STRUCTURE OF HEPARITIN SULFATE IN TISSUES OF THE HURLER SYNDROME*

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The Hurler syndrome, a heritable disease of connective tissue, is characterized by the deposition in tissues and the excretion in urine of heparitin sulfate (HS) and chondroitin sulfate B (CS-B). Unlike their normal location extracellularly, in this disease, the two mucopolysaccharides are present intracellularly (Brante, 1952; Stacey and Baker, 1956; Brown, 1957; Dorfman and Lorincz, 1957; Meyer et al., 1958).

Previous studies in this laboratory have demonstrated that CS-B obtained from liver, spleen, or urine of patients with the Hurler syndrome contains only small amounts of amino acids (predominantly serine) when extracted with H_2O and purified without the use of alkali or proteolytic enzymes. In contrast, CS-B of normal skin is only separated from protein by alkali treatment or exhaustive proteolytic digestion (Dorfman, 1964). Following the original works of Muir (1958), several investigators have shown that a number of sulfated mucopolysaccharides are covalently bound to protein through the hydroxyl group of serine. Lindahl and Roden (1964) have demonstrated that in both heparin and chondroitin sulfate of cartilage, the binding of mucopolysaccharide chains to serine is probably by way of a glucuronylgalactosylgalactosylxylosyl linkage. The presence of serine in a heparitin sulfate fraction obtained from aorta (Jacobs and Muir, 1963) suggested a similar carbohydrate-protein linkage pattern in this molecule.

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Based on the method of Lagunoff and Warren (1962) for the determination of heparitin sulfate, Cifonelli (1965) has developed a method for structural study, utilizing the reaction of HNO_2 with N-sulfated groups. When the HNO_2 reaction is carried out at -20°C in 60% glyme,* N-sulfated 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 DNP-amino derivatives do not react with HNO_2. This method is particularly useful for structural studies of HS, since this compound contains both N-sulfated and N-acetylated amino sugars.

The studies to be reported indicate that HS, extracted from livers of three patients who died of the Hurler syndrome, is heterogeneous. All three samples were resolved into two major fractions by Dowex 1 chromatography. The first fraction was low in N-sulfate, high in N-acetyl and contained serine, galactose and xylose. The second fraction contained larger amounts of N-sulfate, less N-acetyl, only small amounts of amino acids and essentially no neutral sugars.

MATERIALS AND METHODS

Crude HS was extracted with water from minced tissues at 5°C in a Waring Blender followed by dialysis and centrifugation. The HS was precipitated with cetylpyridinium chloride (CPC) from 0.03M NaCl and dissolved in 2.0M NaCl. Following precipitation with three volumes of ethanol, it was dissolved in 0.03M NaCl and reprecipitated with CPC. This product was treated with deoxyribonuclease and ribonuclease and once again precipitated with CPC. Fractionation was carried out on a Dowex 1 X 2 Cl^- column with stepwise elution with NaCl. The principal fractions were reprecipitated with CPC, treated with testicular hyaluronidase, passed through a Sephadex G-75 column and rechromatographed on Dowex 1 until constant chemical analyses including amino acid contents were obtained.

*glyme - 1,2-dimethyoxyethane, purchased from Distillation Products, Inc. Rochester, N. Y.

Analyses for uronic acid, hexosamines, sulfate and nitrogen were performed as previously described (Cifonelli, Ludowieg, and Dorfman, 1958). Number average molecular weights were obtained from osmotic pressure measurements. Amino acids were determined with the Technicon amino acid analyzer. Chromatography for neutral sugars was carried out in two solvents, BuOH:EtOH:H_2O, 10:3:5, and EtAc:Pyridine:H_2O, 8:2:1. Anhydromannose was determined by the indole method of Dische and Borenfreund (1950). The authors are grateful to Dr. Richard Winzler and Mr. Gerald Meyer for the determination of N-acetyl groups using a gas chromatographic method.

RESULTS AND DISCUSSION

Table I summarizes the results of analyses of purified fractions obtained from the livers of 3 male patients. The clinical histories of

TABLE I

ANALYSES OF HEPARITIN SULFATE FRACTIONS

Fraction	Recovered MPS %	N*	UA*	SO_4*	$[\alpha]_D$	Acetyl	N-SO_4*†	MW_N
1.0 TP	54	1.31	1.48	0.99	+66	0.72	0.38	5,500
1.0 GI	31	1.51	1.35	1.08	+55	0.46	0.56	3,200
1.0a GI	21	1.56	1.56	0.72	+70	0.77	0.30	5,500
1.0 GC	63	1.50	1.60	1.2	+70	0.40	0.48	-
1.3 TP	35	1.68	1.35	2.02	+47	0.26	0.80	4,000
1.3 GI	18	1.55	1.38	2.12	+47	0.21	0.78	2,700
1.3 GC	18	1.20	1.30	1.80	+56	0.24	-	-

*All values expressed as molar ratios with hexosamine taken as 1.0.

†N-SO_4 was determined on the basis of difference between original hexosamine values and those obtained after HNO_2 treated. Colorimetric analyses for anhydromannose was also used to follow the HNO_2 reaction but this determination gave spuriously high values and could not be used for quantitative comparisons.

these patients were not adequate to be certain as to whether the patients were of the sex-linked or autosomal recessive type. During fractionation of the material from patient G1, the material which was eluted with 1.0M NaCl was only partially precipitated with CPC. The material which did not precipitate with CPC was precipitated with ethanol and repurified. It is identified as 1.0a G1 in Table 1.

In all cases, the fractions eluted from Dowex 1 with 1.0M NaCl exhibited lower sulfate and N-sulfate content (based on hexosamine determinations after HNO_2 treatment) and a higher optical rotation than those obtained with 1.3M NaCl. An inverse relationship was observed between N-sulfate and N-acetyl contents, the sum of two was approximately equal to the total hexosamine.

The oligosaccharides resulting from the HNO_2 treatment of the 1.0M and 1.3M fractions were separated on Sephadex G-25. The results, which will be reported in detail elsewhere, confirmed the finding that the 1.0M fraction contained much less N-sulfate than did the 1.3M fraction. Of special interest was an oligosaccharide fraction which contains an appreciable amount of free amino groups as witnessed by reaction with HNO_2 at room temperature, but not at -20°C. The presence of free amino groups was confirmed by reaction of the oligosaccharide with trinitrophenylsulfonic acid.

Table 2 illustrates the amino acid analyses of the 1.0M and 1.3M fractions obtained from the three livers. In all cases, serine predominates in the 1.0M fractions, while in the 1.3M fraction, small amounts of other amino acids are present with no predominance of serine. The 1.3M fractions contained galactosamine suggesting the presence of contaminating substances and rendering the interpretation of the amino acid data difficult.

The 1.0M and 1.3M fractions were analyzed for the presence of neutral sugars following hydrolysis with 1M HCl at 100°C for 3 hours. On paper chromatography in two solvents, both galactose and xylose were demonstrated

in the 1.0M fractions while no neutral sugars were found in the 1.3M fractions.

The findings reported in this paper raise important questions regarding both the structure and biosynthesis of HS and the molecular defect in

TABLE 2

AMINO ACID ANALYSES OF PURIFIED HEPARITIN SULFATE FRACTIONS

	1.0M				1.3M		
	TP	GI	GIa	GC	TP	GI	GC
Aspartic acid	0.3	0.4	0.4	0.1	0.2	0.1	0.3
Threonine	0.1	-	0.2	0.1	0.1	0.1	0.2
Serine	1.8	0.9	2.9	1.0	0.2	0.4	0.6
Glutamic acid	0.3	0.5	0.8	0.3	0.4	0.4	0.7
Glycine	0.3	0.5	0.9	0.2	0.3	0.3	0.6
Alanine	0.1	0.1	0.9	0.1	0.2	0.1	0.7
Valine	-	-	-	0.1	-	-	-
Leucine	-	-	-	-	-	-	0.2
Lysine	0.3	0.2	0.4	-	0.3	0.2	-
Histidine	-	0.5	0.5	-	-	0.2	-
Galactosamine	-	Trace	Trace	0.3	2.04	3.4	7.0

Amino acids are expressed as μMole per 100 μMoles of glucosamine.

the Hurler syndrome. A branched structure for HS has been suggested by Linker and Sampson (1960) and Cifonelli (1964, 1965) with the outer branches composed of predominantly N-sulfate glucosamine groups and the inner core containing principally N-acetylated groups. The inner core may be expected to be the site of linkage to protein (via galactose and xylose). It could then be assumed that the 1.0M fraction derives from the inner core and that

the 1.3M fraction originates from the outer branches. Telser, Robinson and Dorfman (1965) have recently reported that, in cartilage, the synthesis of the protein moiety of the CS-protein complex appears to precede the formation of mucopolysaccharide chains. If a similar mechanism is obligatory for HS biosynthesis, the 1.3M fraction could only arise as a result of degradative mechanisms, possibly involving a debranching enzyme. However, since nothing is known concerning the biosynthesis of HS, it is possible that polysaccharide chains not linked to protein may arise normally or in Hurler's disease by some as yet undescribed biosynthetic mechanism. A more serious problem arises with respect to the interpretation of the finding that the 1.0M fraction contains only one serine residue for each 10 polysaccharide chains. Previous studies (Dorfman, 1964) have shown that CS-B isolated from tissues and urine of patients with Hurler's disease is also deficient in amino acids with an average of one serine residue for each five polysaccharide chains. In contrast, CS-B isolated from normal human skin, even after proteolysis, averages one serine for each polysaccharide chain. These considerations highlight the unresolved problem of the molecular defect in Hurler's and related syndromes. Van Hoof and Hers (1964) have suggested that the accumulation of polysaccharide results from the deficiency of a normal lysosomal degradative enzyme. Hutterer (1965) has reported the presence of an enzyme in liver lysosomes which degrades HS. The data presented in this paper together with those previously published concerning CS-B are consistent with the hypothesis of Van Hoof and Hers. The presence in Hurler's syndrome of partially degraded HS and CS-B low in amino acids (including serine) might result from accumulation of the products due to the action of proteolytic enzymes in the absence of an enzyme(s) necessary for final degradation of the polysaccharide chains. However an equally plausible explanation is the overproduction of polysaccharide-peptides characterized by a molecular defect which prevents normal formation of macromolecular structure in tissues. Such soluble

polysaccharide-peptides may be excreted or deposited within cells where they may be subjected to partial degradation in lysosomes with a final result not unlike that postulated by a defective degradative mechanism.

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