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A SUBSTRATE FOR DIRECT MEASUREMENT OF L-IDURONIC ACID 2-SULFATE SULFATASE

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ABSTRACT

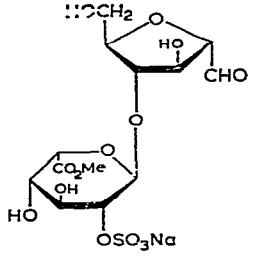
Commercially available sodium heparinate has been sequentially treated with methanolic 0.06M hydrogen chloride and nitrous acid. The nondegraded material was separated by gel filtration from the nonsulfated and monosulfated disaccharides produced. The latter ones, obtained in 10% yield, have been used as a substrate for the direct measurement of the enzyme L-iduronic acid 2-sulfate sulfatase present in human plasma and fibroblast homogenates. Studies of the kinetics and pH optimum of the enzyme, by use of plasma of a patient with mucolipidosis II, indicated an apparent K_m of 2.5mM and a pH optimum of 4.6–4.8. The levels of activity in normal plasma and plasma of a patient with Hunter's disease were found to be 20.4 ± 1.22 units (μmol sulfate/24 h/g protein) and 3.25 ± 0.35 units, respectively. In homogenates of cultured skin fibroblasts, the levels were 137.6 ± 10.7 units for normal controls and 6.4 ± 5.1 for patients with Hunter's disease. The plasma of two obligated heterozygotes gave intermediate levels of activity, whereas the plasma of two possible heterozygotes gave either intermediate levels or entirely normal levels of activity.

INTRODUCTION

The preparation of a suitable substrate for a direct and simple measurement of the enzyme L-iduronic acid 2-sulfate sulfatase, which is defective in Hunter's syndrome (mucopolysaccharidosis II), was studied.

L-Iduronic acid 2-sulfate residues occur in several glycosaminoglycans, such as heparin, heparan sulfate, and dermatan sulfate. These glycans, however, also contain sulfate groups located at other positions of the respective 2-amino-2-deoxyhexose moieties. Thus, in order to measure L-iduronic acid 2-sulfate sulfatase by direct assay of the cleaved sulfate groups, it is necessary to remove from the potential substrate all the unwanted sulfate residues. This paper describes (a) the method for the preparation of, presumably, the sodium salt of *O*-(methyl α-L-idopyranosyluronate 2-sulfate)-(1→4)-2,5-anhydro-D-mannose (**1**) from the commercially available sodium salt of heparin, and (b) the use of this substrate for the measurement of

L-iduronic acid 2-sulfate sulfatase in plasma samples and fibroblast homogenates of normal individuals, Hunter patients, and obligated or possible heterozygotes.



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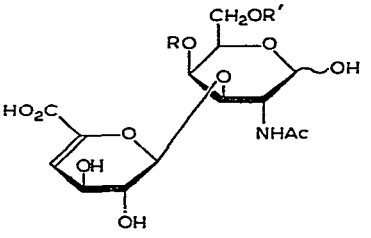

2 R = SO_3H, R′ = H
3 R = H, R′ = SO_3H
4 R = R′ = H

EXPERIMENTAL

Material. — The sodium salt of heparin (Grade B, CalBiochem, La Jolla, CA 92037) was analyzed for hexuronic acid by the method of Bitter and Muir[1] and for sulfate groups with the benzidine method as modified by Ginsberg and Di Ferrante[2]. The molar ratio of sulfate groups to hexuronic acid residues of the starting materials was 2.4:1.

Partial desulfation of heparin. — Methanolic HCl (0.06M) was prepared by the dropwise addition of acetyl chloride (2.5 ml) to ice-cold, absolute methanol (500 ml). Heparin sodium salt (2.5 g) was suspended into the solution (50 ml), and stirred for 24 h at room temperature in a closed container. The heparin was collected by centrifugation at 1500*g* for 10 min and resuspended in fresh methanolic HCl (50 ml). The procedure was repeated three times, each day new methanolic HCl being added and a small aliquot of the suspension (100 µl) taken to measure hexuronic acid residues and sulfate groups. After 96 h, the heparin was recovered from the suspension by centrifugation, washed several times with methanol, and dried with ether (1.66 g).

Degradation to disaccharides with nitrous acid. — The procedure used is a modification of that proposed by Lagunoff and Warren[3]. Aliquots (400 mg) of partially desulfated heparin were each dissolved in 10% acetic acid (40 ml) at 10°. After a sample (10 µl) was taken for hexuronic acid determination, sodium nitrite (1.4 g) was added with stirring. The production of anhydro-D-mannose residues during the reaction was followed with the indole–HCl reagent of Dische and Borenfreund[4] on samples (50 µl) taken every 10 min (Fig. 1). The reaction was considered to have reached completion when the molar ratio of anhydro-D-mannose to hexuronic acid reached a plateau at a value of ~0.9:1. At that time, methanol (40 ml) was added, and the mixture was evaporated to dryness with a rotary evaporator at 25°. The residue was washed twice with additional methanol (30 ml) and evaporated to dryness (1.49 g).

Separation of disaccharides from undegraded material. — Aliquots of the resulting powder, dissolved in water (2 ml), were applied to a first column (90 × 2.5 cm) of Biogel P-2 (50–100 mesh) packed and eluted with water. Fractions (4 ml) were collected and tested for hexuronic acid, anhydro-D-mannose, and sulfate residues. The peak closest to the void volume (Fig. 2), which contained undegraded material and sulfated disaccharides[5], was collected, lyophilized, and applied to a second column (90 × 2.5 cm) of Biogel P-2 (50–100 mesh), packed and eluted with 20mM NaCl. The fractions (4 ml) containing the material having the highest anhydro-D-mannose to hexuronic acid ratio (Fig. 3, Fraction II) were combined, desalted by gel filtration on the first column of Biogel P-2, and eventually lyophilized.

Purification of disaccharides by high-pressure, anion-exchange chromatography. — The desalted disaccharides were purified by high-pressure chromatography on an anion-exchange column (14 × 0.7 cm) of controlled-pore glass (QAE 250, AcO^-, Pierce Chemical Co., Rockford IL 61105). The column was eluted automatically with a gradient of 0.5M sodium acetate (200 ml, pH 4.6) in water (200 ml) at a flow rate of 2 ml/min. The elution was completed within 20 min, at which time the eluting sodium acetate solution had a concentration of 0.35M. The column was standardized with unsaturated disaccharide 4- and 6-sulfates [2 acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-(**2**) and 6-sulfates (**3**)] and with an unsaturated, nonsulfated disaccharide [2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose, **4**] (Miles Laboratories, Inc., Elkhart, Indiana 46514) prior to use. As anhydro-D-mannose prepared from pure 2-amino-2-deoxy-D-glucose (Pfizer & Co., Inc., New York, NY 10017, Lot 86197-05-EPD) also absorbs at 254 nm, a u.v. detector was used to monitor the elution of the standard unsaturated disaccharides and of the products of nitrous acid degradation (Fig. 4). The major peak eluted after 12.5 min was collected, concentrated, and then desalted by gel filtration on a Biogel P-2 column packed and eluted with water. The desalted material was lyophilized, analyzed, and stored as dry powder (0.24–0.30 g), and used for enzyme measurement.

I.r. spectra. — These spectra were recorded with a Beckman IR-9 spectrophotometer. An aliquot of desalted sample (10–20 μl) was applied to an Irtran plate, dried under a stream of N_2, and scanned at 200 cm^{-1}/min. The spectra obtained were compared with those of undegraded chondroitin 6-sulfate (Miles Laboratories, Inc.) and chondroitin 4-sulfate.

Collection of plasma and preparation of fibroblast extracts. — Venous blood (10 ml) was drawn from normal individuals, Hunter patients, and patients' parents, and (0.1 ml) 0.27M ethylenediaminetetraacetic acid was added to prevent clotting. Plasma was recovered by centrifugation for 10 min at 1500*g* at 5°, and each sample was dialyzed overnight at 5° against several changes of 0.15M NaCl (1 l each). The protein content of the retentate solutions was determined[6], and each sample was stored frozen until used.

Cultured skin-fibroblasts, derived from one or two 75-cm^2 plastic plates, were removed by trypsin treatment and collected by centrifugation[7]. The pellet was

suspended in 0.15M NaCl (1 ml) and treated by sonication for 20 sec, in order to disrupt the cells. The suspension was dialyzed overnight at 5° as described for the plasma. The retentate solution was clarified by centrifugation at 20,000*g* for 10 min at 5°; the protein content of the clear supernatant solution was measured, and the extract was kept frozen until used.

Enzyme assays. — These assays were performed in duplicate, as follows: The dry substrate (5 mg) was dissolved in 0.6M sodium acetate buffer (1 ml, pH 4.0) to obtain a 10mM concentration. An aliquot of this solution (50 µl) was added to dialyzed plasma (50 µl) or fibroblast extract (50 µl) in a plastic centrifuge tube (0.4 ml total volume, A. H. Thomas, Philadelphia, PA 19105). The final pH of the incubation mixture was 4.6 with plasma and 4.2 with fibroblast extracts.

Two control tubes were prepared simultaneously to the enzyme assays as follows: one contained only the substrate solution (50 µl), the other the enzyme preparation (either plasma or fibroblast extract) (50 µl) plus the acetate buffer (50 µl). The tubes were capped and immersed in a shaking water-bath for 24 h at 37°. At the end of the incubation, the contents of the two control tubes were combined (total volume 150 µl) and used as a blank. Acetate buffer (50 µl) was added to each enzyme assay (to achieve a total volume of 150 µl), and then to each tube a 20% solution of sulfosalicylic acid in water (20 µl) was added. The contents of the tubes were mixed with a Vortex agitator, and then centrifuged at 20,000*g* for 20 min at 5°.

In order to obtain accurate sulfate group determinations, it was important that the supernatant solutions were completely free of precipitated protein. Those derived from incubation mixtures containing fibroblast extracts could be quantitatively transferred to tubes containing 0.6M sodium acetate buffer solution (0.5 ml). However, those derived from incubation mixtures containing plasma had to be quantitatively transferred to another tube and centrifuged again for an additional 10 min before the clear supernatant could be combined with the 0.6M sodium acetate buffer solution (0.5 ml).

Barium chloride reagent[2] (200 µl) was added to each clear supernatant solution,

TABLE I

MOLAR RATIO OF SULFATE TO HEXURONIC ACID RESIDUES OF HEPARIN TREATED WITH METHANOLIC HYDROGEN CHLORIDE FOR VARIOUS LENGTHS OF TIME

Time (h)	*Experiment*	
	1	*2*
0	2.40:1	2.40:1
24	1.15:1	0.80:1
40	0.80:1	0.80:1
72	0.51:1	0.40:1
96	0.42:1	0.37:1
120	0.37:1	

and the tubes were at first cooled in ice for 15 min, and then warmed to 37° before the rhodizonate reagent (1.5 ml) and absolute ethanol (2 ml) were added. The content of the tubes was mixed, and then incubated for 20 min at room temperature in the dark. The absorbance of each sample was measured at 520 nm and subtracted from that of the corresponding blank solution. The various absorbance values obtained were compared with those of increasing amounts of inorganic sulfate (10–75 nmol), processed in a similar manner.

Properties of L-*iduronic acid 2-sulfate sulfatase.* — The properties of the crude sulfatase were studied with the substrate just described, and with plasma obtained from a patient affected by mucolipidosis II. The elevated plasma levels of this and other lysosomal enzymes occurring in the disease allow multiple measurements of a given enzyme with a single plasma aliquot.

RESULTS

Table I shows the effects of the treatment with methanolic hydrogen chloride on the molar ratios of sulfate to hexuronic acid residues of heparin. After 24 h, the ratio was ~1.0:1, and after 72–96 h, it decreased to 0.4–0.5:1. The residual sulfate residues were not removed, even after additional 24–48 h of treatment.

The partially desulfated polymer was cleaved into disaccharides with nitrous acid. A treatment lasting 20–25 min was sufficient to produce maximal deamination of 2-amino-2-deoxyglucose into anhydro-D-mannose, as indicated by the highest value of the molar ratio (0.95) of anhydro-D-mannose to hexuronic acid (Fig. 1). Longer treatment with nitrous acid caused a decrease of this ratio, probably because of polymerization of the disaccharides. In order to prevent the loss of anhydro-D-

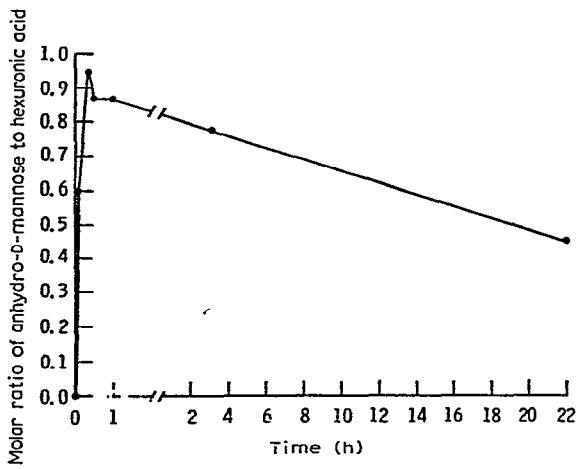

Fig. 1. Production of anhydro-D-mannose from partially desulfated heparin by nitrous acid treatment at 10°. Anhydro-D-mannose was measured with the indole–hydrochloric acid reagent of Dische and Borenfreund[4], and its production related to the amount of hexuronic acid present in the substrate. Maximal production of anhydro-D-mannose was achieved within 20 min, after which loss of anhydro-D-mannose might be due to polymerization of disaccharides produced.

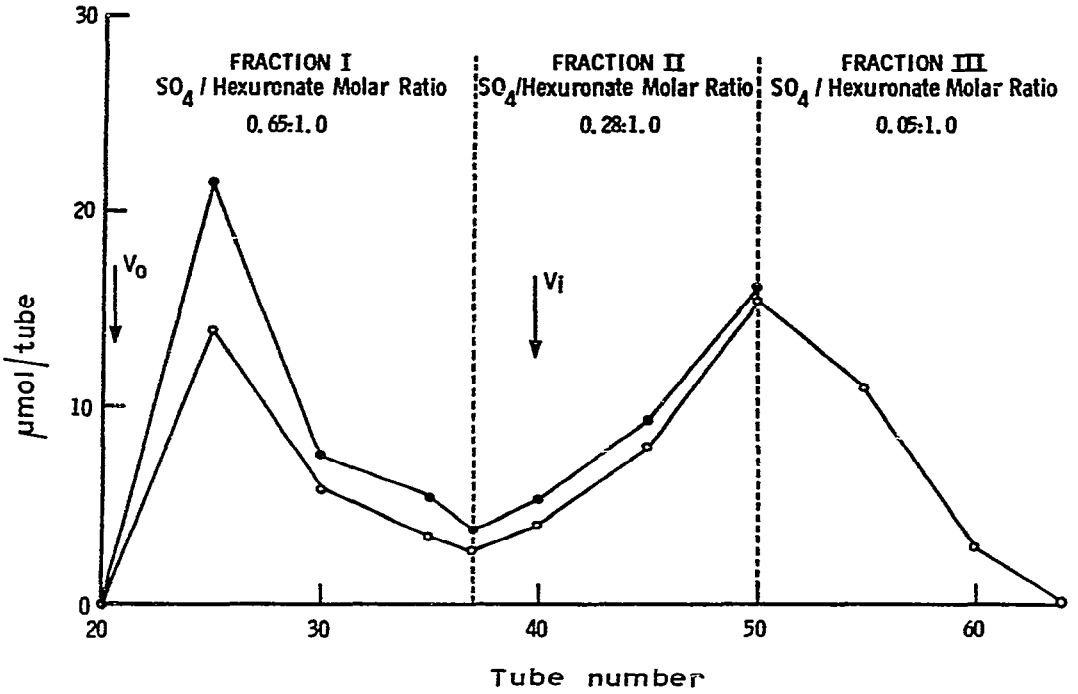

Fig. 2. Elution of the products after nitrous acid treatment of heparin sodium salt from a Biogel P-2 column, packed and eluted with water. With this system, undegraded material and sulfated disaccharides are excluded from the gel and eluted with the void volume. The material eluted was arbitrarily divided into 3 fractions, according to the molar ratio of sulfate to hexuronic acid residues. V_o and V_i are excluded and included volume, respectively. Hexuronic acid (●), anhydro-D-mannose (○).

mannose, the product had to be removed rapidly from the salt mixture produced during rotary evaporation and immediately applied to the column of Biogel P-2 for desalting. Fig. 2 shows the pattern of elution of the degraded material from this column packed and eluted with water. As noted previously[5], when Biogel P-2 is used with water, sulfated disaccharides are eluted with high-molecular-weight, undegraded material, instead of being retarded. The eluted material was tested for anhydro-D-mannose and hexuronic acid, and arbitrarily divided into 3 fractions according to the ratio of sulfate to hexuronic acid residues. Because of the presence of salt after tube 40, the sulfate content of fractions 2 and 3 very probably represents inorganic sulfate.

Fraction I was applied to Biogel P-2 packed with sodium chloride (20mM), and eluted with the same solution, in order to separate the disaccharides from the residual, undegraded material. The elution pattern obtained is shown in Fig. 3. The material present in Fraction II (tubes 35–40) had equimolar amounts of hexuronic acid and anhydro-D-mannose; after being desalted and lyophilized, it was further purified by anion-exchange, high-pressure chromatography. Fig. 4 shows the pattern of elution,

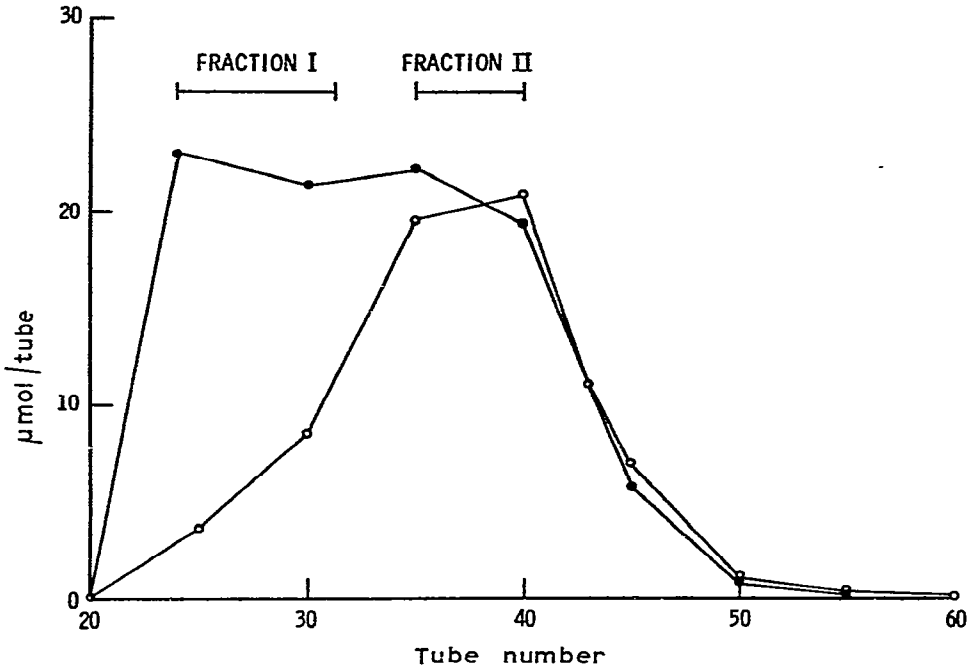

Fig. 3. Elution of the material from Fraction I of Fig. 2 from a Biogel P-2 column, packed and eluted with 20mM sodium chloride. With this system, sulfated disaccharides are retarded and eluted separately from undegraded material. The material eluted was arbitrarily divided into 2 fractions on the basis of the molar ratio of anhydro-D-mannose to hexuronic acid residues. Hexuronic acid (●), anhydro-D-mannose (○).

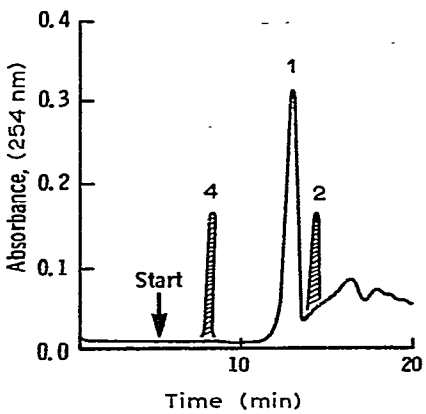

Fig. 4. High-pressure, liquid chromatography of sulfated disaccharide **1** on anion-exchange column of controlled-pore, glass beads. The column was eluted with a gradient of sodium acetate buffer (pH 4.6), and the effluent was monitored at 254 nm for anhydro-D-mannose and for standard unsaturated disaccharides **2** and **4**. The superimposed shaded areas represent the time of elution of nonsulfated (**4**), and 4-sulfated unsaturated disaccharide **2**.

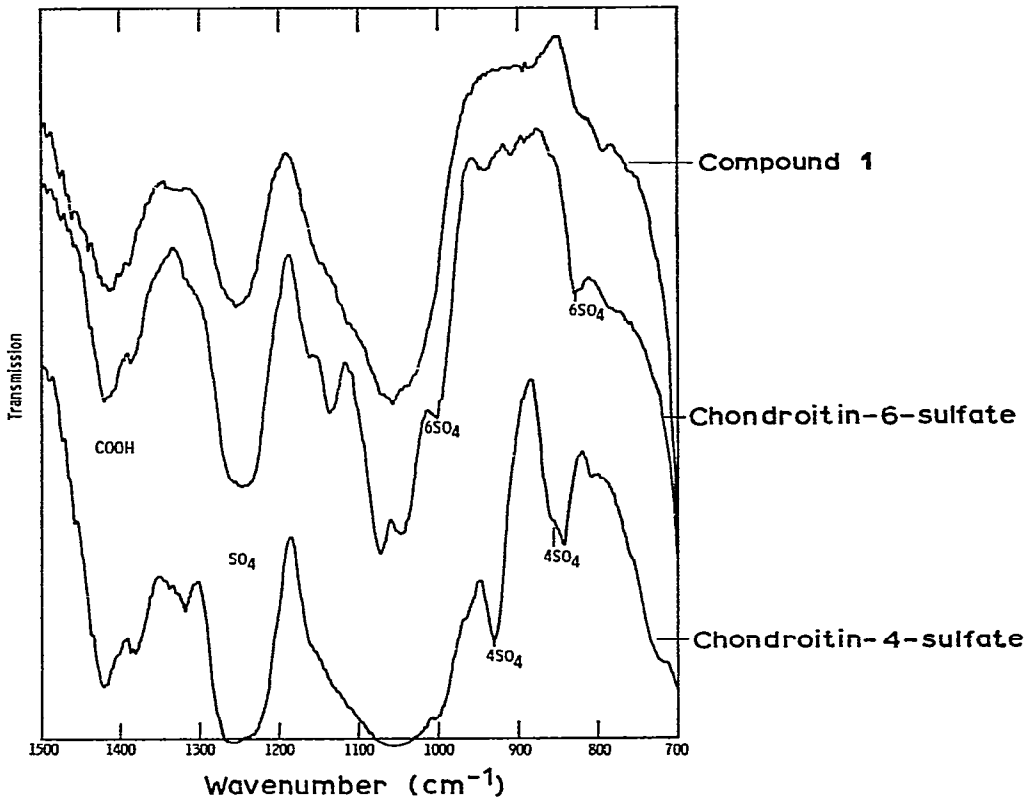

Fig. 5. I.r. spectra of chondroitin 4-sulfate, chondroitin 6-sulfate, and of the monosulfated disaccharide prepared (**1**).

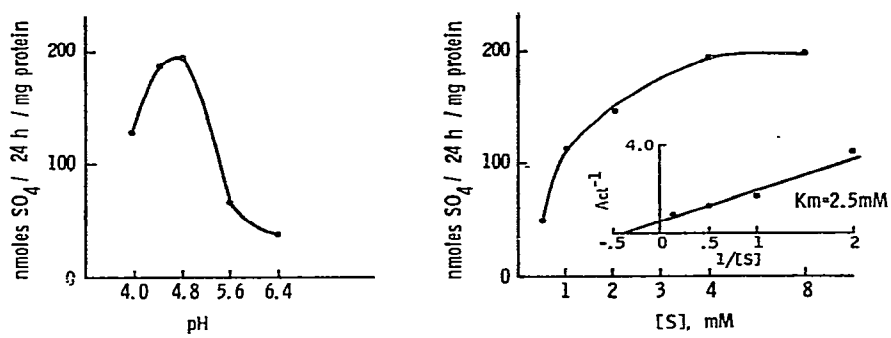

Fig. 6. Release of inorganic sulfate from **1** at different pHs when incubated with plasma of a patient with mucolipidosis II, and at different substrate concentrations.

as monitored with a u.v. detector. The major peak was recovered and lyophilized. It contained 36% of hexuronic acid, 31% of anhydro-D-mannose, and 15.4% of sulfate, in the molar ratio of 20:19:17. From 2.5 g of heparin sodium salt, 0.24–0.30 g (presumably of disaccharide **1**) was obtained.

Fig. 5 shows the i.r. spectrum of the substrate described here, compared with the i.r. spectra of chondroitin 4- and 6-sulfate. All three spectra show a strong absorbance at 1240 cm^{-1}, due to S–O stretching. However, whereas chondroitin 4- and 6-sulfate show a typical absorbance in the fingerprint region (890 and 920 cm^{-1} for chondroitin 4-sulfate; 775, 820, and 992 cm^{-1} for chondroitin 6-sulfate), the monosulfated disaccharide prepared has a single peak at 800 cm^{-1}.

Fig. 6 shows the release of inorganic sulfate from the substrate described when

TABLE II

L-IDURONIC ACID 2-SULFATE SULFATASE ACTIVITY OF PLASMA SAMPLES AND FIBROBLAST HOMOGENATES OF NORMAL CONTROLS, HUNTER PATIENTS, AND OBLIGATED AND POSSIBLE HETEROZYGOTES

Samples of	*Units*[a]
Plasma of normal individuals (13)	mean ± SEM 20.48 ± 1.22
of Hunter patients	
M.H.	3.95
S.R.	3.69
J.P.	3.46
J.P.	3.53 (5.00)
P.M.	5.50
T.J.	3.70 (5.70)
T.Z.	2.45 (3.56)
T.Z.	0.85
E.G.	2.15 (4.51)
	mean ± SEM 3.25 ± 0.35
of obligated heterozygotes	
Mrs. P	13.50
	9.00
Mrs. R.	12.42
of possible heterozygotes	
Mrs. Z.	15.00
	17.40
Mrs. H.	10.86
Fibroblast extracts	
of normal individuals (6)	mean ± SEM 137.6 ± 10.75
of Hunter patients	
J.P.	20.00
E.G.	below detection
D.J.	22.80
M.H.	below detection
S.R.	10.80
	mean ± SEM 6.40 ± 5.16

[a]1 unit = 1 nmol of sulfate residue released/24 h/mg of protein. In parentheses, values obtained with a disulfated disaccharide [(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-mannose 6-sulfate] as a substrate.

dialyzed plasma from a patient with mucolipidosis II was used as a source of enzyme, and the reaction was performed at various pHs and substrate concentrations. The apparent K_m of the enzyme is 2.5mM and its pH optimum lies between 4.6 and 4.8. Accordingly, a 5mM substrate concentration and a final pH 4.6 were adopted for the routine measurements of the enzyme. The results have been expressed in terms of a unit corresponding to 1 μmol of sulfate/24 h/g protein.

Table II shows the results obtained with 13 plasma samples of normal individuals, 7 plasma samples of Hunter patients, and plasma samples of two obligate heterozygotes and two possible ones. In parentheses are listed the units of activity obtained with the identical method, but using as substrate *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-mannose 6-sulfate, a disulfated disaccharide similar to that proposed by Lim *et al.*[8]. This was prepared essentially with the same technique as that described here, omitting however the treatment with methanolic hydrogen chloride. The same Table also shows the enzymic activity determined for extracts of normal and Hunter fibroblasts with the monosulfated disaccharide as the substrate.

DISCUSSION

In 1957, Kantor and Schubert[9] reported that treatment with anhydrous methanolic hydrogen chloride completely desulfates chondroitin sulfates. However, Danishefsky *et al.*[10] found that this treatment would desulfate heparin only partially. No satisfactory explanation for this phenomenon could be provided at that time. The demonstration that residues of L-iduronic acid 2-sulfate are present in heparin[11], however, raised the possibility that the sulfate groups in this position might account for the groups not hydrolyzed with methanolic hydrogen chloride, for the following reasons: (a) its amount roughly corresponds to what would be expected if all the *N*-sulfate and 6-*O*-sulfate were removed; (b) after cleavage with nitrous acid, only nonsulfated and monosulfated disaccharides were found; in fact, disaccharides having a sulfate to hexuronic acid molar ratio higher than 1.0:1.0 were not found; and (d) the monosulfated disaccharides obtained show the specific i.r. absorbance described by Dietrich[12] for the sulfate group at C-2 of L-iduronic acid residue, *i.e.*, strong absorbance at 1240 cm^{-1} and absorbance in the fingerprint region at 800 cm^{-1}.

The preparation of this monosulfated disaccharide provides a convenient substrate for the measurement of L-iduronic acid 2-sulfate sulfatase. Since the only sulfate group present is at C-2 of the L-iduronic acid residue, it is now possible to measure directly the inorganic sulfate released by the enzyme, instead of the products of several concurrent enzyme reactions[8,13]. With the availability of sensitive methods for the measurement of inorganic sulfate[2], the need for a radioactively labeled substrate may be obviated.

The method, as described, is completed within 26 h after dialysis of the samples. The values of enzyme activity obtained, by this method, with plasma samples incubated for 24 h are, on the average, higher than those reported for serum by Liebaers

and Neufeld[13], both for normal controls and for Hunter patients. However, the values obtained by the present method for extracts of normal fibroblasts are slightly lower than those reported previously by these authors[13], while those of Hunter fibroblasts are higher. Whether the values obtained with samples from Hunter patients reflect true residual activity or the activity of nonspecific sulfatases is not known at this time.

Plasma samples of two obligate heterozygotes (with two children affected, or family history, or both) gave intermediate values between those obtained with normal plasma and Hunter plasma; of two plasma samples derived from possible heterozygotes, one had normal values or activity on two different occasions, the other had a heterozygote level of activity.

Of considerable interest is the observation that incubation of plasma samples of Hunter patients with the disulfated disaccharide showed from 40 to 100% more activity than incubation with the monosulfated disaccharide. This suggests that the anhydro-D-mannose 6-sulfate obtained by deamination of 2-amino-2-deoxy-D-glucose 6-sulfate may still serve as a substrate for 6-sulfate sulfatase.

ACKNOWLEDGMENT

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