moiety of chondromucoprotein remains attached to chondroitin sulfate is another possibility. As by the carbazole method for hexuronate, experiments with chondroitin sulfate in the range 250 to 1,000 μg/ml yield recoveries of 60 to 100% in the presence of protein. A similar result using the method of Bollet *et al.*(8) has been reported by Castor(9) for hyaluronate. This variation makes it possible to speak only of chondroitin sulfate-like equivalents in evaluating results of these determinations.

Since dialysis against 1/150 M phosphate buffer at pH 7.0 interfered with turbidity produced by $Co(NH_3)_6^{+++}$, distilled water was used in these studies. Euglobulins, however, precipitate with chondroitin sulfate at low pH in aqueous solution, therefore it was important to rule this out. Euglobulin precipitates under test conditions contained no appreciable amount of S^{35} activity, and indeed were minimal.

During these experiments, it was found that if whole serum of rabbits given chondroitin sulfate alone, or treated with papain, were diluted 1:60 with 0.01 M acetic acid/acetate buffer at pH 4.48, turbidities proportional to amount of chondroitin sulfate present were obtained. This is similar to the "acid-precipitable globulin" method described by Greenspan(10) and may explain his findings of increased turbidity in human heparinised plasma as opposed to plasma oxalated and diluted in like manner. As this latter test was dependent upon secondary protein changes in serum, it was not employed quantitatively. Similarly, cetylpyridinium chloride turbidity is vitiated by such protein changes.

Summary. 1. Serum of rabbits following injection of chondroitin sulfate, crude papain, or several modifications of papain, became turbid following addition of hexamminecobaltic trivalent cation. Turbidity was directly related to amount of chondroitin sulfate-like material present in the serum. 2. The precipitate produced by this cation contained most of the non-dialysable S^{35} activity present in the circulation following injection of crude papain, was metachromatic when redissolved, and did not form after incubation of serum with testicular hyaluronidase. 3. Addition of hexamminecobaltic chloride to dilute, dialysed serum is a useful way of demonstrating sulfated mucopolysaccharides in serum at levels exceeding 250 μg/ml.

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Received July 30, 1959. P.S.E.B.M., 1959, v102.

Sulfated Mucopolysaccharides of Urine and Organs in Gargoylism (Hurler's Syndrome) II. Additional Studies.* (25327)

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In gargoylism or Hurler's Syndrome, 2 characteristic features have been reported: (1) the intracellular storage of one or more sulfated polysaccharides(1,2,3,4), and (2) excessive excretion in urine of 2 distinct muco-

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

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polysaccharides, chondroitin sulfate B and heparitin sulfate, neither of which is found in detectable amounts in normal urine(3,4). In this paper, data are reported on mucopolysaccharide excretion in urine and on the content of various organs obtained at autopsy of additional cases. It is concluded from these data (1) that various organs contain or store both mucopolysaccharides, and (2) that the ratio of these mucopolysaccharides differs in various organs from the same individual.

Methods. Mucopolysaccharides were precipitated from urine at pH of approximately 5 to 6 by addition of an aqueous 5% solution of cetyltrimethyl ammonium bromide or by cetylpyridium chloride as described previously(4). Usually, after purification, only 2 fractions were collected, the Ca salts precipitated at an alcohol concentration of 25% and of 50%. The first fraction consisted mostly of ChS-B and the second of heparitin sulfate although each fraction was contaminated to some extent with the other mucopolysaccharide. The relative concentrations of ChS-B and of heparitin sulfate were calculated from ratios of carbazole and orcinole and from their relative content of galactosamine and glucosamine, respectively. In many instances, an additional criterion of relative concentrations was utilized by determining degree of hydrolysis of each fraction after incubation with a flavobacterium extract adapted to ChS-B, followed by further incubation with heparitin-sulfate adapted enzyme(5). The organs available for study were kept frozen and shipped in dry ice. Specimens for analysis were ground in a tissue grinder, dehydrated in acetone, and defatted with ether. The material was then filtered, washed with acetone and ether, and dried *in vacuo*. Mucopolysaccharides were isolated from tissues after digestion with pepsin at pH 1.5, followed by tryptic digestion at pH 7.5 in the presence of toluene. Further purification and fractionation of the Ca salts was carried out as described previously(6).

Results. Urinary concentration of mucopolysaccharides in additional cases of gargoylism is listed in Table I. This table also lists concentration of mucopolysaccharides of 3 patients who did not have gargoylism. Since urine volume is normal, it can be seen that all

TABLE I. Chondroitin Sulfate B and Heparitin Sulfate of Urine in Gargoylism.

Patient	Sex	Age, yr	Urine conc.: Total quantity (mg/l)	ChS-B (%)	Heparitin sulfate (%)	Remarks
1	♂	8	85	58	42	
2	♂	20	58	74	26	Siblings with all symptoms of the disease, mentally both normal (Dr. L. Abt., Phila.)
3	♀	18	78	78	22	
4	♂	4.5	74	66	34	
5*	♂	25	154	61	39	Sex-linked recessive form, normal I.Q.
6†	♂	10.8	33	~50	~50	
7	♀	3.9	74	~90	~10	
8	♂	2	194	70	30	
9	♀	1	132	72	28	
10	♂	7.5	245	62	38	5 days prior to medication, creatinine 588 mg/l
			179	64	36	5 days on prednisone (30 mg/day), creatinine 515 mg/l
			155	71	29	11 days on prednisone (30 mg/day), creatinine 582 mg/l
11	♂	9	9			All Ch-S, no B, predominantly A, achondroplasia
12	♀	7	16			*Idem*
13	♀	1.2	4			Probably not gargoylism

* Obtained from Dr. G. A. Jervis, Letchworth Village, N. Y.

† This patient of Dr. McKusick, Baltimore, Md., was markedly retarded in his mental development and had skeletal changes consistent with mild Hurler's Syndrome. He had no hepatosplenomegaly and no limitation of motion.

TABLE II. Chondroitin Sulfate B and Heparitin Sulfate Fractions in Gargoylism.

Patient	Sex	Age, yr	Organ	Total quantity	ChS-B (%)	Heparitin sulfate (%)	Remarks
1*	♂	12	Liver	6.7	6	>90	
			Spleen	2.4	65	~25	
			Brain	.7	40	60	
			Kidney	1.5	50	~50	
2†	♂	10	Liver	1.2	56	44	
3‡	♂	7	Liver	6.3	5	~90	
			Brain	.8	~40	~30	
4§	♂	10	Liver	2.5	6	94	
5‡	♀	12	Brain	1.8	85	15	
6‖	♂	.6	Brain	.3		~40	Normal brain; 60% hyaluronate, no ChS

* Obtained from Dr. J. H. Austin, Portland, Oreg. † Obtained from Dr. W. A. Reiley, San Francisco, Calif. ‡ Obtained from Dr. G. W. F. Edgar, Amsterdam, Holland. § Obtained from Dr. G. A. Jervis, Letchworth Village, N. Y. ‖ Obtained from Dr. W. A. Blanc, Babies Hospital, N. Y. City.

cases of gargoylism had an increased excretion of total mucopolysaccharide and that ChS-B was the predominant mucopolysaccharide excreted in majority of instances.

Cases #2 and #3 are of especial interest. These two siblings had advanced and typical clinical symptoms of the disease including cloudy corneas but were of normal mental development. The similar ratio of ChS-B to heparitin sulfate in these siblings is notable. In Case #10, prednisone therapy (30 mg/day) was instituted for 11 days after a control period of 5 days. No appreciable change in creatinine excretion occurred during this period. Following institution of prednisone, a significant decrease in total mucopolysaccharide excretion occurred; however, the ratio of ChS-B to heparitin sulfate was not significantly altered.

Cases #11, 12 and 13 may be regarded as control subjects. Although Case #12 may have an increased polysaccharide excretion, no ChS-B or heparitin sulfate was detected. Infrared spectroscopy showed the fraction to be mainly ChS-A.

The organs of patients with gargoylism investigated included liver, spleen, brain and kidney. The results are listed in Table II.

As seen from this Table all organs of cases of gargoylism contained varying amounts of both ChS-B and heparitin sulfate. In Case #1, a 12 year old boy, the liver contained 6.7% (fat-free dry weight) polysaccharide; the latter consisted of about 90% heparitin sulfate. However, even in this case, a small fraction obtained at low alcohol concentration was identified as ChS-B. It seems remarkable that the spleen of this case contained 65% of ChS-B and only approximately 25% of heparitin sulfate, the rest being composed of other mucopolysaccharides. The liver in Cases #3 and #4 had a mucopolysaccharide partition very similar to that of Case #1 although the concentration in Case #4 was considerably lower. The liver of Case #2, however, had a ChS-B content equal to or higher than that of heparitin sulfate. The brains, including meninges, in Cases #1 and #3 had a low total mucopolysaccharide content while in Case #5, it was increased. It may be significant that this case had a high ChS-B content.

Since no comparisons with normal brain values were available, one brain including meninges of a child with no obvious pathology of the central nervous system, was analysed (Case #6). The mucopolysaccharide content of this brain was lower than any of the cases of gargoylism. On fractionation, no ChS was encountered. The only mucopolysaccharides which could be identified consisted of hyaluronic acid and heparitin sulfate.

Discussion. Our data are similar to those reported by Dorfman and Lorincz(3) and from this laboratory, in urine and liver, in liver by Brown(2), and in liver and spleen by Stacey and Barker(7). The last two labora-

tories apparently isolated from liver and spleen fractions corresponding to heparitin sulfate which apparently, in the majority of cases, occurs in the liver almost to the exclusion of other polysaccharides. It appears most noteworthy that the same individual may contain ChS-B as the major component of some organs and heparitin sulfate as the major component of others.

In all organs investigated from patients with Hurler's syndrome the concentrations of polysaccharides were greater, in most cases excessively so, than those of control organs. In unpublished experiments with organs such as liver, spleen, and kidney, the concentration of polysaccharides did not exceed 0.2% of the defatted dry weight. Amounts of polysaccharides isolated in most cases were insufficient to allow adequate characterization.

It is not known which cells produce and which ingest the polysaccharides from the extracellular fluids. Probably in the normal, some type of differentiated fibroblasts produces ChS-B and some cells of blood vessel walls produce heparitin sulfate. Experimentally, normal human macrophages in tissue culture have been shown by Mr. Morris and Dr. Godman of this school‡ to take up added heparitin sulfate from the culture medium and store it in the protoplasm in the form of discrete metachromatic granules similar in size to those of eosinophil granules. In Hurler's syndrome, polysaccharides contained in some parenchymatous cells may originate through a similar process of uptake from the circulating fluids. What factors determine the preferential storage of one or the other polysaccharide and the sparing of some organs (brain, for example) is not known.

The primary genetic defect of gargoylism remains obscure. As in other storage diseases, the intracellular storage is a gradually increasing process which manifests itself clinically later than the increased urinary excretion(4). The large quantities of polysaccharide excreted daily in these individuals strongly indicates an overproduction of the 2 polysaccharides. At present, our previous hypothesis, namely that the defect may be in a faulty differentiation of fibroblasts, appears still most plausible. According to this hypothesis, the connective tissue cells producing ChS-B and heparitin sulfate are increased possibly at the cost of those producing other mucopolysaccharides. One possible site of this defect is the blood vessel wall where ChS-B and heparatin sulfate occur normally together in relatively high concentration(8).

Summary. 1) Further examples of quantity and types of urinary excretion of sulfated mucopolysaccharides in Hurler's syndrome have been studied in 9 cases. All excreted both ChS-B as the major component and heparitin sulfate as a minor constituent. 2) In one case, excretion of mucopolysaccharides dropped after prednisone administration. 3) From various organs of 5 autopsy cases, the 2 polysaccharides were isolated. 4) Livers of 4 cases contained between 1.2 and 6.7% of total mucopolysaccharides (on fat-free dry wt). In 3 of these, 90% was heparitin sulfate, but from all of these, also some ChS-B was obtained. One liver had approximately equal quantities of the 2 polysaccharides. 5) In one case, spleen, brain and kidney of the same individual also yielded both polysaccharides, but in proportions remarkably different from those of liver. Brain of 2 additional cases also yielded the 2 mucopolysaccharides. One of these contained 85% of ChS-B. The brain of a normal child did not yield any chondroitin sulfate, but hyaluronate and heparitin sulfate.

‡ We thank Dr. G. C. Godman for permission to use these data.

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