Isolation and Identification of Keratosulphate in Urine of Patients Affected by Morquio-Ullrich Disease.* (27668)

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Mucopolysaccharides (MPS) are normally excreted in urine in amounts approximating 3-15 mg/24 hr (young girls 4.8-8.10 mg/24 hr)(1). Chondroitinsulphates A or C (ChS A, C) are present in largest amount(2), but Hyaluronic acid (HA)(3). Chondroitinsulphate B (ChS B) and Heparinmonosulphate (HMS)(4) can also be identified.

Increased amounts of MPS may be found in urine of patients with osteochondrodystrophies of growth. Several workers have shown deposits of MPS in liver, spleen, brain and other tissues of patients with Hurler's disease (gargoylism). Large amounts of MPS, mainly ChS B and HMS are found in the urine of these patients(5,6,7). Increased MPS urinary excretion has been reported recently in patients with Morquio-Brailford's disease, but the type of MPS excreted was not identified(8). An increase of MPS urinary excretion was found in 2 patients with Morquio-Ullrich's disease. The urinary MPS in these patients were identified as ChS A or C, a small amount of ChS B, and a large fraction which appeared to be similar to keratosulphate (KS)(9).

We will present here isolation and identification of KS from the urines of 3 patients with Morquio-Ullrich disease. Clinical findings will be published later.

Materials and methods. The urines from 3 female patients (B.M.T. 18 months old, B.R. 30 months old, and M.L. 8 years old) from the Rizzoli Orthopedic Institute in Bologna, were collected over a period of several days; the urines of M.L. were carefully collected in the hospital, while the urines of B.R. and B.M.T. were collected at home and probably do not represent complete 24-hr samples. At the end of the collection period, during which the samples were kept at -20°C, the urines were thawed, those coming from a single patient were pooled and pH adjusted to 5-6 with 2N HCl. The MPS were precipitated by addition of a warm solution of Cetyltrimethylammonium bromide (Cetavlon) and purified according to the technics of Meyer *et al.*(6) and Di Ferrante and Rich(2) with slight modifications.

The Cetavlon-MPS complexes, still containing proteins and other impurities, were collected by centrifugation and washed several times with sodium acetate-saturated 95% ethanol to dissociate the complexes. The residue, containing the sodium salt of the crude MPS was then dissolved in 10% sodium acetate, the PH adjusted to 9.0 with 3N NaOH, and the insoluble material removed by centrifugation.

The sodium salts of the MPS were precipitated with ethanol, collected by centrifugation, dissolved in 0.1 M phosphate buffer pH 6.6 and subjected to digestion with papain for 24 hr at 67°C. The resulting solution was dialyzed for 24 hr against frequently changed distilled water: The MPS were precipitated with ethanol, treated with chloroform-amylalcohol to remove residual proteins(10) and finally with Lloyd's reagent. The calcium salts of the MPS were fractionally precipitated with ethanol. Three fractions were collected in each case, at alcohol concentrations of 40, 50 and 60%. No precipitate was obtained when the alcohol concentration was below 40%.

On the fractions thus obtained, uronic acid was determined by the carbazol method of Dische(11), neutral sugars by the anthrone method(12), aminosugars by the Elson-Morgan method, as modified by Schloss(13), sulphate content by the method of Dodgson with $BaCl_2$ and gelatin(14). The last 2 determinations were performed after hydrolysis with 4N HCl at 110°C for 3 hr in sealed am-

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poule. Chromatographic analyses of the sugars were performed after hydrolysis of the MPS with Dowex 50 H and resin in a sealed ampoule at 105°C with occasional shaking (15). The chromatographic solvent was composed of pyridine, n-butanol, water (1:3:1.5)(16). The aminosugars were chromatographed after conversion to pentoses (17). The spots were detected with aniline-hydrogen phtalate(18).

Results. The preliminary analyses showed that both fractions precipitated at 50 and 60% ethanol had a high content of a neutral sugar, chromatographically identified as galactose. The paper chromatograms of these fractions showed also the presence of glucosamine together with minor amounts of galactosamine. These first data suggested therefore that these fractions contained a keratosulphate-like MPS, together with contaminating ChS A or C that was probably present in the fractions precipitated with 40% ethanol.

To identify the first component we tried to eliminate the ChS by digestion with hyaluronidase. The fractions having the highest content of galactose were incubated with testicular hyaluronidase (Jalergon Serono, 100 units/mg of MPS) for 3 hours at 37°C in 0.1 M Acetate buffer pH 5.6 containing 0.15 M NaCl. The enzyme was then inactivated by heating for 2 minutes at 100°C and the denatured protein was removed by centrifugation. The supernatant solutions were dialyzed for 6 hours at room temperature against distilled water. Acetic acid and calcium acetate, and then ethanol to a concentration of 60% were added to the solutions to precipitate the MPS that had not been attacked by the enzyme. The purified MPS were analyzed for Uronic acid which was no longer present. The chromatographic analysis showed the presence of galactose and glucosamine only.

The infrared spectra were determined on the fractions purified as described above, using a Perkin-Elmer mod. 21 spectrophotometer with KBr pellets. The spectra of these fractions (Fig. 1) are practically identical with each other and with that of a known sample of KS kindly supplied by Prof.

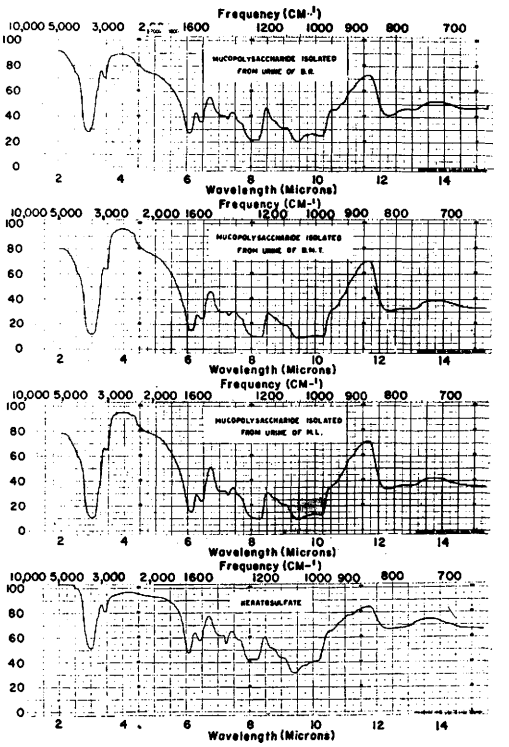

FIG. 1. Infrared spectra of the MPS isolated from the urines of three patients with Morquio-Ullrich disease and of a known sample of KS.

K. Meyer, New York. Since the preliminary analytical values and the infrared spectra were identical for all 3 samples, the small remaining amounts of material from the urines of B.M.T. and M.L. were pooled together, to obtain a sufficient specimen for a complete chemical analysis. The residual material from the urines of B.R., of greater consistency, was analyzed separately. Data of these analyses (Table I) show that both samples are of

TABLE I. Chemical Data of MPS Isolated from Urines of 3 Patients with Morquio-Ullrich Disease.

	MPS isolated from urines of M.L. and B.M.T.*		MPS isolated from urines of B.R.	
	%	Molecular ratio	%	Molecular ratio
Hexosamines	28.21	1.00	30.21	1.00
Uronic acids	1.37	.01	0	0
Neutral Hexoses	30.23	1.06	30.56	1.05
Sulphates	15.50	1.02	14.91	.89

* Sample obtained by mixing residual MPS from urines of patients M.L. and B.M.T.

TABLE II. Amounts of MPS Extracted from Urines of 3 Patients with Morquio-Ullrich Disease and Percentages of KS as Calculated from Content in Neutral Sugars of MPS.

	M.L.	B.R.	B.M.T.
Total MPS/liter of urine	22.1 mg	17.1 mg	30.5 mg
KS %	46.2	37.6	39.4

identical composition and contain approximately equimolar amounts of glucosamine, galactose and sulphate. These results and the infrared spectra (Fig. 1) indicate that MPS fraction studied is KS. Neither ChS B nor HMS were found in the urines of our patients. Amounts of MPS extracted from the urines and percentages of KS, as calculated from the content of neutral sugars of the MPS, are reported in Table II.

Conclusions. This study points to a disorder of the MPS metabolism in Morquio-Ullrich disease different from that found in Hurler's disease. Excretion of KS appears to be pathognomonic of Morquio-Ullrich disease although study of more cases of this and other osteochondrodystrophies is needed to corroborate our finding.

Summary. The MPS present in the urines of 3 patients with Morquio-Ullrich's disease have been studied. In all 3 cases a substantial amount of KS has been identified. The identification has been based on chemical, chromatographic and infrared data. This MPS has hitherto never been identified in urine, either normal or pathological. Neither ChS B or HMS was found in the urines of these children. The metabolic disorder of the MPS in Morquio-Ullrich's disease appears to be different from that observed in Hurler's disease.

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Morphology of the Collecting Tubules in Diuretic and Antidiuretic Rats. (27669)

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The mechanism of action of antidiuretic hormone (ADH) is still obscure. Ginetzinsky has proposed that the action of ADH is mediated through hyaluronidase(1). He showed that in rats during antidiuresis induced either by dehydration or by administration of exogenous vasopressin, the excreted urine contains a large amount of hyaluronidase. Similar results were found during osmotic diuresis. On the other hand, when water diuresis was in-