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*TISSUE STORAGE OF MUCOPOLYSACCHARIDES IN HÜRLER-PFAUNDLER'S DISEASE**

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Conflicting reports have been published on the nature of the storage substance which accumulates in the spleen, liver, and other tissues of patients with Hürler-Pfaundler's disease ("gargoylism"). Brante[1] reported that a sulfated mucopolysaccharide of seemingly simple constitution was present in large amounts in one autopsy liver specimen. Uzman[2] found two principal components—one a polysaccharide fraction having hexoses, hexosamines, and sulfate and the other a glycolipid of complex composition. Stacey[3] has reported the presence of sulfated polysaccharide fractions as well as of nonsulfated fractions, the latter somewhat related to blood group-specific substances.

Isolation of Storage Substance.—In the present work we have examined tissues removed at autopsy from (*a*) one normal child dying from an acute respiratory tract infection, (*b*) and (*c*) two fatal cases of Hürler-Pfaundler's disease in children, and (*d*) liver tissue removed by biopsy from the liver of a child with this disease. It has been found that homogenization of liver or spleen in 10 volumes of cold 5 per cent trichloroacetic acid (TCA) in a Waring Blendor extracts at least 80 per cent of the storage substance, as well as any glycogen which is present. A study of the TCA-insoluble residue has shown that it contains not over 20 per cent additional material which is related in structure to the soluble storage substance described below. Following removal of TCA by ethyl ether extraction, the storage substance and the glycogen are precipitated completely by 2 volumes of ethanol in the presence of 1 per cent potassium acetate and 1 per cent calcium chloride (pH 5.7). In the case of tissue from a gargoyle patient, the voluminous precipitate is mucoid in character. An aqueous solution of the precipitate is centrifuged at 100,000 $\times$ *g* for 2 hours to sediment a large part of any glycogen present. None of the characteristic storage substance is present in the pellet. An additional amount of glycogen can be removed from the supernatant solution by enzymatic digestion with phosphorylase and amylo-1,6-glucosidase, followed by ethanol precipitation of the mucopolysaccharide which remains. This latter material has been subjected to one or more of the following procedures: alcohol fractionation in the presence of calcium acetate; direct column chromatography on Dowex-1

(formate); continuous paper electrophoresis-chromatography. The properties of some of these fractions are shown in Table 1.

TABLE 1

PROPERTIES OF SOME MUCOPOLYSACCHARIDE FRACTIONS FROM HÜRLER'S DISEASE AND NORMAL TISSUES

CASE*	TISSUE	TOTAL CONTENT OF STORAGE SUBSTANCE (PER CENT WET WT.)	RATIOS: No. of Ethanol Fraction	RATIOS: Glucuronic Acid/Glucosamine†	RATIOS: S/N‡	RATIOS: Acetyl/N	SPECIFIC ROTATION $[\alpha]_D^{25°}$ (DEGREES)
K. N.	Autopsy liver	1.4	I-A	1.50	2.00	..	+60.2§
			I-B	1.47	1.0	..	+79.6§
			I-AP-ED	1.37‖	1.00#	0.67#	....
			I-331**	1.11	1.7	0.75‡	+69.5††
	Autopsy spleen	0.11	I-S-A	1.30	1.7	..	....
			I-S-C	1.44	2	..	....
G. H.	Autopsy liver	0.4	II-D	1.35	0.66#	0.62#	+57.0§
D. C.	Biopsy liver	0.9	III-GR	1.29	1.31	0.73#	+35.8††
Normal	Autopsy liver	*Ca.* 0.01‡‡	....	..	..	..	....

* The author is grateful to Dr. C. H. Carter, Florida Farm Colony, Gainesville, Florida, for the tissue from K. N. (female, age 14 years at death); to Dr. B. H. Landing, Children's Hospital, Cincinnati, Ohio, for the tissue from G. H. (female, age 2½ months at death); and to Dr. Gilbert B. Forbes, University of Rochester School of Medicine, Rochester, New York, for the tissue from D. C. (male, age 6 years).

† Uronic acid analyzed by carbazole method (see text) and calculated as glucuronic acid. Glucosamine determined as described in the text.

‡ Nitrogen content calculated from glucosamine content.

§ Determined on aqueous solution of the polysaccharide as the free acid.

‖ Glucosamine content calculated from Dumas N determination (see Table 3).

Nitrogen by Dumas determination (see Table 3).

** Purified by Dowex-1 (formate) column chromatography, followed by ethanol precipitation of calcium salt.

†† Determined on aqueous solution of the calcium salt of the fraction.

‡‡ Obtained from sum of hexosamine contents of all fractions, using an estimated value of 35 per cent for the hexosamine content of the material. The identity of these fractions with any of those from the pathological tissues has not been established.

Identification of Constituent Sugars.—All fractions were hydrolyzed in 4 *N* HCl for 16 hours at 100°C., and the hydrolyzates were analyzed quantitatively for hexosamine by a modification[4] of the Elson-Morgan method. The liberation of hexosamine was found to be complete under these conditions of hydrolysis. In addition, qualitative information has been obtained about the kind of hexosamine present through examination of the hydrolyzates by paper chromatography, using the *n*-butanol–pyridine–water solvent (3:2:1.5) of Jeanes *et al.*,[5] by which glucosamine and galactosamine are well separated. All fractions from all cases had glucosamine as the only hexosamine present. The possible presence of other hexoses in the fractions was investigated by paper chromatography of hydrolyzates prepared in 1 *N* HCl for 3 hours at 100°. In addition, enzymatic assays were done for glucose on these hydrolyzates via the hexokinase–glucose-6-phosphate dehydrogenase–TPN system and, after addition of phosphomannose isomerase, for mannose. By these procedures, the only other sugar found in some fractions was glucose. This was shown to arise in every case from a contamination of the mucopolysaccharide fraction by glycogen, since the glucose content fell to a very low value after enzymatic degradation of 98 per cent of the residual glycogen by phosphorylase and amylo-1,6-glucosidase. Fucose was found to be absent from the mucopolysaccharide by the test of Dische and Shettles.[6]

The only constituent sugar of the mucopolysaccharide other than glucosamine was found to be uronic acid, through use of the carbazole test of Dische[7] as well as by the orcinol test of Khym and Doherty.[8] The molar ratio of apparent glucuronic acid to glucosamine was found to be from 1.3 to 1.5 with the former and

from 0.7 to 0.8 with the latter method.[9] Thus the observed ratio of apparent uronic acid content by the carbazole method to that by the orcinol method is about 2. Hoffman, Linker, and Meyer[10] have found that this ratio is 0.5 to 1 for three types of chondroitin sulfates. We have found that for heparin this ratio is about 4. The question of the type of uronic acid present was studied by subjecting the mucopolysaccharide fractions to partial hydrolysis in 1 *N* HCl at 100°. At this concentration of acid the polysaccharide is cleaved only slowly to smaller fragments. After either 7 hours or 16 hours of such hydrolysis, the only free uronic acid found on a paper chromatogram developed in the solvent of Fischer and Dorfel[11] was glucuronic acid.

The sulfate content of the mucopolysaccharide fractions was determined on 16-hour, 4 *N* HCl (100°) hydrolyzates, using a modification of the method of Dodgson and Spencer[12] for the precipitation and washing of benzidine sulfate, followed by the method of Andersen[13] for the estimation of the benzidine content of the precipitates by spectrophotometric observation at 249 mμ. All fractions were found to be sulfated. After purification the fractions showed ratios of sulfur to nitrogen of either 0.66, 1.0, 1.33, 1.67, or 2.0.

The possible presence of acetyl groups was investigated by *p*-toluene sulfonic acid hydrolysis of the polysaccharide, followed by distillation of acetic acid under reduced pressure according to the method of Elek and Harte.[14] Several samples of the polysaccharide were also analyzed for acetyl groups by Dr. Adalbert Elek. A uniform finding was the presence of a considerable acetyl content in all samples analyzed, the molar ratio of acetyl to nitrogen being 0.67. A study of the yield of acetic acid as a function of time of reflux with toluene sulfonic acid showed that the analysis was performed under conditions of maximum acetyl yield.

Structural Features.—The question of the nature of the linkage of the sulfate to the polysaccharide chain was studied by measuring the sulfate released on mild acid hydrolysis of a fraction with a sulfur-to-nitrogen ratio of 1.0. Approximately one-third of the total sulfate was hydrolyzed in 30 minutes at 100° in 0.04 *N* HCl. After 60 minutes, from 43 to 57 per cent (in duplicate experiments) of the total sulfur had been released as inorganic sulfate, and similar values were obtained after 1 hour and 3 hours of additional hydrolysis. These data suggest that a part of the sulfur may be present in the intact polysaccharide in a sulfamic-type linkage to the nitrogen of some of the glucosamine residues. Jorpes *et al.*[15] have shown that this bond occurs in heparin. In the case of the mucopolysaccharide fractions from Hürler's syndrome, the colorimetric amino-N test of Frame *et al.*[16] was applied to the intact polysaccharide (fraction I-AP-ED, Table 1) and to samples hydrolyzed in 0.04 *N* HCl as discussed above. It was found that the intact polysaccharide gave some color in this test but that the apparent amount of amino-N rose to about three times this value after 15 minutes of hydrolysis and remained unchanged after 2 hours of hydrolysis. It was impossible to calculate the percentage of the total nitrogen set free as amino-N, because of a marked hyperchromic effect of the structure of the polysaccharide on the color yield of the amino groups of the glucosamine residues present in the chain. The fact that there appears to be a concomitant appearance of free amino groups under mildly acidic hydrolysis conditions when inorganic sulfate is also set free supports the suggestion that in the Hürler disease storage substance some of the glucosamine residues have acetylated amino

groups (see above), while others have their amino groups bound in amidosulfonic acid linkage.

Early in the work it was observed that the mucopolysaccharide fractions were slowly dialyzable through Visking sausage casing and were more rapidly electrodialyzable. Brief electrodialysis was occasionally used to prepare the materials in the form of their free acids for analytical study. The relative ease of dialysis suggested that the average size of the molecules is rather small—and, accordingly, aqueous solutions of several fractions which had been extensively dialyzed and, in some cases, electrodialyzed were subjected to physical study in the Model E Spinco analytical centrifuge. A synthetic boundary cell was used, and sedimentation constants and diffusion constants were calculated from the same pictures. In addition, diffusion constants were calculated from runs made at low speed, after it became apparent that the material under study had a very high diffusion constant and a very low sedimentation constant. Partial specific volumes were determined pycnometrically and in a density-gradient tube. The results of these physical studies are shown in Table 2. The molecular weights calculated from

TABLE 2

PHYSICAL PROPERTIES OF THE STORAGE SUBSTANCE FROM LIVER

FRACTION*	ELECTROPHORETIC MOBILITY AT $\Gamma/2 = 0.1$ ($\times 10^{-5}$ CM^2/VOLT/SEC) Acetate (pH 5.0)	Veronal (pH 8.6)	SEDIMENTATION CONSTANT, $S_{20, w}$ (SVEDBERG UNITS)	DIFFUSION CONSTANT, $D_{20, w}$ ($\times 10^{-7}$) CM^2/SEC	PARTIAL SPECIFIC VOLUME	CALCULATED MOLECULAR WEIGHT
I-B	−15.8	−15.6	..	..	..	..
I-A	....	....	0.92	20	0.46	2075
I-AP-ED	....	....	0.76	24.5	0.44	1350
II-D	....	....	0.66	28.3	0.53	1240

* See Table 1.

sedimentation-diffusion data are probably accurate within 20 per cent. These values, as well as the observations on dialyzability mentioned above, suggest that the storage substance might more properly be said to belong to the class of oligosaccharides than to that of the polysaccharides. Each of the fractions examined in the ultracentrifuge sedimented as a single peak. Further, a plot of the apparent diffusion constant versus time was linear and had only a small slope. These criteria speak for the relative homogeneity of the fractions. On the assumptions that each substance is pure, that the materials have generally similar structure, but that they differ from each other chiefly in their degree of sulfation (see Table 1), the storage oligosaccharides appear to consist of seven monosaccharide units. Further discussion of the detailed structure of the substances is given below.

The oligosaccharide storage substances from the livers of the three cases of Hürler's disease resemble heparin in many of their structural features: (1) in being composed exclusively of glucosamine and glucuronic acid units; (2) in being sulfated and apparently having some of the sulfate groups present in amidosulfonic acid linkage; (3) in having a considerable positive optical rotation. The materials differ from heparin in having acetyl groups present, presumably as N-acetyl, and in being of low molecular weight. Assays for anticoagulant activity were made on fractions I-A and I-B by the method of Blomback *et al.*[17] It was found that these substances had, if any, less than 2 per cent of the activity of heparin[18] on a weight basis.

Partial acid hydrolyzates of fraction I-AP-ED and of heparin were prepared in 1 *N* HCl at 100° for 7 hours and for 16 hours. After removal of chloride ion with Ag_2CO_3, the hydrolyzates were examined by paper chromatography, using the pyridine–ethyl acetate–acetic acid–water (5:5:1:3) solvent system of Fischer and Dorfel.[11] Figure 1 shows such a chromatogram. The storage oligosaccharide is

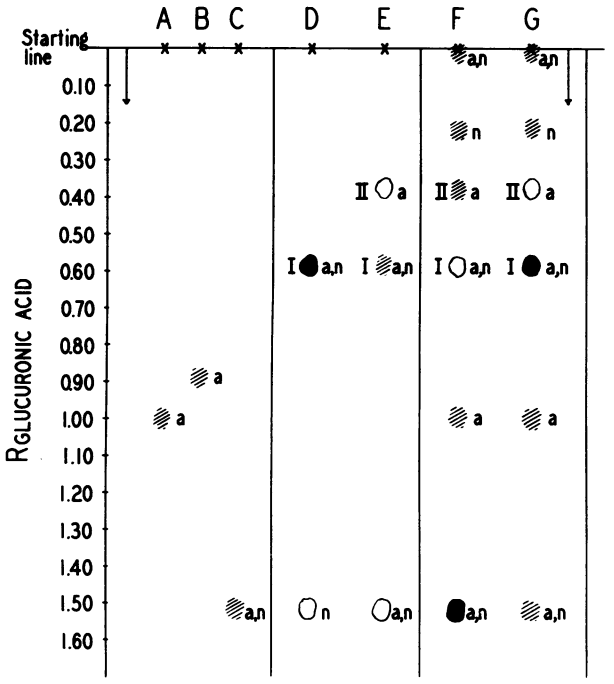
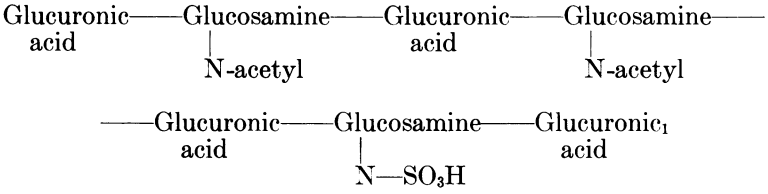


FIG. 1.—Descending paper chromatogram of partial acid hydrolyzates of heparin and of a Hürler's disease oligosaccharide fraction. Strip *A*, glucuronic acid; *B*, galacturonic acid; *C*, glucosamine; *D*, heparin, 7 hours, 1 *N* HCl; *E*, heparin, 16 hours, 1 *N* HCl; *F*, fraction I-AP-ED, 16 hours, 1 *N* HCl; *G*, fraction I-AP-ED, 7 hours, 1 *N* HCl. The relative intensity of the sprayed spots is indicated by the degree of shading. Reaction with an aniline phthalate spray is indicated by *a;* reaction with a ninhydrin spray is indicated by *n*.

similar to heparin in yielding a large amount of the same substance, I, at 7 hours. As shown, the amount of this material was markedly reduced in both cases by further hydrolysis. It is interesting that this fragment appears to terminate on its reducing end with a glucosamine unit, since it reacts with a ninhydrin spray. Such a product would not be expected as a principal component of a partial acid hydrolyzate of heparin in the light of the present ideas about the structure of this polysaccharide. On the other hand, the type of structure postulated below for the storage oligosaccharide would be expected eventually to yield a considerable amount of a disaccharide terminating in glucosamine during acid hydrolysis. Figure 1 also shows that heparin and the storage oligosaccharide are alike in yielding a substance, II, whose amount increases with increasing time of hydrolysis. This fragment, more abundant from the storage oligosaccharide than from heparin, appears to terminate in a glucuronic acid unit at its reducing end. Other substances having lower mobility on paper, and apparently terminating in glucosamine units, were detected in the hydrolyzate of the storage oligosaccharide. Although free glucosamine was present in all hydrolyzates, its amount was considerably greater in those of the storage oligosaccharide than in those of heparin. This is probably due partly to the lower molecular weight of the former substance but chiefly to the presence of N-acetylated glucosamine residues whose hexosaminidic

bonds are cleaved first by acid hydrolysis, followed by liberation of free glucosamine. A distinct feature of the chromatograms from the oligosaccharide was always seen to be the presence of glucuronic acid at all intermediate stages of hydrolysis. No glucuronic acid could be detected from heparin.

It is believed that the observations made can be unified somewhat by postulating that the storage oligosaccharides often are heptasaccharides to which the following partial structure may be assigned:



Most or all of the glycosidic bonds probably have the α-configuration. From this structure one would predict that the least sulfated substance of this class will have a sulfur-to-nitrogen ratio of 0.33. Other substances, in which one or more of the free hydroxyl groups of the glucosamine residues are sulfated, would have sulfur-to-nitrogen ratios of 0.66, 1.00, 1.33, 1.67, 2.00, or 2.33. Thus far, no substance with a ratio of 0.33 or of 2.33 has been encountered from the tissues examined.

Additional information pertaining to the structure proposed is available from the titration curve of 1.92 mg. of fraction I-AP-ED, titrated with standard 0.008 *N* NaOH (CO_2-free) at 27°, using N_2 stirring and a glass electrode. This weight of electrodialyzed sample contained 3.63 μatoms of S and 3.67 μatoms of N (see Table 3).

TABLE 3

ELEMENTARY COMPOSITION OF SOME OLIGOSACCHARIDE FRACTIONS*

	FRACTION					
	II-D (Mol. Wt. 1450) (Per Cent)		I-AP-ED (Mol. Wt. 1530) (Per Cent)		I-331 (Mol. Wt. 1766) (Per Cent)	
ELEMENT OR GROUP	Calc.†	Found	Calc.‡	Found	Calc.§	Found
C	..	..	35.79	35.62	..	..
H	..	..	4.61	5.27	..	..
N	2.90	3.01	2.72	2.68	2.38	2.28‖
S	4.42	4.54	6.23	6.07	9.07	9.16
Acetyl	5.94	5.74	5.58	5.47	4.87	5.25

* Microanalyses by Dr. Adalbert Elek, Los Angeles, California. All analyses on samples dried to constant weight at 78° *in vacuo* over P_2O_5. The analyses found have been corrected for a small contamination by glycogen determined microenzymatically as glucose (see text).

† Calculated for the free acid form of a heptasaccharide with S:N = 0.66 (see text and Table 1).

‡ Calculated for the free acid form of a heptasaccharide with S:N = 1.00 (see text), correcting for the presence of 1.6 per cent ash as K. Sample was electrodialyzed before analysis.

§ Calculated for the calcium salt (2 Ca) of a heptasaccharide with S:N = 1.67 (see text and Table 1).

‖ Calculated from glucosamine analysis.

From its ash content (1.6 per cent), it can be calculated that it contained 0.45 μatom of cation (calculated as K). The titration curve showed the consumption of 4.90 μmoles of NaOH from a small rise at pH 2.97 (corrected to zero volume change) to the major rise at pH 6.50. This quantity of NaOH may be taken to measure the glucuronic acid carboxyl groups. The midpoint of their titration (corrected to zero volume change) was at pH 3.45 (pK′). The consumption of NaOH for their titration indicates that the number of carboxyl groups is 1.33 times as great as the number of glucosamine residues. The heptasaccharide structure assigned has such a ratio.

Table 3 gives analytical data on the elementary composition of some of the oligosaccharide fractions from two of the cases of Hürler's disease which have been studied. The composition of each has been calculated with reference to a heptasaccharide having the structure given above and with a varying number of sulfate groups as indicated. The molecular weight of the proposed heptasaccharide structure is shown for comparison with the molecular weights calculated from sedimentation-diffusion data (Table 2).

While this manuscript was in preparation, a report appeared[19] on the finding in the urines of two patients with Hürler's disease of a considerable amount of chondroitin sulfate B and of a small amount of a poorly characterized mucopolysaccharide fraction which may be related in structure to the storage oligosaccharides which are described in this paper. Meyer has reported[20] on the occurrence in aorta and amyloid of a type of mucopolysaccharide ("heparitin sulfate") which also may have some structural features in common with the oligosaccharides described herein.

The structure of the family of sulfated oligosaccharides accumulated in the tissues of patients with Hürler-Pfaundler's disease is only partially clarified by the work reported here. Further studies are under way to extend this information. It is impossible to state at present whether these substances of low molecular weight are normal constituents of human tissues, present in very small amounts, or whether they are abnormal products of synthetic mechanisms peculiar to Hürler's disease. The three cases of this disease from which tissues have been available were found to be qualitatively similar in that they all possessed a large amount of this new class of oligosaccharides.

Summary.—The characteristic storage substance which accumulates in the liver and spleen of patients with Hürler-Pfaundler's disease ("gargoylism") has been isolated from tissues removed at autopsy and by biopsy. By suitable fractionation procedures, this water-soluble material has been shown to consist of a family of oligosaccharides each of which is composed exclusively of D-glucosamine and D-glucuronic acid units united in glycosidic linkage. Two-thirds of the glucosamine residues of the molecule are acetylated, and the remaining one-third most probably is united with sulfate in an amidosulfonic acid linkage. The oligosaccharides are all further sulfated and differ from one another in the degree of this sulfation. The substances are of low molecular weight, as shown by their slow dialyzability and by calculation from determinations of sedimentation and diffusion constants. Elementary analyses support the assignment of a heptasaccharide structure to many of the fractions studied. In such a structure the ratio of glucuronic acid to glucosamine residues is 1.33. The substances all have a considerable positive optical rotation. One possible detailed structure of the molecule is discussed.

* A preliminary report of these findings was made at the Conference on the Metabolism of Mucopolysaccharides held at the Retina Foundation, Boston, Massachusetts, June 1 and 2, 1956. This work has been supported in part by a grant (RG 4761) from the National Institutes of Health, United States Public Health Service.

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*VACCINE FOR THE PREVENTION IN HUMANS OF COLDLIKE SYMPTOMS ASSOCIATED WITH THE JH VIRUS**

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The discovery of the adenoviruses[1, 2] constituted a major advance in leading to the identification of agents responsible for common respiratory disease of presumed viral etiology in the human population. These agents have been shown to be of particular importance in military populations.[3–4] However, studies from this laboratory[5] as well as from others[7, 8] have indicated that these agents produce little clinical disease in the civilian populations studied, although the significance of the adenoviruses in causing disease in infants and children has not yet been determined.

In agreement with the findings of Dingle and co-workers,[9] it has been our experience that respiratory illness makes up about 70 per cent of all illnesses seen in families and various groups we have studied.[6] No etiologic agent has as yet been isolated which accounts for the major share of such illnesses, which clinically are of the common-cold variety.

In a recent paper[10] from this laboratory, evidence was presented for the isolation of a new virus (JH) which was associated with mild upper-respiratory illness in humans. In this paper we wish to report the development of a vaccine which protects individuals against the coldlike symptoms associated with the JH virus.