LIPID ABNORMALITIES IN A VARIANT OF THE HURLER SYNDROME*

By Reuben Matalon†, J. Anthony Cifonelli, Hans Zellweger, and Albert Dorfman

Department of Pediatrics and Biochemistry, Larabida-University of Chicago Institute, and the Joseph P. Kennedy, Jr., Mental Retardation Research Center, University of Chicago, and Department of Pediatrics, University of Iowa

Communicated by George W. Beadle, February 12, 1968

Numerous studies have established that the excretion in urine and the deposition in tissues and in cultured fibroblasts of acid mucopolysaccharides (AMPS) are markedly increased in Hurler's disease.1-4 Previous reports have indicated an increase in ganglioside content in brain and visceral organs of patients with the Hurler syndrome.5-9 The relationship of the increase in glycolipids to that of AMPS is not clear. In the course of a study of two brothers with clinical evidence of Hurler's syndrome, fibroblasts obtained from one of the brothers were strikingly abnormal but did not show typical metachromasia. However, after the specimens were treated with chloroform:methanol (2:1, v/v), metachromatic granules could be demonstrated, thus suggesting that excess lipids were present which interfered with staining. It is the purpose of this communication to report details regarding the occurrence of abnormal quantities of certain lipids in fibroblasts of this patient as compared with the fibroblasts of a patient with typical Hurler's disease and those of a normal child.

Materials and Methods.—Carrier-free H$_2$S$^{18}$O$_4$ was obtained from New England Nuclear Corporation. Sodium acetate-C$^{14}$ (53 mc/m mole) was obtained from Nuclear Chicago. Monosialogangliosides, GM$_1$, GM$_3$, and disialoganglioside GD$_1$ (nomenclature of Svennerholm$^{10}$) were a gift from D. Kaufman and S. Roseman, Johns Hopkins University.

Tissue culture: Fibroblast cultures were established from skin biopsies of a previously studied Hurler patient (B. H.), a normal child, and the proband J. S. and his parents. Cells were grown in 100-mm plastic Petri dishes as previously described.$^7$

Isolation of lipids: Cells were removed from the Petri dishes by using a rubber policeman, homogenized, and lyophilized, and lipids were extracted 4 times with chloroform:methanol (2:1, v/v) with the addition of KCl.$^{15}$

Isolation of AMPS: Following digestion of the cells with crystalline papain, the solution was dialyzed and AMPS were precipitated with cetylpyridinium chloride (CPC) as previously described.$^{16}$ In the case of J. S., a copious precipitate was obtained from CPC which was found to include lipids in addition to AMPS. Addition of 2.0 m LiCl, which dissolves AMPS-CPC complexes, did not clear the solution. However, the subsequent addition of 3 vol of ethanol resulted in a precipitate containing AMPS and a clear supernatant solution containing the lipids.

Fractionation of AMPS: Separation of AMPS was accomplished by chromatography on Dowex 1-X2, Cl$^-$ columns utilizing stepwise sodium-chloride elution.$^{17}$

Thin-layer chromatography: Thin-layer chromatography (TLC) was carried out on silica-gel plates. Plates 1 mm thick were used for preparative studies. For analysis of the crude lipid fraction, the solvent was chloroform:methanol:H$_2$O (16:6:1, v/v) and the stain was iodine vapor. In this solvent, gangliosides remain close to the origin, neutral fats migrate with the solvent front, and other components including phospholipids display an intermediate mobility. For further study, the gangliosides were eluted from the silica gel with chloroform:methanol (1:1, v/v) and rechromatographed on silica-gel...
plates with n-propanol:H$_2$O (7:3, v/v). Resorcinol reagent was used to visualize the gangliosides.

**Paper chromatography:** Sugar components were identified by paper chromatography. After hydrolysis in 1 N H$_2$SO$_4$ for 3 hr at 100°, the samples were neutralized by addition of BaCO$_3$ and chromatographed on Whatman no. 1 paper by using ter-amyl alcohol: isopropl alcohol:H$_2$O (8:2:3, v/v) as solvent. Sugar spots were visualized with a silver nitrate stain.

**Analytical methods:** Sialic acid was determined by the methods of Aminoff and Warren. Uronic acid was determined by the carbazole method of Dische and by the orcinol method. Hexosamine was determined by the Boas modification of the Elson-Morgan method that omits the resin treatment. Neutral sugars were determined by the phenol method. Sulfate was estimated according to Muir's modification of the method of Dodgson and Spencer. Protein was precipitated with hot trichloroacetic acid (TCA) and determined quantitatively by the method of Lowry et al.

The intrinsic viscosity and osmotic pressure of AMPS fractions were kindly determined by Dr. M. B. Mathews, who used a method previously described.

**Labeling experiments:** Cells were incubated for 2 hr with acetate-C$^{14}$ or H$_2$S$^{35}$O$_4$ with an activity of 50 μc per 100-mm plate containing 10 ml of medium. Following digestion with papain, AMPS and lipids together with the dermatan-sulfate carrier were precipitated with CPC. The AMPS were dissolved in 2.0 M LiCl and reprecipitated with 3 vol of ethanol. The alcohol fraction which contained the lipids was evaporated to dryness, and the lipid material was extracted with chloroform:methanol (2:1, v/v). Radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer as previously described.

**Staining of cells:** Cells grown in monolayers on cover slips were washed with Earle's balanced salt solution, fixed with tetrahydrofuran:acetone (1:1), and stained with toluidine blue.

**Results.—Morphology of fibroblasts:** Observation by phase microscopy of cells obtained from patient J. S. indicated a large amount of dense, refractile inclusions. On staining with toluidine blue, the cytoplasm stained dark blue. This abnormal appearance differed strikingly from the intense metachromasia regularly observed in the typical Hurler cells. When cells of J. S. were treated with chloroform:methanol (2:1, v/v) prior to staining, metachromatic granules could be demonstrated. Examination of fibroblasts of the mother of J. S. showed cells with metachromatic granules similar to those seen in Hurler heterozygotes. In contrast, cultures from the father showed some cells which resembled those of the proband, but metachromatic granules were also seen.

**AMPS and lipids in cultured cells:** Suspecting that the refractile material might be lipid in nature, we extracted the cells from five 10-mm plates (5th transfer, approx. 12 × 10$^6$ cells/plate) for lipids and compared them with similar extracts of normal fibroblasts and typical Hurler fibroblasts. The dried residue was redissolved in chloroform:methanol (2:1, v/v) and again evaporated to dryness for dry-weight determinations. Sialic acid was determined on the total lipid extract. The comparative analyses on the three cell types presented in Table 1 indicate a marked increase in total lipids in J. S. fibroblasts and an even more striking increase in sialic acid. The typical Hurler cells also show an increase in sialic-acid content.

Thin-layer chromatography on silica-gel plates revealed eight distinct fractions in extracts from all types of cells with increased intensity of staining of all components in the material from the cells of J. S. The ganglioside frac-
tion was extracted four times from the silica gel with chloroform:methanol (1:1, v/v). The molar ratio of sialic acid to hexosamine for ganglioside preparations of normal, Hurler, and J. S. cells were 0.9, 1.65, and 2.0, respectively.

Preliminary chromatographic studies of the gangliosides using an n-propanol:H₂O (7:3, v/v) solvent showed rapidly moving spots in the Hurler extract. In the fraction isolated from J. S. fibroblasts, there was a marked increase in intensity in a slower-moving spot in the region expected for GM₁ and GD₁. Following hydrolysis of the ganglioside fraction with 1.0 N H₂SO₄ for 3 hr, paper chromatography revealed hexosamine, glucose, and galactose.

In order to determine the nature of the metachromatic inclusions in the cells of patient J. S., fibroblasts from 100 plates (100-mm) were harvested and 55 mg of AMPS was isolated. On electrophoresis, two major spots with the mobility of hyaluronic acid and dermatan sulfate were observed as well as a minor spot with the mobility of chondroitin sulfate A/C. On chromatography on Dowex 1-X2, Cl⁻ columns, 23.5 mg of pure dermatan sulfate and 11 mg of hyaluronic acid were isolated. The analyses shown in Table 2 were similar with respect to composition, optical rotation, and molecular weight to those observed for AMPS isolated from fibroblasts of a typical autosomal Hurler homozygote.²²

Incorporation of radioactivity: Experiments were carried out to determine the extent of incorporation of acetate-C¹⁴ and S³⁵O⁴⁻ into the lipid fractions. No appreciable incorporation of S³⁵O⁴⁻ into the lipid fractions of the different cell types was noted. However the data in Table 3 clearly indicate markedly

**Table 1. Analyses of the lipid fraction from fibroblasts.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lipids (mg/mg protein)</th>
<th>Total sialic acid (µg)</th>
<th>Sialic acid (µg/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.18</td>
<td>45</td>
<td>0.78</td>
</tr>
<tr>
<td>Hurler (B. H.)</td>
<td>0.22</td>
<td>83</td>
<td>1.58</td>
</tr>
<tr>
<td>J. S.</td>
<td>0.50</td>
<td>315</td>
<td>5.25</td>
</tr>
</tbody>
</table>

**Table 2. Analyses of AMPS isolated from J. S. fibroblasts.**

<table>
<thead>
<tr>
<th>AMPS fraction</th>
<th>Molar Ratio*</th>
<th>Uronic acid</th>
<th>Sulfate</th>
<th>Carbazole/orcinol</th>
<th>[α]D/t</th>
<th>Intrinsic viscosity</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatan sulfate</td>
<td>0.53</td>
<td>1.30</td>
<td>0.32</td>
<td>-68°</td>
<td>0.74</td>
<td>2.50</td>
<td>81,000</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>1.12</td>
<td>—</td>
<td>—</td>
<td>-62°</td>
<td>2.00</td>
<td>2.50</td>
<td>21,500</td>
</tr>
</tbody>
</table>

* Molar ratio is based on hexosamine equal to 1.0.
† Optical rotation based on dry weight basis. The dermatan sulfate contained 25.4% hexosamine and the hyaluronic acid, 23.6% hexosamine.
‡ Viscosity, average molecular weight.
§ Number, average molecular weight determined by osmotic pressure.

**Table 3. Incorporation of acetate-C¹⁴ into lipid fraction of J. S. fibroblasts as compared with Hurler and normal cells.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Specific activity of cells*</th>
<th>Specific activity of medium*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>250,000</td>
<td>8,500</td>
</tr>
<tr>
<td>Hurler (B. H.)</td>
<td>447,000</td>
<td>33,900</td>
</tr>
<tr>
<td>J. S.</td>
<td>1,550,000</td>
<td>102,000</td>
</tr>
</tbody>
</table>

* Specific activity expressed as cpm/1 X 10⁴ cells.
enhanced incorporation of radioactivity in the cells of J. S. as well as some increase above normal in the Hurler cells of B. H.

Discussion.—It is clear from the data presented in this communication that the fibroblasts derived from the skin biopsy of patient J. S. are markedly different morphologically and chemically from both normal fibroblasts and those derived from a patient (B. H.) with typical autosomal recessive Hurler's disease. The data also indicate a definite increase above normal in ganglioside concentrations in fibroblasts of the typical Hurler patient, B. H. Preliminary studies on TLC indicate that the increase in the Hurler fibroblast is primarily due to the rapidly moving gangliosides with a mobility similar to GM3 and GM2. These glycolipids have been reported to be increased in the brains of Hurler patients.12, 13, 33, 34

The lipid increase observed in patient J. S. is both qualitatively and quantitatively different. There is an excessive amount of lipids including not only gangliosides but phospholipids as well. Preliminary fractionation of the gangliosides indicate that the chromatographic pattern is quite different from that of the extract of the typical Hurler cells and shows a marked increase of gangliosides migrating in the region of GM1 and GD1. The sialic acid:hexosamine ratio suggest that a major portion must contain more than one sialic acid residue per hexosamine.

In attempting to define the nature of the disease process in this unusual patient, several previously recognized syndromes should be considered. A number of reports now indicate that late infantile amaurotic familial idiocy may be distinguished chemically from Tay-Sachs disease.12, 35—37 The primary ganglioside increase appears to be in GM1, the major normal monosialic ganglioside of the brain. Most of the available data concern the nervous system. What might be a related syndrome has been described as neurovisceral gangliosidosis, a disease which shows important clinical overlap with Hurler's syndrome.38, 39 Such cases, which have also been called "pseudo-Hurler's disease," show a marked increase in GM1. However, in none of the cases in which ganglioside analyses have been performed have excessive acid mucopolysaccharides been reported. The cases most closely resembling J. S.'s are those described by Leroy and Crocker40 and Leroy and DeMars.41 These authors described the occurrence of cells in tissue cultures which may be similar to those of J. S. They also noted similar cells in cultures obtained from the father and a paternal uncle of one of their patients but found metachromatic granules in cells derived from the mother. We have observed a similar pattern in the fibroblasts grown from biopsies of the parents of J. S.

The results reported in this paper raise the interesting question of the relationship of lipid abnormalities in Hurler's syndrome to the now well-established mucopolysaccharide disturbances. In view of the clearcut genetics involved in typical autosomal-recessive Hurler's disease, it seems unlikely that two different gene products are involved. More reasonable is the supposition that a single gene product results in a metabolic abnormality involving both mucopolysaccharides and glycolipids and that one or both of the apparent abnormalities is secondary to a primary defect. Although the AMPS excreted in urine and extracted from tissues are degraded, it seems as if degradation is secondary, since
recent studies indicate that the dermatan sulfate and hyaluronic acid accumulated in Hurler fibroblasts are of high molecular weight and are formed as protein complexes.\textsuperscript{32} Electron microscopic studies suggest that inclusions of AMPS are bound by membranes.\textsuperscript{42} It seems possible that the increase in intracellular lipids may be derived from the membrane systems surrounding mucopolysaccharide-protein complexes.

Such an explanation leaves open the metabolic defect in the particular variant represented by J. S. Particularly difficult to explain is the difference in appearance of the maternal and paternal cells. Since J. S. had a similarly affected sibling and both mother and father are phenotypically normal, it seems most likely that this condition represents an autosomal recessive disease with the proband as a homozygote. However, the difference between the cells of the mother and those of the father suggests a more complicated situation. DeMars and Leroy\textsuperscript{43} have suggested that their proband might be a double heterozygote. Heterozygosity for two nonallelic genes might result in the expression of the phenotype represented by the proband. This appears to be an unlikely explanation, since metachromatic granules were observed not only in the mother's cells but in some of those of the father as well. A more likely possibility might be that the father is a double heterozygote and the proband is homozygous for Hurler's disease and heterozygous for a lipid disturbance. Such situations might be expected to occur with some frequency. If our supposition with regard to the relationship of lipid and AMPS metabolism is correct, then the abnormality in J. S. would be expected to be both qualitatively and quantitatively different from typical Hurler's disease. Such a formulation may have pertinence to the occurrence of a number of variants of other genetically determined metabolic diseases.

In view of the fact that recent studies on the biosynthesis of AMPS clearly indicate that the enzymes involved are localized on membranes,\textsuperscript{44} a possible relationship of lipid storage to biosynthesis and excretion of AMPS may exist. Failure to complete the normal excretion of a product synthesized within a membrane system may be due to a defect in the mechanism of membrane lysis with secondary accumulation of both product and membrane components. Since it is known that there appears to be a turnover of membranes in secreting cells,\textsuperscript{45} the metabolism of membrane lipids may play a critical role in the normal biosynthesis and excretion of other macromolecules. Such a concept would seem to have obvious implications for the explanation of storage diseases.

The authors are grateful for the helpful discussions with Dr. Lennart Rodén. The skillful technical assistance of Mrs. Bonnie B. Sipe and Mrs. Louise Roth is gratefully acknowledged.

\* This investigation was supported by grants from the U.S. Public Health Service (AM-05996) (FR-00305).
\dagger This work was completed during the tenure of the Junior Philanthropic Society fellowship.  
\textsuperscript{3} Brown, D. H., these \textit{Proceedings}, \textbf{51}, 783 (1957).  
Aronson and (1965).
Matalon, R., and A. Dorfman, these PROCEEDINGS, 56, 1310 (1966).
Matalon, R., and A. Dorfman, unpublished data.
Horwitz, A., and A. Dorfman, personal communication.