Mucopolysaccharide Excretion in Patients with Hurler's Syndrome, Their Families, and Normal Man.* (31292)

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Since Brante(1) suggested that Hurler's syndrome was a mucopolysaccharidosis, numerous reports have appeared on the abnormal urinary excretion of mucopolysaccharides in these individuals; and it is well established that chondroitin sulfate B and/or heparitin sulfate are excreted in the urine in abnormal amounts. Hurler's syndrome is not a single entity, and several different types occur. Genetic studies differentiate two hereditary forms: an autosomal recessive and sex-linked recessive, and mucopolysaccharide excretion patterns indicate the existence of 4 or 5 different types. Clinical, radiological, biochemical and genetic findings in the various types have recently been reviewed(2,3). The heterozygotes of Hurler's syndrome are asymptomatic and the reports(4,5,6,7,8,9) concerned with the detection of heterozygotes by abnormal urinary mucopolysaccharides are not consistent. Disorders similar to Hurler's syndrome may exist in other mammals as Lorincz(10) and McIlwain and Eveleth(11) have reported abnormal mucopolysaccharide excretion in snorter dwarf cattle, but Mayes *et al*(12) and Tyler *et al*(13) were unable to measure any qualitative or quantitative difference in mucopolysaccharide excretion in cattle within the same age group.

The excretion of mucopolysaccharides in the urine of normal man appears to be more complex than originally reported. Chondroitin sulfate A and/or C was first demonstrated to be the major mucopolysaccharide in normal human urine(14), but more recently heparitin sulfate(6,15,16,17), kertosulfate(6,17), chondroitin sulfate B(6,16,17), and products of partial and complete desulfation of chondroitin sulfate A or C(16,17) have been identified in normal human urine.

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In this study, chondroitin sulfates and total mucopolysaccharides were measured in urine specimens from normal humans of various ages and of both sexes. Mucopolysaccharides were also measured and identified in patients with Hurler's syndrome and in families with affected children.

Materials and methods. Urine samples from patients with Hurler's syndrome and from other patients for age comparisons were obtained from state schools for the mentally retarded. Urine was also obtained from 3 families which had a child with Hurler's disease. Urine specimens from humans of both sexes and of various ages were also obtained from a local hospital. For isolation of the mucopolysaccharides, the amount of urine varied from 5 to 40 ml depending upon availability, age, and whether from Hurler's patients or normal subjects.

Mucopolysaccharides were isolated from urine by a modification of the method by DiFerrante and Rich(18) as previously described(12). The amount of total mucopolysaccharides was estimated by determining the uronic acid content by the carbazole method as modified by Bitter and Muir(19). Chondroitin sulfate B was estimated by the ratio of carbazole to naphthoresorcinol color reactions for uronic acid (C/N ratio) as described by Teller *et al*(5). The method described by Pelzer and Staib(20) was employed for the naphthoresorcinol color reaction for uronic acids. Total chondroitin sulfates were determined by the chondroitinase method of Mayes and Hansen(21). Creatinine was determined by the picrate method(22). Paper chromatography of mucopolysaccharides was carried out by the method of Spolter and Marx(23) employing their Type III (60% 0.04 M ammonium formate buffer, pH 4.3, and 40% isopropyl alcohol) solvent system.

Results. Mucopolysaccharide excretion in normal man. There was some variation in the amount of mucopolysaccharides in differ-

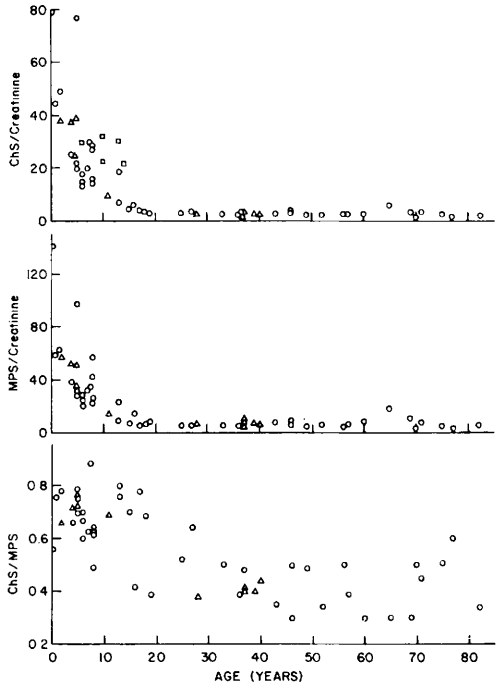

FIG. 1. Urinary mucopolysaccharides. ○—Controls, △—Parents and sibs, □—Hurler's. 1A (top). Amount of chondroitin sulfates (ChS) in relation to creatinine in urine samples from humans of various ages. 1B (middle). Total quantity of mucopolysaccharides (MPS) in relation to creatinine in urine samples from humans of various ages. 1C (bottom). Ratio of chondroitin sulfates (ChS) to mucopolysaccharides (MPS) in urine samples from humans of various ages.

ent urine samples, but the data revealed a marked decrease in mucopolysaccharide excretion with increased age of children and a close correlation with creatinine levels. A uniform excretion pattern is evident when the amount of chondroitin sulfates (Fig. 1A) or total mucopolysaccharides (Fig. 1B) per unit of creatinine is plotted against age. The decrease with increased age is apparent until the late teens, after which time the level of mucopolysaccharide excretion remains constant. Total mucopolysaccharides per unit of creatinine varied from 60 to 140 in the very young and became constant at a value of about 6 in adults. The chondroitin sulfates per unit of creatinine varied from 50 to 80 in the very young and became constant at a value of about 2.5 in adults.

In addition to the decrease in the amount of mucopolysaccharides with age, a decrease in the chondroitin sulfate/mucopolysaccharide (ChS/MPS) ratio occurs (Fig. 1C). This ratio varied from 0.60 to 0.80 in children to 0.30 to 0.50 in adults.

Mucopolysaccharides in urine from patients with Hurler's syndrome. Mucopolysaccharide excretion levels in urine samples from Hurler's patients are given in Table I. Five of the six patients showed high C/N ratios, a small amount of chondroitin sulfates (also see Fig. 1A), a high level of total mucopolysaccharides and a low ChS/MPS ratio. All of these criteria indicate that these patients excrete abnormal amounts of heparitin sulfate. The other patient excreted abnormal amounts of both chondroitin sulfate B and heparitin sulfate as indicated by a low C/N ratio and the excessive amount of chondroitin sulfates and mucopolysaccharides.

Sample No. 6 showed 3 spots on paper chromatography which had mobilities similar to chondroitin sulfate B, heparitin sulfate and chondroitin sulfate A and/or C. The other samples showed only 2 spots with mobilities similar to those of heparitin sulfate and chondroitin sulfate A and/or C. The solvent system for chromatography does not separate chondroitin sulfate A and C. On paper chromatography all samples showed a spot indi-

TABLE I. Mucopolysaccharides in Urine from Patients with Hurler's Syndrome.

Patient	Family	Age	Sex	C/N	ChS/Cr	MPS/Cr	ChS/MPS	Paper chromatography
1	1	10	♂	19.9	25.4	185	13.7	ChS-A and/or C, HS
2	1	14	♂	15.5	23.4	203	11.5	*Idem*
3	1	13	♀	13.4	31.3	301	10.4	"
4	2	6	♀	—	29.5	234	12.6	"
5	3	10	♂	8.0	35.0	421	8.3	"
6	4	10	♂	1.7	141.7	278	50.8	ChS-A and/or C, HS, ChS-B

Age in years. C/N is the carbazole hexuronic acid/naphthoresorcinol hexuronic acid ratio. ChS/Cr is μg of chondroitin sulfate (chondroitinase assay) per mg of creatinine and MPS/Cr is μg of total mucopolysaccharides (carbazole hexuronic acid) per mg of creatinine.

TABLE II. Mucopolysaccharides in Urine from Parents and Siblings of Hurler's Syndrome.

Subject	Family	Relation	Age	C/N	ChS/Cr	MPS/Cr	ChS/MPS
7	1	Father	40	7.1	2.25	5.04	.44
8	1	Mother	37	8.9	1.71	4.19	.41
9	1	Brother	11	8.8	9.6	13.9	.69
10	1	Sister	4½	14.2	25.5	35.1	.73
11	1	Brother	2	12.8	37.9	57.4	.66
12	2	Father	28	7.9	2.61	6.78	.38
13	2	Brother	5	13.0	38.8	51.2	.76
14	4	Father	39	11.0	2.58	6.51	.40
15	4	Mother	37	8.9	3.04	7.53	.40
16	4	Sister	4	9.7	37.5	52.3	.75

Age in years. C/N is the carbazole hexuronic acid/naphthoresorcinol hexuronic acid ratio. ChS/Cr is μg of chondroitin sulfates (chondroitinase assay) per mg of creatinine and MPS/Cr is μg of total mucopolysaccharides (carbazole hexuronic acid) per mg of creatinine.

cating sufficient amounts of chondroitin sulfate A and/or C which was also observed by Sanfilippo and Good(24). Patients that excreted abnormal amounts of heparitin sulfate also excreted slightly more chondroitin sulfates as determined by the chondroitinase assay (Fig. 1A). Since this was especially true in older patients, it is possible that Hurler's patients do not mature chronologically as fast as normal individuals.

Mucopolysaccharides in urine from families with an affected child. Family 1 had 3 affected children, and all excreted abnormal amounts of heparitin sulfate. The affected child in family 2 also excreted abnormal amounts of heparitin sulfate, but the affected child in family 3 excreted abnormal amounts of both heparitin sulfate and chondroitin sulfate B. By all criteria employed, the mucopolysaccharides in urine samples from all parents and siblings were within the normal range (Table II). When the samples from parents and siblings of affected patients are compared with the appropriate controls, the amount of chondroitin sulfate (Fig. 1A) and total mucopolysaccharides (Fig. 1B), and the ChS/MPS ratio (Fig. 1C) were in the same range as the controls.

Discussion. With increased age of children, a marked decrease in the amount of mucopolysaccharides excreted per unit of creatinine was observed in this study. This is in agreement with the observations of Teller *et al*(25) and Rich *et al*(26). After the late teens, the amount of mucopolysaccharides per unit of creatinine becomes constant with age. The amount of chondroitin sulfates in the urine is also a function of age, and a pattern which was similar to the total mucopolysaccharide excretion has been found. The ChS/MPS ratio decreases with increasing age indicating that a decrease in the amount of chondroitin sulfates in relation to total mucopolysaccharides takes place. Consequently, the types as well as the total amount of mucopolysaccharides at various ages should be considered in assessing urine specimens. Similar changes in mucopolysaccharides and chondroitin sulfates have been observed in cattle (12).

Excretion of abnormal amounts of mucopolysaccharides in heterozygous carriers of Hurler's disease is equivocal. Teller *et al*(5) found low C/N ratio in partially purified mucopolysaccharides from urine of suspected heterozygous carriers indicating an excretion of chondroitin sulfate B in abnormal amounts. A normal ChS/Cr ratio was observed in carriers which indicates that the increase in total mucopolysaccharides was not detectably different from the normal. However, Linker and Terry(6) observed no significant differences in either C/N ratio or urinary mucopolysaccharides in the carrier and normal individuals and other workers(7,8,9) employing various techniques, have not detected any differences. Recently, Berggard and Bearn (4), comparing urinary mucopolysaccharide patterns by electrophoresis in carriers and normal individuals, did not obtain conclusive evidence of abnormal amounts of urinary mucopolysaccharides in carriers, but in some cases the patterns appeared to deviate from pooled normal urine. We have found that the

mucopolysaccharides from urine of parents and siblings of Hurler's patients were within the normal range by all criteria employed. The mucopolysaccharides have been measured in urine samples from 3 families which have at least one child with Hurler's syndrome. In 2 families the affected children excreted abnormal amounts of heparitin sulfate. Since this defect is probably inherited as an autosomal recessive trait, it was important to test the parents and possibly some of the siblings to determine whether they excrete an excess of heparitin sulfate. The ChS/MPS ratio and total mucopolysaccharides appeared to be within the normal range. In another family the affected child excreted both heparitin sulfate and chondroitin sulfate B. Again the mucopolysaccharides from the urine of both parents and sibling showed a normal C/N ratio and normal mucopolysaccharides and chondroitin sulfates in the urine. Possibly the methods are not sensitive enough to detect a very small amount of a given mucopolysaccharide, but it has been established that the parents and siblings do not excrete a large amount of abnormal mucopolysaccharides.

In Hurler's syndrome it has now been found that 5 out of 6 patients excrete abnormal amounts of heparitin sulfate. Although 3 of these patients were in the same family, it is still a large percentage of the patients and excretion of only heparitin sulfate is not uncommon. From the available data it seems that the heparitin sulfateuria type is inherited as an autosomal recessive and appears to differ from the other types of Hurler's syndrome (*cf.*2,3). With the methods now available to distinguish the various types of Hurler's syndrome this latter group may be found to be more prevalent than was previously thought.

Summary. Mucopolysaccharide levels have been measured in urine specimens of normal persons, patients with Hurler's syndrome and their relatives. In the samples from normal humans a marked decrease was observed in the amount of chondroitin sulfates and mucopolysaccharides per unit of creatinine with age until the late teens, after which the amount became constant. In normal humans a decrease in the chondroitin sulfate/mucopolysaccharide ratio with increased age was also observed. After making appropriate allowances for age and creatinine levels in the urine, mucopolysaccharide excretion in parents and siblings of Hurler's patients was found to be similar to normal individuals by all criteria used. Several patients with the heparitin sulfateuria type of mucopolysaccharidosis were found in this survey.

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Characterization of an Established Line of Canine Kidney Cells (MDCK).*† (31293)

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During the last few years, an established line of canine kidney cells (MDCK) has been used in several laboratories to study virus-host cell relationships(1-4). The line was established in 1958 by Madin and Darby(5) but to our knowledge has never been characterized. This report describes the growth, immunologic and cytogenetic properties and preliminary data on its virus susceptibility.

Materials and methods. The MDCK cells were grown in lactalbumin medium(6) or Eagle's MEM containing 10% calf serum and nonessential amino acids. These cells were obtained from D. T. Imagawa of the University of California Medical Center and were designated MDCK-USD. Cultures incubated for 7 days at 35-36°C developed confluent monolayers of epithelial cells which were dispersed with trypsin-versene (TV) solution (6). The cells from one 7-day culture were resuspended in growth medium with a split ratio of 1:10 which was gradually increased to 1:40. The medium was changed after 3 and 6 days of incubation. The cells were grown in antibiotic-free medium after the 50th passage and were tested for the presence of *Mycoplasma* at periodic intervals. We also obtained an MDCK line from M. D. Hoggan at National Institutes of Health which we designated MDCK-NIH and a line from the American Type Culture Collection which we designated MDCK-ATCC. The latter 2 lines were grown in Eagle's MEM as described with a split ratio of 1:40.

The plating efficiency of MDCK-USD cells was determined by placing a known number of cells in growth medium and incubating them in 60 mm petri dishes for 2 weeks at 35.3°C in an atmosphere of 5% CO_2. The colonies formed were stained with crystal violet and counted.

Virus suspensions were prepared in MDCK-USD, KB and chick fibroblast (CF) monolayer cultures or in 9-day embryonated eggs. To determine virus susceptibility of MDCK-USD cells, Influenza (PR8), Vesicular Stomatitis Virus (VSV), Western Equine Encephalomyelitis (WEE), Sindbis, Semliki Forest Virus (SFV), Poliovirus Type 1 and Vaccinia Virus were inoculated into 7-day monolayer cultures at virus multiplicities of at least 1. After a suitable period for adsorption, the inoculum was removed and replaced with Eagle's MEM without serum and the cultures incubated for 5-7 days.

Anti MDCK-USD serum was prepared by disrupting 1.2×10^6 cells/ml in phosphate buffered saline (PBS) and injecting the debris into several guinea pigs *via* the intramuscular and intraperitoneal routes. Two additional injections of antigen were given at 1 and 2 months and the animals bled 1 week after the last injection. All sera were heated at 56°C for 30 minutes and stored at —62°C. We used the method of Brand and Syverton(7) to confirm the species of origin of this cell line. Preimmunization sera were used as controls.

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