Urinary Acid Mucopolysaccharides in Normal Man and in Hurler's Syndrome.* (28478)

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The metabolism of acid mucopolysaccharides seems to be disturbed in several diseases of connective tissue(1,2). This disturbance is reflected in abnormal tissue storage and urinary excretion of these compounds. In most cases much larger than normal amounts of polysaccharides are excreted; however, there are indications that in some disorders the excretion pattern is abnormal, while the total amount is only slightly higher than normal. As there were also indications of an abnormal excretion pattern in carriers of Hurler's syndrome(3), it would appear that urinary excretion may reflect the tissue metabolism of mucopolysaccharides.

Therefore, it was thought to be of interest to investigate the exact mucopolysaccharide composition of urine from normal individuals, from cases of Hurler's syndrome, and from carriers of this disease.

Di Ferrante *et al.*, had shown(4) that the major mucopolysaccharide of normal urine was most likely chondroitin sulfate A; minor constituents would not have been detected by his method.

Experimental and results. The following methods for uronic acids were used: carbazole(5), orcinol(6), and naphthoresorcinol (7). Paper chromatography of polysaccharides was carried out by the method of Spolter and Marx(8) with slight modifications. The papers were stained with alcian blue or methylene blue(9). For enzymatic digestion products a butanol-acetic acid solvent(10) was used, and the papers were sprayed with an aniline(11) or silver reagent(12).

Normal urine. Total acid mucopolysaccharides were isolated from 8 liters of pooled normal urine(4); 45 mg of relatively pure polysaccharide with the following analysis were obtained. Uronic acid (carbazole) = 24%, uronic acid (orcinol) = 13%. Paper chromatography of the sample was carried out in an ammonium formate solvent system. When the original system(8) is slightly modified by using solvent No. 1.5 on top and solvent No. 2 in the jar, chondroitin sulfate A and chondroitin sulfate C‡ migrate at different rates. The major component of the isolated mucopolysaccharide migrated like CSA in this system. Weak spots, indicating the presence of other compounds, were also detected. To verify the excretion of CSA, a small-scale digestion of the isolated compound with testicular hyaluronidase was carried out, and the solution analyzed for acetylglucosamine(13) (Table I).

TABLE I. Comparison of Color Values.

Compound	Acetylglucosamine (γ/ml)
CSA	6.0
CSC	26.0
Urinary MPS	8.0*

* Corrected for uronic acid content.

To identify small amounts of other polysaccharides, the presence of which was indicated by the paper chromatography, the CSA was removed by digestion with testicular hyaluronidase. The crude polysaccharide (20 mg) was digested in 2 ml of 0.1 M acetate buffer, pH 5.0 with 600 TRU of testis hyaluronidase (California Biochemical Co.) at 37°. After 24 hours the same amount of enzyme was again added and the incubation continued for 2 days. The solution was then dialyzed for 3 days against 100 ml of distilled water. The dialyzate was evaporated *in vacuo* to 2 ml, and 0.01 ml aliquots were

* Supported in part by a U. S. Public Health Service Grant.

† Taken in part from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philoscphy, Univ. of Utah.

‡ Abbreviations: CSA, chondroitin sulfate A; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; HS, heparitin sulfate; MPS, mucopolysaccharide.

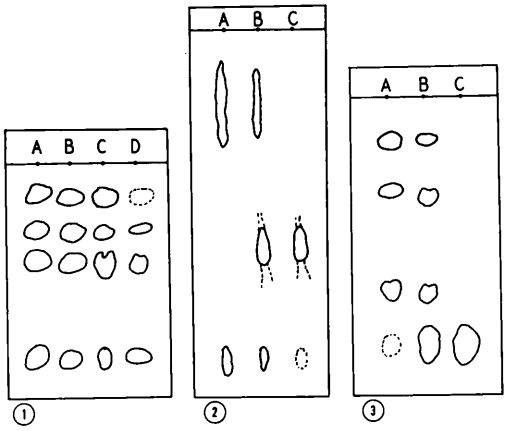

FIG. 1. Reproduction of paper chromatogram of testis hyaluronidase digests. (A) Dialyzate obtained from digest of MPS from normal human urine. (B) Control, CSA oligosaccharides. (C) Hyaluronidase digest of MPS from Hurler's urine. (D) Hyaluronidase digest of MPS from Hurler's urine.

FIG. 2. Reproduction of chromatogram of MPS in ammonium formate solvent. (A) Standard CSB. (B) MPS from normal urine. (C) Standard HS.

FIG. 3. Reproduction of chromatogram of flavobacterium enzyme digests. (A) Hydrolyzate of standard HS. (B) Hydrolyzate of MPS from normal urine by flavobacterium heparinase. (C) Acetylglucosamine standard.

chromatographed on paper with a testis hyaluronidase digest of known CSA as control (Fig. 1). It can be seen that the oligosaccharide spots of the urinary compound are identical with those of the control. No oligosaccharides corresponding to a hyaluronic acid digest could be detected.

To the non-dialyzable portion a small amount of Lloyd's reagent was added, the solution stirred and centrifuged. The supernatant was brought to 5% in sodium acetate, and 0.5 N in acetic acid and 2 volumes of ethanol were added. The solution was kept in the refrigerator for 1 day, centrifuged and the precipitate washed with 80%, 95%, absolute ethanol and ether and dried in a desiccator over P_2O_5. The amount obtained was 6 mg, about one-third of the starting material. Analysis: uronic acid (carbazole) = 23%, uronic acid (orcinol) = 13%, ratio carbazole/orcinol = 1.8. Paper chromatography of this material in the ammonium formate solvent showed the presence of CSB, heparitin sulfate, and a faster moving component (Fig. 2). Paper chromatography of an acid hydrolyzate showed the presence of glucosamine and galactosamine in a ratio of 3:2(14).

To confirm the presence of HS, a small-scale 24-hour digestion with flavobacterium heparinase was carried out under conditions used previously(15). A chromatogram of the digest is shown in Fig. 3, with a control digest of known HS.

As not enough material remained for fractionation of the mixture containing CSB and HS, the same procedures as above were carried out starting with 7 liters of pooled urine. The final product isolated after hyaluronidase digestion weighed 6.0 mg. This material was dissolved in 5% sodium acetate, 0.5 N in acetic acid, and fractionated with ethanol. The fractions shown in Table II were obtained. The carbazole-orcinol ratios and the paper chromatography show all fractions to be mixtures of CSB and HS. The fast CSB spot will be discussed. These data indicate that about 20% of the total MPS excreted consists of a mixture of CSB and HS, containing about equal amounts of each. A small amount of kerato sulfate may also be present. Polysaccharides from the urine of 2 children, 5 and 8 years old, were isolated by the same method as above. Though the total amount isolated (10 mg per liter) was higher than in adults, the excretion pattern was the same.

Urine from patients with Hurler's syndrome. In Hurler's syndrome, large amounts of CSB and/or HS are excreted in the urine. Total MPS excretion is from 10 to 40 times the normal amount. As the large quantity of these 2 polysaccharides would overshadow the excretion of small amounts of others, the following experiment was carried out.

MPS was isolated from 2 cases of Hurler's syndrome by a described method(16). In 1 case, mostly CSB was excreted; in the other, mostly HS. Isolated polysaccharide (5 mg) from each case was incubated with testicular hyaluronidase as above. Increase in reducing sugar was found to be about 7% of that obtained with pure CSA. An aliquot of each digest was chromatographed on paper (Fig. 1). These data show that CSA is present as a minor component of the polysaccharides excreted in this disease.

MPS excretion in carriers of Hurler's syn-

TABLE II. Analyses of Ethanol Fractions.

Fraction	Weight (mg)	Carbazole (%)	Orcinol (%)	Carbazole/ orcinol	Paper chromatography*
40% EtOH	1.0	20	13	1.5	CSB, HS
50% "	1.5	37	34	1.1	HS, f. CSB
60% "	2.0	22	21	1.0	HS, f. CSB, KS?
Pure CSB		13	45	.3	CSB, f. CSB
" HS		45	15	3.0	

* Ammonium formate solvent. f. CSB is a fast spot associated with CSB. KS is a spot migrating at the same rate as kerato sulfate.

drome. It had been reported that carriers of this disease could be detected by an abnormal MPS excretion pattern(3), as measured by the ratio of 2 color reactions for uronic acids. The carbazole and naphthoresorcinol reactions were carried out(3) on urine samples from the father of an autosomal recessive patient and from the mothers of 2 other patients. No really significant differences from normal urine could be detected. The ratios of the carriers, including 2 cases where the affected offspring excreted mainly CSB, varied from 1.6 to 3.2, and of several normals from 1.1 to 5.6. The naphthoresorcinol method worked well on pure samples of MPS, giving the values reported; however, it seemed to give unreliable results on crude urinary samples. The reaction appeared to be non-linear, giving only 25-50% increase in color when the aliquot (obtained from urine) taken was doubled. All attempts to improve the method were unsuccessful.

In addition, MPS were isolated from the urine of 2 mothers of Hurler's patients by the above methods. The total amounts of polysaccharide obtained (6 mg/l) in both cases, and the relative amounts of CSB, HS, and CSA appeared to be the same as in normal urine. Small differences could, of course, not be detected by these relatively crude methods.

Mittwoch(17) had also indicated that carriers could be detected by counting the number of segments of neutrophil nuclei. Smears from 3 mothers of Hurler's patients showed no differences from normal.

Discussion. The excretion pattern of MPS in normal urine is of interest for several reasons. First, knowledge of the normal pattern is needed for comparison with the excretion in connective tissue disorders such as Hurler's and Marfan's syndromes, Morquio and Morquio-Ullrich diseases, and others. Also, the excretion may reflect the metabolism of these compounds in connective tissue, a view supported by the finding that total excretion is much higher in children than in adults (18). It is interesting, therefore, that only CSA, CSB, and HS could be detected, particularly as HS occurs in only relatively small amounts in connective tissue. This may indicate that HS, a compound of unknown biological function, may be quite active metabolically.

HS has been identified in normal urine by the carbazole-orcinol ratio, paper chromatography, the presence of glucosamine in a testis hyaluronidase resistant residue, and by the products obtained by flavobacterium digestion. After a preliminary report of this work had been presented(19), King *et al.* confirmed the presence of HS in normal urine(20).

The presence of CSB has been shown by carbazole-orcinol ratios, paper chromatography, and the presence of galactosamine in a testis hyaluronidase resistant residue. As the fast moving component associated with CSB is present after testis hyaluronidase digestion, it cannot be CSA, from which it cannot be differentiated by chromatography. Also, this fast component occurs in CSB, with good analysis, isolated from other sources such as pig skin, Hurler's urine, and β-heparin. Preliminary fractionation studies indicate a similar, though not identical, analysis to CSB. These data reopen the problem of whether CSB is a single polysaccharide or a family of compounds as suspected previously(21).

The finding that Hurler's syndrome urine contains small amounts of CSA in addition to CSB and HS and that normal urine contains

small amounts of HS and CSB in addition to CSA, allows a somewhat different viewpoint of this disease. It would appear that an abnormally high excretion of a given MPS is superimposed on a basically normal excretion pattern, emphasizing more strongly that the metabolic defect involves only 1 or 2 out of 7 possible MPS.

It had been reported that kerato sulfate is excreted in Morquio-Ullrich disease(22). As shown above, there are only small amounts of this MPS in normal urine. What is of additional interest is that the total excretion of MPS in this disorder is not much higher than normal and that the abnormal excretion is again superimposed on a normal pattern.§

Methods proposed for detection of carriers in Hurler's syndrome(3,17) have so far not been sufficiently reliable to be used in genetic studies, which would be of considerable importance.

Paper chromatography of MPS in the ammonium formate system of Spolter and Marx (8) seems to be very sensitive to slight changes in conditions. It is therefore advisable to use standards in each run and to vary solvent compositions slightly to obtain desired results under different environmental conditions.

Summary. Chondroitin sulfate A comprised 80% of the mucopolysaccharides excreted in normal human urine. The remaining 20% consisted of approximately equal amounts of chondroitin sulfate B and heparitin sulfate. In Hurler's syndrome the urine contained an excessive amount of chondroitin sulfate B and/or heparitin sulfate but the same amount of chondroitin sulfate A as normal urine. No significant differences from the normal excretion pattern were detected in carriers of this disease.

§ K. Terry, A. Linker and M. Robbins, in preparation.

The authors wish to thank Drs. E. M. Lahey and P. F. Bray, Dept. of Pediatrics, Univ. of Utah, and Dr. E. C. Burke, Mayo Clinic, Rochester, Minn., for help in obtaining urine from the Hurler's patients, and Miss K. Seymour for excellent technical assistance.

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Received April 16, 1963. P.S.E.B.M., 1963, v113.