Hurler's, Hunter's and Morquio's Syndromes

A BIOCHEMICAL STUDY IN THE LIGHT OF CURRENT VIEWS OF THE UNDERLYING DEFECTS

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Glycosaminoglycans were isolated from the urine of three patients with Hurler's, Hunter's and Morquio's syndromes and also from the liver and spleen of the case of Hurler's syndrome by a procedure avoiding further degradation. A method of determining the proportions of dermatan sulphate, heparan sulphate and chondroitin sulphate in each preparation is described. The relative proportions of these glycosaminoglycans in the urine and organs of the case of Hurler's syndrome were very similar. Glycosaminoglycans from the organs were of much lower molecular weight than normal, consisting of single chains of molecular weight about 5000 together with multiples of up to four such chains attached to peptide moieties. The linkage region normally attaching glycosaminoglycan chains to protein in whole protein-polysaccharides of connective tissue was degraded progressively towards serine. The total output and relative proportions of abnormal glycosaminoglycans in the urine were compared in two brothers with Hunter's syndrome examined on two occasions 4 years apart. At comparable ages they excreted about the same amount, and the relative proportions of each glycosaminoglycan remained essentially constant. The composition and chromatographic behaviour of the glycosaminoglycan in the urine from the case of Morquio's syndrome indicated that it consisted of material containing about one-third keratan sulphate and two-thirds chondroitin sulphate as part of the same molecule, as in proteoglycans of cartilage. The total output of glycosaminoglycans, although higher than normal, was considerably less than in other types of Mucopolysaccharidoses.

The Mucopolysaccharidoses are a group of diseases in which sulphated glycosaminoglycans are excreted in the urine in large amounts and stored within cells of the body, particularly of organs such as liver and spleen. Several types that differ in clinical picture and in the pattern of excretion of glycosaminoglycans have been recognized and tentatively classified (McKusick, 1966). They are autosomal recessive diseases, apart from Hunter's disease which shows recessive inheritance linked to the X-chromosome (X-linked). Their recessive nature implies that a mutation of a single gene has occurred, giving rise to a biochemical defect which is presumably different in each distinct type of Mucopolysaccharidosis. Current knowledge of these diseases has been reviewed in a symposium (Bearn, 1969). The most widely held explanation is that degradation of glycosaminoglycans is defective but in exactly what way remains obscure.

In examining possible explanations, it is of interest to know (1) how far the abnormal glycosaminoglycans in the organs are degraded; (2) whether the relative proportions of the various glycosaminoglycans are the same in the organs as in the urine, which would suggest that those in the urine are derived from those in the organs; (3) whether the output and relative proportions of glycosaminoglycans change with age, which would indicate that growth and development affect the metabolism of the particular glycosaminoglycans that are involved in the diseases.

The first two questions are here examined in a boy with Hurler's syndrome. The third question has been investigated in two brothers with Hunter's syndrome examined on two occasions 4 years apart. Finally the question of whether keratan sulphate and chondroitin sulphate, which are normally part of the same complex macromolecule in cartilage,

were both affected was examined in a case of Morquio's syndrome.

MATERIALS AND METHODS

All chemicals were of analytical grade with the exception of anthrone, 9-aminoacridine hydrochloride, galactose, glucuronolactone, glucosamine hydrochloride and xylose (BDH Chemicals Ltd., Poole, Dorset, U.K.). Acetone and ethanol were RR grade (James Burroughs Ltd., London S.E.11, U.K.) and water was glass-distilled.

Urine samples were collected in vessels containing a few millilitres of toluene and then frozen. Portions of liver and spleen, obtained at post-mortem, were stored frozen until required.

Clinical material

Hurler's syndrome. A West Indian boy who died at the age of 4 years showed the clinical signs of Hurler's syndrome, first diagnosed at 16 months of age when being operated on for inguinal hernia. At 3½ years of age the diagnosis was confirmed after a complete radiographic survey and by the presence of metachromatic inclusions in lymphocytes (Muir, Mittwoch & Bitter, 1963) and a high output of glycosaminoglycans in the urine. At autopsy the heart valves showed marked nodular thickening and the liver and spleen were enlarged although no enlargement had been noted at 18 months of age. At the time of death most clinical signs of the disease were evident. Severe mental deterioration had developed by 3½ years, although the child had appeared mentally normal at 2 years of age. There was no evidence from the family history to suggest recessive inheritance linked to the X-chromosome.

Hunter's syndrome. The genealogy and clinical description of the two brothers investigated here have been published (Emmanuel, 1970). On two occasions 4 years apart 24h urine samples were collected from each boy. During the interval of 4 years their mental condition had not deteriorated but their clinical state had worsened, particularly that of the cardiovascular system.

Morquio's syndrome. An Asian boy aged 9½ years showed the classical radiographic features of Morquio's syndrome (Morquio, 1929, 1935), particularly of the hands, pelvis and the head and neck of the femurs. He had all the characteristic deformities including short stature, knock knees, ulnar deviation of both hands, pectus carinatum and flat feet. He also had diffuse opacity of the cornea (Von Noorden, Zellweger & Ponseti, 1960) but his hearing and intelligence were normal and he had no visceral enlargement.

Analytical methods

Determination of sugars. Hexuronic acid was determined by the procedure of Bitter & Muir (1962) or by an automated modification (D. Heinegård, personal communication) when column effluents were analysed. Glucuronolactone was used as a standard. Hexose was determined by the method of Trevelyan & Harrison (1952) with galactose as a standard. Control tubes containing amounts of hexosamine (glucosamine) and glucurono-

lactone similar to those in the sample were used to account for interference which normally amounted to between 5 and 10%. Neutral sugars were determined by g.l.c. with a modification of the procedure of Clamp, Dawson & Hough (1967). Hexosamine was determined as described by Kraan & Muir (1957) after hydrolysis in a small volume of 8M-HCl at 95°C *in vacuo* for 3h. The acid was neutralized and the solution made up to a known volume from which samples were taken for the determination of hexosamine. Glucosamine hydrochloride was used as a standard. Glucosamine/galactosamine molar ratios were determined after hydrolysis as described by Tsiganos & Muir (1969) with a Locarte amino acid analyser.

Amino acids. Amino acid analysis was carried out as described by Tsiganos & Muir (1969) with a Locarte amino acid analyser.

Determination of pentose before and after digestion with highly purified hyaluronidase. The amounts of pentose present in a sample of spleen glycosaminoglycans were determined by the method of Tsiganos & Muir (1966) before and after digestion of a 6mg portion with 200i.u. of sheep testicular hyaluronidase, purified by the method of Borders & Raftery (1968) (a gift from Miss E. Baxter). After digestion the glycosaminoglycans were precipitated with ethanol to 80% (v/v), washed with ethanol and acetone and dried.

Cellulose acetate electrophoresis. Electrophoresis was performed as described by Wessler (1968). Samples (about 5µg) of glycosaminoglycan in 1µl of water were applied to cellulose acetate strips (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.), moistened in 0.1M-barium acetate, pH 7.0, and a current of 5V/cm was applied for 3h in the cold. The strips were stained with 0.05% Alcian Blue in 95% (v/v) ethanol saturated with NaCl.

Chromatographic methods

Gel chromatography. Samples (10mg) of glycosaminoglycans in 1.0ml of 0.2M-sodium acetate, pH 6.8, were applied to a column (170cm × 1.7cm) of Sephadex G-200 and eluted with the same solvent. The uronic acid contents of 5ml fractions were determined. Glycosaminoglycans were also chromatographed after digestion with crystalline papain (EC 3.4.4.10; BDH Chemicals Ltd.). The papain suspension (40µl, containing 4 units) was added to solutions of the glycosaminoglycans in 0.2M-sodium acetate buffer, pH 5.7, containing 4mM-EDTA and 20mM-cysteine-HCl and incubated at 60°C for 8h. A further 50µl of suspension was then added and the digestion continued overnight. The solution was clarified by centrifugation and then applied directly to the column.

Ion-exchange chromatography. Fractionation of glycosaminoglycans was carried out as described by Di Ferrante (1967). A column (23cm × 1.8cm) was packed with a slurry of ECTEOLA-cellulose (0.6mequiv./g) suspended in 0.9% NaCl. Samples (about 3mg) of glycosaminoglycan in 0.9% NaCl were applied to the column and eluted in a stepwise manner, with 3 column volumes each, firstly of 0.9% NaCl followed by 0.5M-, 1.0M-, 2.0M- and 4.0M-NaCl. The uronic acid and hexose contents of 5ml fractions were determined.

Fractionation of cetylpyridinium-glycosaminoglycan complexes. Solubility profiles of the cetylpyridinium-glycosaminoglycan complexes before and after digestion

of glycosaminoglycans with hyaluronidase (see below) were determined by the procedure of Antonopoulos, Gardell, Szirmai & De Tyssensk (1964) as described in detail by Dean & Muir (1970) but the concentration of Na_2SO_4 was increased to 10 mM (Dorner, Antonopoulos & Gardell, 1968).

Preparative procedures

Isolation of glycosaminoglycans from urine samples. Samples of urine (100 ml) were dialysed for 48 h at 4°C against four changes of 3 litres of water in dialysis tubing which had been previously heated to decrease the pore size (Callanan, Carroll & Mitchell, 1957). Glycosaminoglycans were then precipitated with 9-aminoacridine hydrochloride (Muir & Jacobs, 1967) and converted into their soluble sodium salts by shaking an aqueous suspension of the 9-aminoacridine complexes with Dowex-50W (8% cross-linked; Na^+ form). Samples were reprecipitated, re-exchanged to sodium salts and the glycosaminoglycans finally precipitated in 80% (v/v) ethanol containing 1–2 ml of a solution consisting of 30 g of anhydrous sodium acetate and 15 ml of acetic acid made up to 100 ml with water. The precipitated glycosaminoglycans were washed with ethanol, then with acetone and finally dried. In the case of Morquio's syndrome the glycosaminoglycans were prepared both by the method described above and by the method of Di Ferrante (1967).

Extraction of glycosaminoglycans from liver and spleen. Fresh post-mortem samples of liver and spleen were stored at -20°C until ready for use. The tissue was allowed to thaw, cut into small pieces and pulverized in a steel die after freezing in liquid nitrogen. Batches (10 g) were then suspended in 50 ml volumes of 0.15 M-sodium acetate, pH 6.8, at 4°C and homogenized for 1 min at setting 3 with a Sorvall omni-mixer. The homogenates were centrifuged, the supernatant solution was decanted and the residues extracted twice more in the same manner. The extracts were all pooled, recentrifuged and the polyanions precipitated with 9-aminoacridine hydrochloride. Conversion into the sodium salt was effected as described above and the solution centrifuged to remove any denatured protein. The clarified solution was reprecipitated with 9-aminoacridine hydrochloride and the procedure repeated, followed by a final precipitation with ethanol. Nucleic acids were removed as described by Olsson & Gardell (1967) and any contaminating protein by cetyltrimethylammonium bromide-cellulose column chromatography as described by Heinegård & Gardell (1967). Purified glycosaminoglycans were then converted to the sodium salt by redissolving in a few ml of 0.15 M-sodium acetate and reprecipitation with 5 vol. of ethanol. The precipitate was washed with ethanol and acetone and samples for analysis were dried to constant weight at 80°C *in vacuo* over P_2O_5 . Glycosaminoglycans remaining in the tissue were isolated after digestion with papain (Dean & Muir, 1970).

Composition of glycosaminoglycans in the tissues and urine

Digestion with hyaluronidase. Two samples (10 mg) of glycosaminoglycans, whose glucosamine/galactosamine molar ratio had been determined, were dissolved in 3 ml volumes of 0.1 M-sodium acetate, containing 0.15 M-NaCl

adjusted to pH 5.0 with a few drops of acetic acid, and a sample (100 μl) removed for the determination of total uronic acid content. The remainder was incubated at 37°C with 3000 turbidity-reducing units of testicular hyaluronidase (EC 3.2.1.35; Hyalase; Fisons Pharmaceuticals Ltd., Loughborough, Leics., U.K.) under a layer of toluene as a preservative. A further 1500 units of enzyme was added 24 h later and a final 1500 units after 36 h. Digestion was assumed to be complete after 48 h. Any denatured protein was removed by centrifugation and the supernatant solution dialysed for 48 h at 4°C in previously heated dialysis tubing (Callanan *et al.* 1957) against four changes of water. The undigested glycosaminoglycans that remained were then precipitated with 9-aminoacridine hydrochloride, converted into their soluble sodium salts and made up to a known volume with water. Total uronic acid content was again determined. Repeated estimations agreed to within $\pm 5\%$. The glycosaminoglycans thus obtained were precipitated with 5 vol. of ethanol, washed with acetone and dried *in vacuo* before analysis and determination of glucosamine/galactosamine molar ratios.

When chondroitin sulphate from pig cartilage was treated as described above nothing remained that was precipitated by 9-aminoacridine hydrochloride, whereas 96–98% of the total dermatan sulphate was recovered from artificial mixtures in normal urine of chondroitin sulphate and dermatan sulphate (a gift from Hoffmann-La Roche Ltd., Basle, Switzerland) or dermatan sulphate alone.

Calculation of composition. The glucosamine/galactosamine molar ratio before hyaluronidase digestion indicated the proportion of heparan sulphate plus any hyaluronic acid in the mixture. The decrease in total uronic acid after digestion with hyaluronidase was taken as a measure of the contents together of chondroitin 4-sulphate, chondroitin 6-sulphate and any hyaluronic acid. The uronic acid content of the resistant glycosaminoglycan was taken as an estimate of dermatan sulphate with heparan sulphate, their relative proportions being calculated from the galactosamine/glucosamine molar ratio. From the composition of the glycosaminoglycans before and after hyaluronidase digestion, it appears that there were only trace amounts of hyaluronic acid present, which was therefore ignored. No corrections were made for differences in colour-yield of the various glycosaminoglycans in the uronic acid determinations. In the Bitter & Muir (1962) procedure dermatan sulphate, which predominated in all preparations, gives 83% of the colour of chondroitin sulphate. The proportion only of chondroitin sulphate was estimated from uronic acid analysis alone.

RESULTS AND DISCUSSION

Hurler's syndrome

Almost the same amounts of material containing uronic acid were extracted from liver and spleen, namely 28.9 and 31.3 mg/100 g wet wt. respectively, and only a further 3.8 mg and 3.7 mg/100 g wet wt. were obtained from the residues after proteolysis, the total being 32.7 mg/100 g wet wt. in the liver and 35.0 mg/100 g wet wt. in the spleen.

The material extracted without proteolysis from liver and spleen had almost identical glucosamine/galactosamine molar ratios, similar to that of the material obtained from urine, which contained 73.8 mg of uronic acid/litre. These ratios were respectively 1:77.7, 1:77.0 and 1:72. Table 1 shows that dermatan sulphate was predominant in all three preparations, and chondroitin sulphate represented one-third of the total material extracted from the organs, with heparan sulphate as only a minor constituent. On cellulose acetate electrophoresis (Wessler, 1968) the glycosaminoglycans from the organs behaved as a mixture of dermatan sulphate with a minor component having the higher mobility of chondroitin sulphate.

The similarity in the composition of the mixture of pathological glycosaminoglycans in the urine and in the liver and spleen suggests that both the latter may be entering the circulation and passing into the urine. Plasma concentrations of glycosaminoglycans are raised in Hurler's syndrome (Calatroni, Donnelly & Di Ferrante, 1969). The relative proportion of chondroitin sulphate, however, was somewhat less in the urine than in the extracts of liver and spleen (Table 1) suggesting that this patient could break down more chondroitin sulphate than dermatan sulphate.

The structural glycosaminoglycans of connective tissue are covalently linked to protein, forming very large composite macromolecules that are not readily extracted by neutral iso-osmotic salt solutions (reviewed by Muir, 1969). In contrast, most of the material containing uronic acid in the liver and spleen of this patient was extracted without proteolysis by iso-osmotic sodium acetate. This suggests that the pathological material, which is presumably stored in the membrane-bound vesicles within cells that were first observed in the liver by Van Hoof & Hers (1964), is not present as normal multichain protein-polysaccharides. It did not behave as such on Sephadex G-200 chromatography,

when it should have been wholly excluded from the gel, whereas only 1.9% of that from liver and 2.7% of that from spleen was excluded (Fig. 1). Together with the low amino acid contents (Table 2) these results are similar to previous reports (Dorfman, 1964; Knecht, Cifonelli & Dorfman, 1967). From amino acid analysis, the material from liver contained only 0.75% protein and that from spleen 0.78%, and serine accounted for 15 and 25% of the total amino acids respectively.

In structural protein-polysaccharides, the chains of chondroitin sulphate and dermatan sulphate are attached to protein through an atypical sequence of neutral sugars consisting of two galactose residues attached to a xylose residue, which is in turn attached to the hydroxyl group of a serine residue forming a 'linkage' region (reviewed by Muir, 1969). After exhaustive proteolysis the serine residue remains as the principal amino acid attached to the carbohydrate chains (Muir, 1958). The preponderance of serine, the behaviour on Sephadex G-200 and the low protein content together indicate that the pathological material had undergone considerable degradation, as if by proteolysis, and also by some further breakdown as discussed below.

Proteolytic breakdown would not appear to be entirely complete, however, because the elution profile on Sephadex G-200 of the material from liver, which was asymmetric (Fig. 1), indicating a molecular weight at the position of maximum uronic acid of about 10000, after papain digestion became symmetrical, indicating a number-average molecular weight of about 5000. Similarly, the material from spleen showed a trimodal elution profile, with approximate molecular weights of 21000, 15000 and 10000, which after papain digestion likewise became symmetrical, with the same number-average molecular weight of about 5000 (Fig. 1). This value is considerably less than the number-average molecular weights, after exhaustive proteolysis, of skin dermatan sulphate

Table 1. *Percentage distribution of purified glycosaminoglycans from liver, spleen and urine of a patient (A.F.) with Hurler's syndrome*

	Uronic acid content		Distribution (%)		
	(mg/100 g wet wt. total in tissue)	(mg/l of urine)	Chondroitin sulphate	Dermatan sulphate	Heparan sulphate
Liver	32.7	—	27.8	69.0	3.2
Spleen	35.0 (6.35)*	—	33.4 (75)*	64.1 (0)*	2.5 (25)*
Urine	—	73.8 (1.9 and 2.3)†	16.9 (83 and 85)†	81.8	1.3

* Values in parentheses are for a normal spleen from a 14-year-old boy (Bitter & Muir, 1966).

† Values in parentheses are for normal urines of two boys, 5½ and 10 years of age (Bitter, Muir, Mittwoch & Scott, 1966).

(about 17000; Fransson & Rodén, 1967a) or of cartilage chondroitin sulphate (20000–25000; Luscombe & Phelps, 1967; Wasteson, 1969). These results suggest that the pathological glycosaminoglycans exist partially as single chains with an average molecular weight of 5000 together with

multiples of up to perhaps four such chains, attached to a small portion of peptide that can be cleaved by further proteolysis to give single chains. It is notable that heparan sulphate obtained from livers of patients with Hurler's syndrome was of comparably low molecular weight (Knecht *et al.* 1967).

The solubility profiles of the cetylpyridinium complexes of the materials extracted directly from liver and spleen without proteolysis also indicated that they were more degraded than normal dermatan sulphate and chondroitin sulphate obtained after proteolysis. These are eluted in 0.45–0.6M-magnesium chloride solutions (Fransson & Rodén, 1967a; Fransson, 1968a; Antonopoulos *et al.* 1964), whereas the pathological materials were mostly eluted in 0.35M-, 0.4M- and 0.45M-magnesium chloride (Figs. 2a and 2c) resembling the solubility profiles of dermatan sulphate after treatment with hyaluronidase (Fransson, 1968a).

Less than 5% of the total hexosamine was eluted in 1% cetylpyridinium chloride, indicating that there was only a small amount of very short glycosaminoglycan chains (Antonopoulos *et al.* 1964; Laurent & Scott, 1964) in these preparations. After hyaluronidase digestion, however, almost 25% of the hexosamine was eluted in 1% cetylpyridinium chloride with corresponding decreases in all other fractions (Figs. 2b and 2d), suggesting that

Fig. 1. Gel chromatography on a column (170 cm × 1.7 cm) of Sephadex G-200 of glycosaminoglycans (10 mg) from the liver and spleen of a patient with Hurler's syndrome. Fractions (5 ml) were collected and the uronic acid contents determined, of glycosaminoglycans from the liver before (▲) and after (△) proteolysis and those from the spleen before (●) and after (○) proteolysis.

Table 2. Chemical analysis of purified glycosaminoglycans from liver and spleen of a patient (A.F.) with Hurler's syndrome

Experimental details are given in the text. n.d., Not determined.

Amino acid	Liver glycosaminoglycans		Spleen glycosaminoglycans	
	(µmol/g)	(residues/1000)	(µmol/g)	(residues/1000)
Asp	6.06	88.8	3.90	52.34
Thr	2.80	41.03	1.90	25.53
*Ser	10.03	146.9	18.54	248.9
Glu	8.42	123.4	5.56	74.66
Pro	3.21	47.03	2.03	27.18
Gly	8.20	120.16	10.59	142.10
Ala	4.54	66.52	2.79	37.40
Val	3.13	45.86	3.07	41.18
Ile	3.41	49.97	0.87	11.69
Leu	4.37	64.03	4.78	64.08
Lys	7.88	115.47	10.09	135.40
His	6.19	107.0	10.39	139.47
Sugar	(µmol/g)	(% of dry wt.)	(µmol/g)	(% of dry wt.)
*Xylose	23.98	0.36	26.70	0.40
*Galactose	81.03	1.46	77.7	1.40
Hexuronic acid	1847.0	32.50	1653.0	29.10
Hexosamine	n.d.	n.d.	1433.0	25.7

* Calculated values for normal skin dermatan sulphate of mol.wt.17000 (Fransson & Rodén, 1967a) with the linkage region intact: galactose, 116.6 µmol/g; xylose, 58.6 µmol/g; serine, 59 µmol/g.

Fig. 2. Solubility profiles on cellulose micro-columns (Antonopoulos *et al.* 1964) of cetylpyridinium complexes of glycosaminoglycans from the liver and spleen of a patient with Hurler's syndrome. About 100 μ g of glycosaminoglycans was applied. The columns were eluted in stepwise fashion as shown. Glycosaminoglycan from liver (a) before and (b) after digestion with hyaluronidase; those from spleen (c) before, (d) after digestion with hyaluronidase. CPC is cetylpyridinium chloride.

chondroitin sulphate was probably distributed throughout all fractions.

Although it would appear that some of the pathological material extracted directly from the liver and spleen existed as rather short glycosaminoglycan chains attached to a residual portion of peptide, the linkage region of much of the rest of the glycosaminoglycans had itself undergone degradation. The amount of serine remaining attached to the glycosaminoglycans from liver and spleen was very much less than that left attached to normal skin dermatan sulphate (Dorfman, 1964) or cartilage chondroitin sulphate (Muir & Jacobs, 1967) after extensive proteolysis. The xylose contents (Table 2) were also much less than would be expected in glycosaminoglycans of normal length. This suggests that the dermatan sulphate, which

was the predominant pathological glycosaminoglycan, had been acted on by hyaluronidase (Fransson & Rodén, 1967b), which has been identified in liver lysosomes (Aronson & Davidson, 1967). The hexuronic acid of dermatan sulphate is mainly iduronic acid, but there are some glucuronic acid residues and where these occur the glycosidic linkages are susceptible to testicular hyaluronidase, with a resultant decrease in molecular weight (Fransson & Rodén, 1967a) and partial loss of the linkage region (Fransson, 1968b) as originally suggested by Matalon & Dorfman (1966). The xylose contents (Table 2) were not only much less than would be expected, but after hyaluronidase digestion the pentose content of the glycosaminoglycans from the spleen was unchanged. Assuming that the pentose was in the surviving linkage

regions, it would appear that dermatan sulphate, the predominant glycosaminoglycan, had already undergone degradation by hyaluronidase as far as was possible and no further loss of the linkage region could take place.

The galactose contents, on the other hand, were higher (Table 2) and approached values expected for glycosaminoglycans of normal chain length. The chains were abnormally short, however (Fig. 1), and if each possessed a linkage region, the galactose contents should have been much greater. Again this suggests that hyaluronidase-susceptible linkages had been cleaved in chains that may originally have been of normal length, producing a number of short chains without linkage regions. The action of hyaluronidase may not have been exhaustive, however, because although it is susceptible to hyaluronidase, there was some chondroitin sulphate in the glycosaminoglycan preparations from both organs, which like the dermatan sulphate was not attached to protein as in normal protein-polysaccharides.

Stepwise degradation of the linkage regions that had survived appeared also to have taken place. When this region is intact the serine/xylose/galactose molar proportion is 1:1:2, as in structural dermatan sulphate and chondroitin sulphate (Fransson, 1968b; Rodén & Armand, 1966; Lindahl & Rodén, 1966; Rodén & Smith, 1966) as well as in a glycosaminoglycan from certain lymphoid cells (Dean & Muir, 1970). The pathological glycosaminoglycans, however, contained less serine than xylose and less xylose than galactose. The serine/xylose/galactose molar proportions were 0.25:0.59:2 for the liver glycosaminoglycans and 0.48:0.69:2 for the glycosaminoglycan from spleen. This stepwise degradation of the linkage region from the serine residues was somewhat less for the spleen than for the liver glycosaminoglycans, which moreover appeared to be less degraded as judged from the elution profile from Sephadex G-200 (Fig. 1). This contrasts with the findings of Knecht *et al.* (1967) on heparan sulphate from livers of patients with Hurler's syndrome, where the surviving linkage regions appeared to be intact.

The galactose values (Table 2) determined by g.l.c. are probably reliable, since values for total hexose determined by this method, which included galactose and lesser amounts of glucose and mannose, were 1.97 and 2.01 for liver and spleen respectively, in good agreement with values of 2.04 and 2.1 determined by an anthrone method (Trevelyan & Harrison, 1952). Degradation of the galactose portion of the linkage region was therefore least but this cannot be attributed to a partial deficiency of an isoenzyme of β -galactosidase that is frequently observed in Hurler's syndrome (reviewed by Muir, 1971) because this enzyme is an

exoglycosidase that acts on non-reducing terminal galactose residues, whereas the galactose residues of the linkage region are towards the reducing end of the chain.

Although it has been suggested that degradation of glycosaminoglycans in Hurler's and Hunter's syndromes is defective, the present study shows that in this one patient at least the glycosaminoglycans that had accumulated in the liver and spleen were already extensively degraded, both in the linkage region and in the chains themselves. The enzymes responsible for these degradative reactions are as yet unknown apart from hyaluronidase, which degrades chondroitin sulphate and to some extent dermatan sulphate as discussed above.

It is difficult to explain merely on grounds of defective degradation why the pathological glycosaminoglycans accumulate, because they are easily soluble, and those in pathological urine are more degraded even than those in normal urine (Constantopoulos, 1968; Constantopoulos, Dekaban & Carroll, 1969). Further, the linkage region appears to be largely intact (Wasteson & Wessler, 1971) in normal urinary glycosaminoglycans, where the average chain length determined by gel chromatography (Wasteson, 1969) was about 11 000 (M. F. Dean & H. Muir, unpublished work).

Hunter's syndrome

Hunter's syndrome has X-linked recessive inheritance, so that presumably a common mutant gene is shared by brothers with this syndrome, the expression of which should be similar in both. Indeed, Table 3 shows that at comparable ages the total output and distribution of the urinary glycosaminoglycans were similar (i.e. patient P.P. at 6½ years of age and patient T.P. at 5½ years of age), dermatan sulphate accounting for about half and heparan sulphate for about 40% of the glycosaminoglycans.

The elution profiles from cetylpyridinium chloride micro-columns of the glycosaminoglycans in the urine, determined on the second occasion only, were also very similar (Figs. 3a and 3b), both profiles being affected in the same way by hyaluronidase digestion (Figs. 3c and 3d), showing that hyaluronidase-susceptible linkages were present in most fractions. Chondroitin sulphate was estimated to comprise about 10% or less of the total glycosaminoglycans (Table 3) by a method that involved dialysis after digestion with hyaluronidase. This estimate may be too high, as hyaluronidase caused some degradation of all cetylpyridinium fractions (Fig. 3) and some fractions might have become small enough to be lost during the dialysis step. The hyaluronidase-susceptible linkages are presumably chiefly present in the dermatan sulphate

Table 3. *Total excretion and percentage distribution of the glycosaminoglycans in the urines of two brothers with Hunter's syndrome examined on two occasions 4 years apart*

Experimental details are given in the text.						
Patient	Age (years)	Excretion of uronic acid		Distribution of excreted glycosaminoglycans (%)		
		(mg/litre)	(24 h output) (mg)	Dermatan sulphate	Heparan sulphate	Chondroitin sulphate
P.P.	2½	86	32.7	38.4	40.7	20.9
	6½	58.1	50.3	55.7	39.7	4.6
T.P.	5½	205	53.5	51.7	41.0	7.3
	9½	98.15	73.1	51.9	36.5	11.6

(Fransson & Rodén, 1967a). Nevertheless, about 25–30% of the total hexosamine was eluted in 1% cetylpyridinium chloride, showing that the glycosaminoglycans before hyaluronidase digestion appear already to be considerably degraded (Antonopoulos *et al.* 1964; Laurent & Scott, 1964). The low hexose/uronic acid molar ratio (1:16.7) indicated little contamination by glycoprotein.

The output of glycosaminoglycans increased with age in both brothers, presumably owing to increase in body mass. The composition of the glycosaminoglycans in the urine of the elder boy (T.P.) was essentially similar at both times (Table 3), whereas the heparan sulphate content of the younger (P.P.) also remained constant, even though at 2½ years he excreted a somewhat higher proportion of chondroitin sulphate relative to dermatan sulphate than when he was 6½ years old. By this time the distribution of glycosaminoglycans in the urine resembled that of the elder boy at 5½ and 9½ years of age (Table 3), showing that at least in the latter half of the first decade in Hunter's syndrome the stage of development of the child does not affect the distribution of glycosaminoglycans in the urine, as might be expected with a recessively inherited metabolic defect.

Morquio's syndrome

The total output of glycosaminoglycan was considerably less than that of the present or reported cases of Hurler's or Hunter's syndromes, when determined as either uronic acid or hexose (Table 4). The amount of glycosaminoglycan recovered from the urine by the present procedure or that of Di Ferrante (1967) was almost the same (Table 4) showing that in this instance at least the present procedure did not result in losses, as has been suggested (Di Ferrante, 1967).

The molar proportion of hexose was equivalent to that of uronic acid in the glycosaminoglycans from the urine, in contrast with other Mucopolysaccharidoses, where uronic acid is in considerable

excess, for example in the case of Hunter's syndrome studied here. In Morquio's syndrome the excretion of keratan sulphate is increased (Pedrini, Lennzi & Zambotti, 1962; Robins, Stevens & Linker, 1963; Marateaux & Lamy, 1963). This glycosaminoglycan contains galactose and glucosamine in roughly equal proportions (Hirano, Hoffman & Meyer, 1961). When the glycosaminoglycans from the urine were subjected to ion-exchange chromatography on ECTEOLA-cellulose (Di Ferrante, 1967) no hexose was eluted in 0.9% sodium chloride, indicating an absence of glycoprotein in the preparation. Most of the uronic acid and hexose were eluted in 0.5M-sodium chloride, with lesser amounts in 1M-sodium chloride and none in 2M- and 4M-sodium chloride. If keratan sulphate and chondroitin sulphate were separate, much of the hexose due to keratan sulphate should have been eluted by 2M-sodium chloride (Antonopoulos, Fransson, Heinegård & Gardell, 1967) rather than by solutions of lower ionic strength. However, as hexose and uronic acid were eluted together, keratan sulphate and chondroitin sulphate would appear to be part of the same molecule, as they seem to be in other cases of Morquio's syndrome (Kaplan, McKusick, Trebach & Lazarus, 1968).

That keratan sulphate and chondroitin sulphate were part of the same molecule was also shown by comparing the elution from micro cellulose columns (Antonopoulos *et al.* 1964) of the glycosaminoglycan-cetylpyridinium complex from the urine before and after papain digestion, and measuring the uronic acid and hexose contents of the effluent fractions (Table 5). Should keratan sulphate and chondroitin sulphate be separate, the first and third fractions would contain mainly keratan sulphate and the second fraction chondroitin sulphate (Antonopoulos, Borelius, Gardell, Hamnström & Scott, 1961). Before digestion, however, both hexose and uronic acid were eluted together in all three fractions, whereas after digestion the distribution of hexose changed, so that there was none in the second fraction and a larger proportion than

Fig. 3. Solubility profiles on cellulose micro-columns of cetylpyridinium complexes of glycosaminoglycans isolated from the urine of two brothers (P.P. and T.P.) with Hunter's syndrome. Details are as in Fig. 2. Glycosaminoglycans (a) from patient P.P. at 6½ years of age; (b) from patient T.P. at 9½ years of age. Glycosaminoglycans from patient P.P. at 6½ years of age (c) after digestion with hyaluronidase; (d) from patient T.P. at 9½ years of age after digestion with hyaluronidase. CPC is cetylpyridinium chloride.

Table 4. Uronic acid and hexose excreted as glycosaminoglycans in the urine of a patient (T.M.) with Morquio's syndrome

Experimental details are given in the text. Values in parentheses, recovered by the procedure of Di Ferrante (1967).

	24 h output (mg)	Concentration (mg/l)
Hexose	4.59 (4.22)	5.88 (5.41)
Uronic acid	4.32 (4.13)	5.54 (5.30)

before in the third fraction (Table 5), showing that proteolysis had liberated keratan sulphate from chondroitin sulphate. Keratan sulphate varies in chain length (Laurent & Scott, 1964) and sulphate

content (Mathews & Cifonelli, 1965; Seno, Meyer, Anderson & Hoffman, 1965) and since both factors influence the solubility of cetylpyridinium complexes in salt solutions, 6M-hydrochloric acid was used in place of 2M-magnesium chloride to ensure that all keratan sulphate was eluted. Although normal skeletal keratan sulphate is largely soluble in 1% (w/v) cetylpyridinium chloride (Antonopoulos *et al.* 1961), the free chains of keratan sulphate liberated here by papain digestion were not, as the amount in this fraction did not increase (Table 5), despite the fact that the chains were rather short. When assessed by the elution of hexose from a column of Sephadex G-200 (Wasteson, 1969), they varied in size below about mol.wt. 5000. This suggests that the keratan sulphate in this patient's

Table 5. *Solubilities of cetylpyridinium complexes of glycosaminoglycans isolated from the urine of a patient (T.M.) with Morquio's syndrome, before and after proteolysis*

The material (2mg/ml) was dissolved in 0.2M-sodium acetate buffer, pH5.7, containing EDTA and cysteine, and 50 μ l of the solution applied to micro-cellulose columns (Antonopoulos *et al.* 1964) in duplicate. The columns were eluted with 1ml each of 1% (w/v) cetylpyridinium chloride, 0.6M-MgCl₂ and 6M-HCl. The remainder of the sample was incubated overnight at 37°C with 25 μ l of crystalline papain (for details see the text) and the procedure repeated. The hexose and uronic acid contents of 0.5ml portions of each fraction were determined.

Solvent	Content (% of total)			
	Before papain digestion		After papain digestion	
	Hexose	Uronic acid	Hexose	Uronic acid
1% (w/v) Cetylpyridinium chloride	40	39	28	41
0.6M-MgCl ₂	25	46	0	44
6M-HCl	35	15	72	15

urine may not be normal, possibly because it is abnormally sulphated. Indeed on cellulose acetate electrophoresis in 0.1M-sodium phosphate buffer, pH 7.2, with staining with Alcian Blue, most of the papain-digested material moved much faster than skeletal keratan sulphate (international reference standard; a gift from Dr A. J. Cifonelli, University of Chicago, Ill., U.S.A.).

By contrast with that of hexose, the distribution of uronic acid in the three fractions did not change after papain digestion (Table 5). Part of the material containing uronic acid would appear to be considerably degraded, since it was eluted in the first fraction (Table 5). Most of the rest was eluted by 0.6M-magnesium chloride and a small amount by 6M-hydrochloric acid, since no intermediate steps of molarity above 0.6M-magnesium chloride were used. The latter may consist of longer chains or be more sulphated than that eluted in the second fraction where normal chondroitin sulphate would appear (Antonopoulos *et al.* 1964).

Keratan sulphate and chondroitin sulphate form part of the same molecule in protein-polysaccharides of cartilage (Tsiganos & Muir, 1967, 1969). Not all the hexose will be attributable to keratan sulphate, because it also occurs in the linkage region of chondroitin sulphate (Rodén & Smith, 1966) and were the chains to be rather short a significant proportion of the hexose would be in this region. Moreover, the glucosamine/galactosamine molar ratio was 1:2.42, and not 1:1 as might be expected from the equimolar proportions of uronic acid and hexose that were found, indicating that only about one-third rather than half of the glycosaminoglycan was keratan sulphate. This is comparable with the proportion of keratan sulphate in normal protein-polysaccharides of cartilage of children of about 10 years of age (Gower & Pedrini, 1969). The proportion of keratan sulphate may be rather higher, however, because not all the

galactosamine will be attributable to chondroitin sulphate, since galactosamine occurs in the linkage region of skeletal keratan sulphate (Bray, Lieberman & Meyer, 1967), and as the chains were rather short this might account for an appreciable amount. The molar ratios thus do not provide exact estimates of the proportions of each glycosaminoglycan.

Keratan sulphate has not been identified elsewhere than in cartilage, nucleus pulposus and cornea, and hence disturbance of its metabolism should have less widespread effects than a disturbance of the metabolism of other more widely distributed glycosaminoglycans, which are affected in other types of Mucopolysaccharidosis (McKusick, 1966). Skeletal development is primarily affected in Morquio's syndrome, abnormalities developing where ligaments and tendons are inserted into cartilage (Ponseti, 1971). Normally there is little keratan sulphate in cartilage during the first decade of life, but it increases progressively with age (Kaplan & Meyer, 1959; Mathews & Glagov, 1966; Gower & Pedrini, 1969). Its seemingly premature appearance in Morquio's disease is detrimental to normal skeletal development, possibly because it may be structurally abnormal as suggested, in the present single example of the disease, by the small proportion of the keratan sulphate from the urine that was soluble in 1% cetylpyridinium chloride.

GENERAL DISCUSSION

Possible explanations of the underlying biochemical defects of the Mucopolysaccharidoses have been reviewed (Muir, 1969, 1971). It is unlikely that there is some defect in the formation of the linkage region because Helting & Rodén (1969a,b) showed that this is the first stage in the biosynthesis of glycosaminoglycans. Because of the specificities of the glycosyltransferases and the sequential

addition of the constituent sugars any defect in the formation of the linkage region would interrupt the production of glycosaminoglycan chains, which would lead to a deficiency rather than an excess.

Defective degradation of glycosaminoglycans has been widely assumed in the Mucopolysaccharidoses, particularly as there is often decreased activity of certain β -galactosidase isoenzymes in the organs (see Muir, 1971). Fratantoni, Hall & Neufeld (1969) showed that fibroblasts from patients lacked some factor present in the cells of other genotypes which corrected the abnormal accumulation of newly synthesized glycosaminoglycans by these cells when in culture for some time. The factors lacking in Hurler's and Hunter's syndromes were different and hence the same degradative enzyme is unlikely to be deficient in both diseases. The factor could not be identified with β -galactosidase, which moreover has not been shown to act on the non-terminal galactose residues of the glycosaminoglycans that accumulate in these diseases; nor does a deficiency of β -galactosidase explain why certain glycosaminoglycans accumulate rather than all those having galactose in the linkage region.

Recent work has shown that the hybrid structure of dermatan sulphate varies from one tissue to another (Fransson, Anseth, Antonopoulos & Gardell, 1970). A defect in the formation of the appropriate structure might seriously affect the metabolism of dermatan sulphate. Dorfman & Matalon (1969) have pointed out that little is known about how protein-polysaccharides are assembled and exported from the cell. The process may not be identical for all protein-polysaccharides. In different types of Mucopolysaccharidosis the export of one rather than another compound may be affected, resulting in its intracellular accumulation. Thereafter it would be broken down by the degradative enzymes in the cell. In pathological organs the glycosaminoglycans were degraded (Dorfman, 1964; Knecht *et al.* 1967). The great extent to which they were degraded in the liver and spleen of one case of Hurler's syndrome has been shown here. This evidence suggests that the degradation of glycosaminoglycans is not defective in the Mucopolysaccharidoses.

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