Pattern of Urinary Acid Mucopolysaccharide Excretion in Normal Male Children

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ORII, T., MINAMI, R. and NAKAO, T. *Pattern of Urinary Acid Mucopolysaccharide Excretion in Normal Male Children.* Tohoku J. exp. Med., 1972, 108 (4), 369-375 — Qualitative and quantitative determination of urinary acid mucopolysaccharide has been studied by several methods including ECTEOLA-cellulose column chromatography, Dowex 1×2 column chromatography, electrophoresis, and enzymatic digestion with testicular hyaluronidase and chondroitinase. To avoid the possibility of loss of urinary acid mucopolysaccharides, especially of keratosulfate, cetylpyridinium chloride as an acid mucopolysaccharide precipitant and dialytic procedure were not used. 1) The normal male children excreted 8.0 mg of uronic acid and 8.5 mg of hexosamine/liter of urine, and the uronic acid (mg)/creatinine (g) ratio was 12.8 and the hexosamine (mg)/creatinine (g) ratio was 13.8. 2) The urinary acid mucopolysaccharides in the normal male children comprised mostly chondroitin sulfate A (47.6%) and chondroitin sulfate C (34.2%). 3) Non-sulfated acid mucopolysaccharides comprised 10.4% of the total acid mucopolysaccharide excreted, consisted of hyaluronic acid (3.3%) and chondroitin (7.1%) respectively. 4) The remaining acid mucopolysaccharides consisted of heparitin sulfate (3.4%), chondroitin sulfate B (2.8%) and keratosulfate (1.6%). ——— urinary acid mucopolysaccharide; normal children

Urinary acid mucopolysaccharides in normal adults and children were quantitatively studied by Berenson and Dalferes (1965), Varadi *et al.* (1967), Orii (1968 a) and Lee (1970). However, these works with acid mucopolysaccharides have been done using quaternary ammonium salts, such as cetyltrimethyl ammonium bromide or cetylpyridinium chloride (CPC) and dialytic procedure. High solubility mainly of keratosulfate in the presence of excess quaternary ammonium salt and loss of urinary acid mucopolysaccharides during dialysis are indicated by several workers (Antonopoulos *et al.* 1961, Di Ferrante 1967, Muir *et al.* 1963, Thompson and Castor 1966, Constantopoulos 1968). For these reasons, pattern of urinary acid mucopolysaccharide excretion in normal male children has been also quantitatively studied using ECTEOLA-cellulose and Sephadex G-25 columns instead of quaternary ammonium salt and dialytic procedure.

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MATERIALS AND METHODS

Nine liters of pooled urine were collected with thymol as a preservative from 75 healthy children (3–5 years) and kept frozen until assay. The procedure for separation and isolation of various acid mucopolysaccharides is summarized in Fig. 1.

Isolation of acid mucopolysaccharides.—The urine was centrifuged at 1,200 g for 10 min and each 3 liters of supernatant were applied to a column (6.0×140 cm) of ECTEOLA-cellulose (capacity, 0.38 mequiv/g, Serva Co.). The column was washed with 12 liters of 0.9% NaCl, and subsequently, acid mucopolysaccharides were eluted with 12 liters of 2.0 M NaCl by the Di Ferrante procedure (1967) respectively. Each eluate was concentrated in a flash evaporator and further desalted on Sephadex G-25 column. Each fraction containing uronic acid was combined and acid mucopolysaccharides were precipitated by the addition of 4 volumes of sodium acetate-saturated 95% ethanol and washed 3 times with 80% ethanol and ether. The total acid mucopolysaccharide obtained, was dissolved in a small volume of water.

Fig. 1. Procedure for the isolation of urinary acid mucopolysaccharides.

Digestion with testicular hyaluronidase and chondroitinase.—These procedures were carried out as described by Saito *et al.* (1968) with some modifications. An aliquot of the total acid mucopolysaccharide was incubated at 37°C for 24 hours with 2,000 units of testicular hyaluronidase (770 units/mg, Sigma Co.) per mg of uronic acid in NaCl-acetate buffer (NaCl 8.0 g and 1.35 g of sodium acetate were dissolved in 100 ml of 0.23% (v/v) aqueous acetic acid), pH 5.4. The undigested acid mucopolysaccharides were recovered and purified by sodium acetate-ethanol procedure as described above. Furthermore, an aliquot of the testicular hyaluronidase-undigested acid mucopolysaccharides was incubated at 37°C for 24 hours with 5 unit of chondroitinase-ABC (Seikagaku Kogyo Co., Tokyo) per mg of uronic acid in Tris buffer (3 g of trishydroxymethylaminomethane, 2.4 g of sodium acetate, 1.46 g of NaCl and 50 mg of crystalline bovine serum albumin in 100 ml of 0.13 N HCl) pH 8.0, and the undigested acid mucopolysaccharides were precipitated and purified as sodium salts using the same technique described above.

Enzymatic assay of isomeric chondroitin sulfates.—In order to determine relative proportions of chondroitin sulfates, A, B, C, hyaluronic acid and chondroitin, the method 1 of Saito *et al.* (1968) using chondroitinases ABC and AC was employed. The reaction mixtures contained, in 50 μ l, 20 μ l of total acid mucopolysaccharide (containing 80 μ g as uronic acid), 10 μ l of Tris buffer, and either 10 μ l (0.9 unit) of chondroitinase AC or chondroitinase ABC 10 μ l (0.3 unit). The blank mixture differed in that it lacked the enzymes. These mixtures were incubated at 37°C for 3 hours and then spotted on a paper (Toyo Roshi No. 51A). After desalting by chromatography in n-butanol-ethanol-water (52:32:16 by vol.) for 48 hours, descending paper chromatography was performed in n-butyric acid–0.5 M ammonia (5:3, by vol.) for 36 hours. The separated spots of the unsaturated disaccharides were detected with a Mineralight. The regions containing disaccharides were cut out and eluted with 0.01 N HCl solution. After centrifugation, the absorbance of the supernatant solution was measured at 232 m μ against the paper and reagent blank. The proportions of chondroitin sulfates A, B, C, hyaluronic acid and chondroitin were calculated as described by Saito *et al.* (1968).

Fractionation of chondroitinase-ABC-undigested acid mucopolysaccharides by column chromatography on Dowex 1 $\times$ 2.—The chondroitinase-ABC undigested acid mucopolysaccharides were applied on to a column (1.0 $\times$ 44 cm) of Dowex 1 $\times$ 2 (Cl⁻) 200–400 mesh which was eluted with only both 1.75 M and 4.0 M NaCl because the amount of acid mucopolysaccharides applied was very small. Each fraction eluted was desalted on Sephadex G-25 column respectively and concentrated to dryness in a flash evaporator, and then residue was dissolved in a small volume of water.

A number of analytical determinations was carried out: uronic acid (Dische 1947), hexosamine (Svennerholm 1956), neutral carbohydrate (Smith and Montgomery 1956), total sulfate (Dodgson and Price 1962), N-sulfate (Lagunoff and Warren 1962), quantitative estimation of glucosamine/galactosamine ratio (Gardell 1958), creatinine (Bonsnes and Taussky 1945), electrophoretic studies on cellulose acetate (Seno *et al.* 1970) with alcian blue stain and paper chromatographic identification of uronic acid (Radhakrishnamurthy and Berenson 1963).

Acid mucopolysaccharide samples were gifts from Dr. J.A. Cifonelli, University of Chicago, and Professor K. Meyer, Yeshiva University in New York.

RESULTS

Acid mucopolysaccharide-uronic acid and -hexosamine contents in 9 liters of urine were 72.5 mg and 77.4 mg, respectively, and uronic acid (mg) to creatinine (g) ratio and hexosamine (mg) to creatinine (g) ratio were 12.8 and 13.9. Table 1 indicates that most of the acid mucopolysaccharides excreted consists of galactosamine and is very susceptible to testicular hyaluronidase. However, it

TABLE 1. *Analytical data on urinary acid mucopolysaccharides of normal children before and after digestion with testicular hyaluronidase, and after digestion with chondroitinase-ABC*

	Recovery		Uronic acid (molar ratio*)	Glucosamine: galactosamine
	% of hexosamine	% of uronic acid		
Before digestion with testicular hyaluronidase	100.0	100.0	0.86	14.5:85.5
After digestion with testicular hyaluronidase	7.8	6.3	0.69	64.4:35.6
After digestion with chondroitinase-ABC	4.9	3.6	0.82	

* Molar ratios based on hexosamine=1.00

TABLE 2. *Fractionation of urinary chondroitinase-ABC undigested acid mucopolysaccharides of normal children on Dowex 1 resin*

Fraction (M NaCl)	Recovery % of hexosamine	Uronic acid (molar ratio*)	Total sulfate (molar ratio*)	N-Sulfate (molar ratio*)	Neutral carbohydrate (molar ratio*)
1.75	68.7	0.80	1.21	0.44	1.34
4.0	31.3	trace	1.76	trace	1.79

* Molar ratios based on hexosamine=1.00

also indicates that 7.8% of the hexosamine in the total acid mucopolysaccharide was stable to testicular hyaluronidase, of which 35.6%, that is, approximately 2.7% of the hexosamine in the total acid mucopolysaccharide was galactosamine. Furthermore, testicular hyaluronidase-undigested acid mucopolysaccharides were not digested by chondroitinase-AC at all, however, 62.8% of those based on hexosamine, that is, 2.9% of the total acid mucopolysaccharide-hexosamine was digested by chondroitinase-ABC. The presence of iduronolactone was also demonstrated in an acid hydrolysate of the testicular hyaluronidase-undigested acid mucopolysaccharides by means of paper chromatography. From these data it appears that chondroitin sulfate B content is approximately 2.8% of the total acid mucopolysaccharide. As shown in Table 2, 1.75 M NaCl fraction from Dowex column which contained 68.7% of the acid mucopolysaccharide-hexosamine eluted, had a molar ratio of uronic acid to hexosamine to total sulfate to N-sulfate to neutral carbohydrate of 0.80:1.00:1.21:0.44:1.34 and the 4.0 M NaCl fraction showed values of trace:1.00:1.76:trace:1.79. The electrophoretic pattern in chondroitinase-ABC undigested acid mucopolysaccharides showed two fragments by alcian blue staining, but these fragments, probably containing heparitin sulfate and keratosulfate, migrated more slowly than standard heparitin sulfate and keratosulfate. These findings indicate that 1.75 M NaCl fraction is composed mostly of heparitin sulfate, whereas 4.0 M NaCl fraction contains mostly keratosulfate. The relative proportions of chondroitin sulfates, A, B, C, hyaluronic acid

TABLE 3. *Acid mucopolysaccharide composition of normal urine*

Acid mucopolysaccharide constituent	Berenson & Dalferes (Adults, 1965) %	Varadi <i>et al.</i> (Adults, 1967) %	Orii (3-5 yrs, 1968) %	Lee (4-16 yrs, 1970) %	Authors (3-5 yrs) %
Hyaluronic acid	-	1	0.5	?	3.3
Heparitin sulfate	14-27	8	5.4	15.7	3.4
Non-sulfated chondroitin sulfate	12-22	25	5.7	?	7.1
Chondroitin sulfate A	4-20	31	38.5	48.6	47.6
Chondroitin sulfate B	4-22	1	1.9	-	2.8
Chondroitin sulfate C	22-39	34	40.4	29.8	34.2
Keratosulfate	1-11	1	7.9	+	1.6

and chondroitin by method 1 of Saito *et al.* (1968) were 50.0:3.1:35.9:3.5:7.5. The percentage of chondroitin sulfate B in total acid mucopolysaccharide was based on the percent recovery of the undigested-galactosamine containing acid mucopolysaccharide after testicular hyaluronidase digestion. The relative proportions of chondroitin sulfates, A, B, C, hyaluronic acid and chondroitin in total acid mucopolysaccharide were calculated from the percentage of the chondroitin sulfate B. Furthermore, the relative proportions of heparitin sulfate and keratosulfate were also determined from the percent recovery of the chondroitinase-ABC undigested acid mucopolysaccharides and each acid mucopolysaccharide-hexosamine content eluted with 1.75 and 4.0 M NaCl respectively (Tables 1 and 2). Table 3 gives the approximate proportions of the various acid mucopolysaccharides in normal human urine studied by several workers and authors.

DISCUSSION

Orii (1968 a) indicated that all of the acid mucopolysaccharides found in normal adult urine could also be found in urine of normal male children. Varadi *et al.* (1967) reported that approximately 25% of the total acid mucopolysaccharide in normal adult urine was non-sulfated chondroitin sulfate (chondroitin). In contrast, Orii (1968 a) showed that chondroitin constituted only approximately 6% of the total acid mucopolysaccharide in normal male children. Furthermore, Orii (1968 b) indicated that approximately 20.2% of the total urinary acid mucopolysaccharide in normal adults and approximately 6.6% of that in normal male children were non-sulfated acid mucopolysaccharides, and that the proportions of the non-sulfated acid mucopolysaccharides from normal adults are much higher than those from normal children. The data presented confirm with different procedure that approximately 7.1% of chondroitin is also present in urine of normal Japanese male children. As shown in Table 3, the present study indicates that the recovery rates of the chondroitin sulfates A, C, hyaluronic acid and keratosulfate differ from those reported by Orii (1968 a). However, it also indicates that the present work is similar to the results reported by Orii (1968 a),

in respect of the proportions of the other various acid mucopolysaccharides. Thus, under optimal conditions, the results obtained by the ECTEOLA-cellulose column method seem to be in good agreement with those obtained with the precipitation method. To elucidate the pathogenesis of genetic mucopolysaccharidoses, the complete structures of both urinary and visceral acid mucopolysaccharide-protein complexes should be accurately analysed.

Acknowledgment

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