) appeared to consist of a mixture of heparitin sulfate and dermatan sulfate as estimated by the

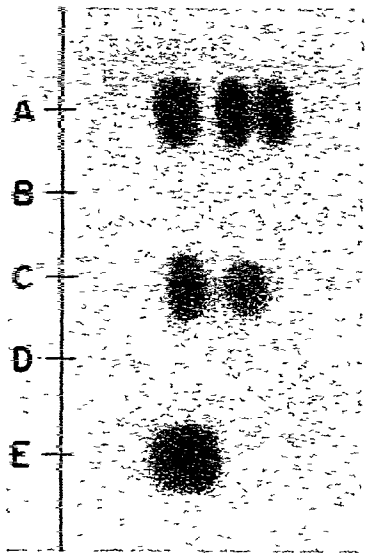

Fig 5. Electrophoresis patterns of heparitin sulfate fractions isolated from human intestine. in 0.2M calcium acetate A, Standards as in Fig 2 and 3; B, intestine heparitin fraction digested with specific heparitinase, C, intestine heparitin fraction; D, 1 2M heparitin fraction from heparin byproduct material treated with heparitinase; E, 1 2M heparitin fraction from heparin byproduct material.

electrophoresis pattern. This material was therefore digested with chondroitinase ABC (Miles Co.) and the resistant GGS reisolated. No change in electrophoresis pattern could be detected; two bands were still present, one migrating like the heparitin sulfate standard, the other like dermatan sulfate (Fig. 5). When the amino sugar of this material was examined only D-glucosamine could be detected, and when a portion was digested with specific heparitinase[36], both electrophoretic bands disappeared. This 30%-ethanol fraction contained 30% of uronic acid by the carbazole and 21% by the orcinol method Insufficient material for further characterization was available. Despite the fact that several species contain significant amounts of intestinal heparin[42,43], we could detect none in the human.

Molecular weight and content of protein-linkage regions. — The general chemical heterogeneity of heparitin sulfates obtained from different tissue sources raises the question of their molecular weight and of their content of neutral sugars of the protein-linkage region. The molecular weights were determined, in some cases, by sedimentation velocity and in other cases by a gel-filtration method[44,45] on Sephadex G-150. Viscosity and partial specificic volume determinations were also carried out on some samples (Table VI). The molecular weights vary over a considerable range, the lung-derived fractions having lower average values than those from other tissues, a finding also observed by others[6,45], and the amyloid heparitin fraction having a relatively high molecular weight. The results of the viscosity determination in general agree with the gel filtration and sedimentation data.

TABLE VI

MOLECULAR WEIGHTS AND PHYSICAL PROPERTIES OF HEPARITIN SULFATES

Heparitine sulfates	*Mol. wt.*[a]	*Mol. wt.*[b]	η[c]	$\bar{v}$[d]
Byproducts 0.5	6,000	6,000 ±500	0.34	0.56
Byproducts 0.9	10,600	10,600 ±1,000	0.52	0.53
Byproducts 1.2		11,500 ±500	0.45	0.45
Byproducts 1.4	11,600	14,000 ±500	0.38	0.50
Lung 0.9		8,500[e] ±1,500	0.46	
Lung 1.2		11,500[e] ±1,000	0.49	
Aorta A-2		22,000 ±500	0.53	
Aorta A-3		24,500[e] ±2,000		
Amyloid L-1-1.2		11,500 ±500	0.33	
Amyloid L-1-1.4		31,000 ±1,000	0.78	
Urine (Unfractionated)		<3,000[c]	0.10	

[a]Sedimentation velocity. [b]Sephadex G-150 chromatography of the sample in 0.2M sodium chloride in 10% ethanol. The column was calibrated with the 0.5 and 0.9 byproduct fractions and three fractions of chondroitin 6-sulfate with known molecular weights as standards. [c]Intrinsic viscosity. [d]Partial specific volume. [e]These fractions showed fairly broad elution peaks indicating polydispersity. As the selection of elution volume is somewhat arbitrary, a range of values is indicated.

Neutral sugars of the linkage region. — D-Galactose and D-xylose were determined, as described by Cifonelli *et al.*[46], except that paper chromatography in 7:1:2 propanol–ethyl acetate–water and densitometry was used for quantitation.

TABLE VII

CONTENT OF NEUTRAL SUGAR RESIDUES OF THE LINKAGE REGION OF HEPARITIN SULFATES

Heparitin fraction	D-*Galactose*[a]	D-*Xylose*[a]
Aorta (A-2)	0.033 (1.5)	0.020 (1.0)
Amyloid (L-1-1.4)	0.034 (2.1)	0.018 (1.2)
Byproduct (1.2)	0.016 (0.7)	0.007 (0.4)
Byproduct (0.5)	0.016 (0.4)	0.005 (0.2)
Lung (0.9)	0 040 (0.8)	0.010 (0.3)

[a]As moles per mole of total hexosamine residues. In parenthese, approximate number of D-galactose or D-xylose residues per chain of polysaccharide based on the molecular-weight values of Table VI.

The results for representative heparitin sulfate fractions are shown in Table VII. When the results of the molecular-weight determinations are taken into account, less than 2 D-galactose residues per polysaccharide chain are present for all of the fractions, except the amyloid heparitin. The byproduct heparitin fractions, which also have a fairly low molecular weight, have considerably less D-galactose residues per chain, *i.e.* they have fewer chains with an intact linkage region. The D-xylose content, in general, is about one half that of the D-galactose, which is in agreement with the postulated structure of the linkage in most proteoglycans.

DISCUSSION

An understanding of the role of compounds of biological importance requires a knowledge of the detailed structure, distribution within the same organism or among different organisms, variations in composition or structure when isolated from different sources, quantitative or qualitative changes during disease processes, and identity in the sense of dealing with a single species rather than a mixture of related compounds. This last point, though relatively easily resolved with low-molecular-weight materials or proteins becomes much more difficult to establish for some polysaccharides. The heterogeneity of animal glycosaminoglycuronans in particular, makes identification difficult. This paper attempts to clearly identify heparitin sulfate and, in addition, describes significant differences of structure when this polymer is isolated from various sources. No general method could be used because of the greater heterogeneity of the heparitins or the presence of other GGS in some sources as compared to others. It should be noted that polysaccharides which can definitely be assigned to the family of heparitin sulfates are distributed over a fairly wide range of fractions extracted by various concentrations of ethanol, column eluates, and CPC precipitates and that they may have quite variable electrophoretic mobilities. Identification by position of peaks of elution or electrophoresis pattern, as is frequently done when only small amounts are available, should therefore be interpreted with caution.

Considerable effort has been made here to detect all heparitin sulfate fractions in a given source, including those present in small amounts which may be hidden in mixtures with other GGS and difficult to separate.

As seen in Table I, the heparitin sulfate isolated from the Upjohn heparin byproduct material was subfractionated into several fractions varying in particular in sulfate content, from essentially no sulfate to 17%. This is accompanied by variations in the proportion of *N*-acetyl to *N*-sulfated D-glucosamine and of D-glucuronic to L-iduronic acid, which reflects the arrangement of heparin-like blocks to nonsulfated blocks in the polymer.

The 0.5 fraction, though obtained in a low yield, is of considerable interest as it contains practically no sulfate groups, is almost fully *N*-acetylated, and contains mainly D-glucuronic acid with only 10% of L-iduronic acid. Though the molecular weight is fairly low (Table VI), this fraction is still of considerable size (15 disaccharides), and its very presence supports the hypothesis of a block type rather than an alternating structure for heparitin sulfate[5,6,15].

The two major fractions (0.9 and 1.2) conform to the general description of heparitin sulfate. They contain roughly equivalent amounts of *N*-acetyl and *N*-sulfate groups, but the total of their molar ratios to hexosamine residues (Table I) is higher than 1.0; this discrepancy has been observed previously[14]. The *O*-sulfate groups content is about 30%, and represents the upper limit of any possible disulfated repeating unit. The third major fraction (1.4) contains considerably more total sulfate, more *N*-sulfate and less *N*-acetyl groups, and more L-iduronic acid residues; about 40% of the total sulfate groups are *O*-linked.

The electrophoresis patterns (Fig. 1) demonstrate the individuality of the fractions quite clearly. That is, the intermediate fractions are certainly not mixtures of high- and low-sulfated polymers, though some heterogeneity within each fraction cannot be excluded. The pyridine–formic acid buffer worked best for separation on the basis of charge, while calcium acetate seemed best for the separation of different types of GGS from each other (Fig. 2).

It is curious that the uronic acid groups content, as measured by the orcinol reaction, appears to decrease as the L-iduronic acid groups content of the fractions increases. This is particularly obvious when heparin is present, and it is exactly the opposite effect one would have predicted from data obtained with dermatan sulfate and with L-iduronic acid itself. It should also be pointed out that the presence of L-iduronic acid in heparitin sulfate, first reported by Cifonelli *et al.*[47], certainly seems real; however, the actual proportions are rather difficult to estimate. The method used in the present paper[26] is a considerable improvement, but the total yields of uronic acid residues after hydrolysis are still quite low, and as the rate of decomposition of L-iduronic and D-glucuronic acid are also different and not easily corrected, it is uncertain whether the ratio of the uronic acid residues in the original polymer is accurate.

Our data for the heparin byproduct material, obtained originally from beef lung, agree very well with those of Cifonelli *et al.*[14]. They do not agree with those of Dietrich *et al.*[31] who used the same material and found a substantial proportion of a low-sulfated *N*-acetylated fraction similar to the Heparitin 0.5 described here, but the other polysaccharides isolated containing no *N*-acetyl groups and being essentially heparin-like except for their low anticoagulant activity.

The heparitin sulfates isolated directly from lung (Table II) are very similar to those obtained from the commercial preparation. The nonsulfated fraction appears to be present but was difficult to purify adequately.

Beef aorta contains 4 or 5 polysaccharides, namely chondroitin 4-sulfate, dermatan sulfate, hyaluronic acid, heparitin sulfate[37,38] and, as indicated here, probably also some chondroitin 6-sulfate. Therefore, purification of an individual GGS was difficult to achieve. Isolation of heparitin sulfate *per se*, though a minor component, did not present special problems, but to ascertain the exact proportion of heparitin required a fairly laborious fractionation. It should be noted, in particular, that after precipitation with CPC of GGS from the papain digest, the heparitin sulfate was left in the supernatant. We had noticed previously[39] that during precipitation of GGS from urine with CPC or cetyltrimethylammonium bromide considerable losses of heparitin sulfate could occur when the urine was not dialyzed prior to precipitation. This appeared to be due to the low molecular weight or low sulfate content of the urinary material. In the case of the aorta polysaccharide, the fairly low sulfate content (see Table III) is probably responsible. In addition, the sulfate groups in heparitin sulfate do not seem to be evenly distributed throughout the molecule, as in other GGS, but appear to occur in blocks. As the arrangement of these groups may vary in different heparitins, anomolous behavior with CPC could be expected. In

general, the aortic heparitin sulfate is a minor component and shows little heterogeneity. Sulfate group values are quite low and in a narrow range. It is not clear why 3 distinct fractions were obtained, but variation in molecular weight (see Table VI) may be a factor. The data are similar to those for a single heparitin fraction obtained from human aorta by Knecht *et al.*[15] and Manley *et al.*[48].

The presence of mucopolysaccharides in amyloid deposits has been recognized for some time[49] and heparitin sulfate, in particular, has been isolated from a variety of deposits of both human and animal origin[10–12]. It should be noted that papain digestion alone did not liberate all the GGS from amyloidic liver. Of the total GGS obtained, 80% was heparitin sulfate, which is a much higher proportion of this compound than that isolated from any other tissue source. This in itself may speak for a more significant and specific role of GGS in the etiology of amyloidosis than generally assigned[50]. In addition, one of the fractions has a higher molecular weight (see Table VI) than any of the heparitins described here, and it also contains a full complement of the neutral sugars of the linkage region, implying that this material is most likely formed *in situ* and is quite different from the GGS accumulated in the mucopolysaccharidoses. The major fractions show little heterogeneity, except for size; they have an intermediate sulfate group content and resemble the lung Heparitin 1.2. The fractions (L-2-0.5 and L-2-0.9), which show a high content of free amino groups, may be quite significant, but this presence is difficult to interpret as *N*-sulfate groups may have been lost during the purification.

The heparitin isolated from the urine of a patient suffering from Sanfilippo's disease was included in this study mainly for comparison purposes. As described previously and confirmed here, it is of quite low molecular weight[15,45,51]. The fractions isolated have a fairly high content of sulfate groups and several fractions were obtained from the ion-exchange column, but no significant differences between material eluted at different sodium chloride concentrations could be detected.

The molecular weights of GGS are difficult to determine because of the high charge density and polydispersity. The agreement between such different methods as sedimentation velocity, light scattering, viscosity, and osmometry has not been good[52,53]. Determination of the molecular weights of heparitin sulfate presents an even more difficult problem, since considerable variation in charge distribution occur and the usual correction factors are difficult to apply. Though the gel-filtration method is not very accurate, it is easy to use, requires small amounts of material, and appears to be internally consistent. Three chondroitin 6-sulfate samples of known molecular weight and 2 heparitin sulfate samples, for which sedimentation velocity values had been obtained, were used to calibrate the column. When Kav (gel partition coefficient[44]) was plotted against log mol. wt. a straight line was obtained for these 5 compounds.

As can be seen in Table VI, considerable heterogeneity in size exist for the various heparitin sulfates, though the overall range is similar to that of other GGS. The Heparitin 0.5 (heparin byproduct) has the lowest molecular weight of the tissue materials, but, as it is nonsulfated, the number of repeating units is actually not much

smaller than in the other lung-derived fractions. This heterogeneity of size, associated with charge variations, is probably responsible for the difficulties experienced in isolation, separation from other GGS, and fractionation of the heparitin sulfates. The viscosity data agree with the molecular weights in some instances, but not in others. This had also been observed previously for other GGS[53].

In conclusion, the heparitin sulfates constitute a family of closely related polymers which differ in size and charge content as well as charge distribution. Aside from their composition and optical rotation they can be characterized by an *N*-acetyl value of more than 3%, a sulfate value of up to 20%, a D-glucuronic to L-iduronic acid ratio of more than 2, and a carbazole to orcinol ratio of less than 2. Of course, these criteria cannot be applied directly to metabolic fragments, as those obtained, for instance, from urine. The data indicate that individual chains are probably less heterogeneous than the complexity of the family would indicate. As the similarity to heparin has been stressed previously, perhaps it should be noted here that heparin is a distinct polymer and does not belong to the heparitins, even though it may contain a fair proportion of *N*-acetyl groups[46]. There is good evidence that the heparitin sulfates have a block-type structure[5,6], that is areas free or low in sulfate groups and containing *N*-acetyl-D-glucosamine units; areas intermediate in sulfate groups and *N*-sulfated-D-glucosamine units; and areas high in sulfate groups (probably similar to heparin) containing *N*-sulfate and *O*-sulfate groups. The proportions of these blocks seem to vary in the various polymers isolated from different sources. The heparitin sulfate isolated from umbilical cord appears to be similar to that from aorta[16] whereas that obtained from rat brain has a fairly high proportion of blocks containing *N*-acetylated residues[54]. The potential for structural variation in conjunction with differences depending on tissue source indicate that the heparitin sulfates have a quite specific role in an individual tissue.

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