Globoid cell leukodystrophy (Krabbe's disease): isolation of myelin with normal glycolipid composition

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ABSTRACT Myelin was isolated from the brain of a patient with Krabbe's globoid cell leukodystrophy at 0.4% of the normal yield. Despite the exceedingly low yield, the fraction appeared morphologically clean, and consisted mostly of well-preserved myelin lamellae and few contaminating structures. Total lipid and cholesterol were slightly lower than in normal myelin. Total phospholipid was normal, but the ratio of ethanolamine phospholipid to lecithin was reversed. Total galactolipid was normal, and consisted only of cerebroside and sulfatide in normal proportions. The only sugar in cerebroside and sulfatide was galactose. The fatty acid composition of cerebroside and sulfatide was essentially normal with no deficiency of long-chain fatty acids and only with a reversed ratio of $C_{24:0}$ to $C_{24:1}$ in cerebroside. These data appear to exclude the previous postulate that abnormally rapid breakdown of myelin occurs in this disorder as the result of the formation of chemically abnormal myelin, deficient in sulfatide.

SUPPLEMENTARY KEY WORDS cerebroside · sulfatide · cerebroside–sulfatide sulfotransferase · galactocerebroside β-galactosidase · inherited metabolic disease

GLOBOID cell leukodystrophy (Krabbe's disease) is a rare, fatal metabolic disorder of infants and is transmitted as an autosomal recessive trait. The affected homozygous patients exhibit signs of severe and progressive white matter involvement before the age of 1 yr and rarely survive beyond the second year of life. At the terminal stage, white matter is characterized by almost total absence of myelin and oligodendroglia and severe astrocytic gliosis. The massive infiltration of the characteristic multinucleated globoid cells in white matter is pathognomonic. A disproportionate lack of sulfatide has been noted in white matter (1, 2), and the recent report of deficient cerebroside–sulfatide sulfotransferase activity (3) appeared to provide the enzymatic basis for the analytical findings.

Increased sulfatide content has been found in isolated myelin in another hereditary metabolic disorder, metachromatic leukodystrophy (4, 5), in which deficient sulfatase A activity results in accumulation of sulfatide in the brain (6, 7). In both metachromatic leukodystrophy and Krabbe's disease, accelerated breakdown of chemically abnormal myelin had been suggested to account for the profound lack of myelin. In the former, the abnormal lipid composition of the myelin is consistent with this hypothesis. We tested this hypothesis in globoid cell leukodystrophy by isolation and chemical analysis of myelin. Contrary to what is expected from the hypothesis of chemically abnormal myelin, we found normal glycolipid composition in the myelin of globoid cell leukodystrophy. The findings reported here actually provided us with the clue for our more recent study which demonstrated a profound deficiency of galactocerebroside β-galactosidase in globoid cell leukodystrophy (8).

MATERIALS AND METHODS

The brain was obtained after death of a 1-yr-old boy with globoid cell leukodystrophy. The histological and ultrastructural aspects of a biopsy specimen obtained from the same patient at age 7 months have been reported elsewhere (9). Histological examination of the postmortem

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brain showed complete absence of myelin in deep white matter; stainable myelin was only rarely encountered in subcortical areas. Myelin was therefore isolated from total brain tissue by the method of Norton, Poduslo, and Suzuki (10, 11), which yields clean myelin even from gray matter alone (12). The essential outline of the standard isolation procedure is as follows. 5% homogenate of whole brain prepared in 0.32 M sucrose was layered over 0.85 M sucrose, and centrifuged at 75,500 *g* for 30 min in a Spinco SW-27 rotor. The material at the interface of the two layers was collected, diluted with water, and centrifuged at 75,500 *g* for 15 min. The resultant pellet was suspended in water and centrifuged at 12,000 *g* for 10 min. This osmotic shock was repeated. The final pellet was suspended in 0.32 M sucrose, layered over 0.85 M sucrose, and centrifuged at 75,500 *g* for 30 min. The myelin fraction at the interface was removed and washed with distilled water five times by repeated suspension and centrifugation. All centrifugations were carried out at 4°C.

During the preparation of myelin from the brain of the patient with Krabbe's disease, the layer above the myelin band after the discontinuous gradient centrifugations appeared unusually turbid, both before and after the osmotic shocks. Therefore, the 0.32 M sucrose layer above the myelin band was carefully removed, and also the same discontinuous gradient centrifugation was carried out the third time immediately following the second gradient centrifugation. All the 0.32 M sucrose layers from the three gradient centrifugations were pooled and then washed with distilled water five times. This careful elimination of the lighter fraction was critical because it contained fragments of the abnormal inclusions but no myelin.

Approximately 30 mg of myelin was collected by repeating the isolation procedure many times. Portions of the myelin sample and the lighter fraction were fixed in osmium tetraoxide, dehydrated through increasing concentrations of ethanol, and embedded in Araldite for the purpose of electron microscopic examination of the fractions. The isolated myelin was analyzed according to the methods previously described (13).

RESULTS

Electron Microscopy

The myelin fraction was subjected to an extensive electron microscopic survey because the purity of the fraction is critical for chemical analysis, and because the probability of contamination is much greater when the amount of myelin is exceedingly small compared with the bulk of the tissue. The fraction was clean, despite the very small yield and consisted mostly of electron-dense lamellar structures, recognizable as myelin by alternating major and minor period lines (Fig. 1). Unlike myelin preparations from normal brains, there were occasional nonmyelin contaminants; the amount of these contaminants, however, was judged to be sufficiently small for reliable chemical analysis. On the other hand, the electron microscopic appearance of the lighter fraction was entirely different. It was quite homogeneous and consisted of fine fragments of tubular or paracrystalline structures (Fig. 2). Extensive search did not reveal any lamellar structures that can be recognized as myelin or its fragments. From the known ultrastructure of the brain tissue of globoid cell leukodystrophy *in situ*, the abnormal tubular or polygonal inclusions found in globoid cells are the most likely source of this abnormal lighter fraction.

Lipid Analysis (Table 1)

The total lipid content of myelin was nearly normal, although there was a higher proportion of the chloroform–methanol insoluble residue and less proteolipid protein. Cholesterol was slightly lower than normal, and total phospholipid was normal. Ethanolamine phospholipid was lower and lecithin higher than normal, resulting in a reversed ratio of these two phospholipids. There appeared to be no preferential loss or preservation of phosphatidal-ethanolamine, which constituted 83% of ethanolamine phospholipid. The most unexpected finding, however, was the normal proportions of cerebroside and sulfatide as indicated by visual inspection of the thin-layer chromatogram (Fig. 3). Sulfatide was actually slightly higher than normal (Table 1). The hexose composition of cerebroside and sulfatide was determined by gas–liquid chromatography (14), and only galactose was found in both cerebroside and sulfatide. No glycosphingolipids other than cerebroside and sulfatide were detected in the lower-phase lipids by thin-layer chromatography after treatment of total lipid with mercuric chloride and saponification (13, 15), despite the presence of significant amounts of several other glycosphingolipids in whole white matter (16). Although gangliosides were detected in the myelin fraction, and visual inspection of the thin-layer chromatogram showed preponderance of ganglioside-G_{M1}[1] as in normal myelin (11, 12, 18), the total amount was too small for quantitative determination of individual gangliosides.

Fatty Acid Composition of Cerebroside and Sulfatide

The fatty acid compositions of both cerebroside and sulfatide in myelin of globoid cell leukodystrophy were very similar to those reported for these glycolipids in myelin of normal human infants (18) (Tables 2 and 3). There was

[1] Nomenclature of gangliosides is according to Svennerholm (17).

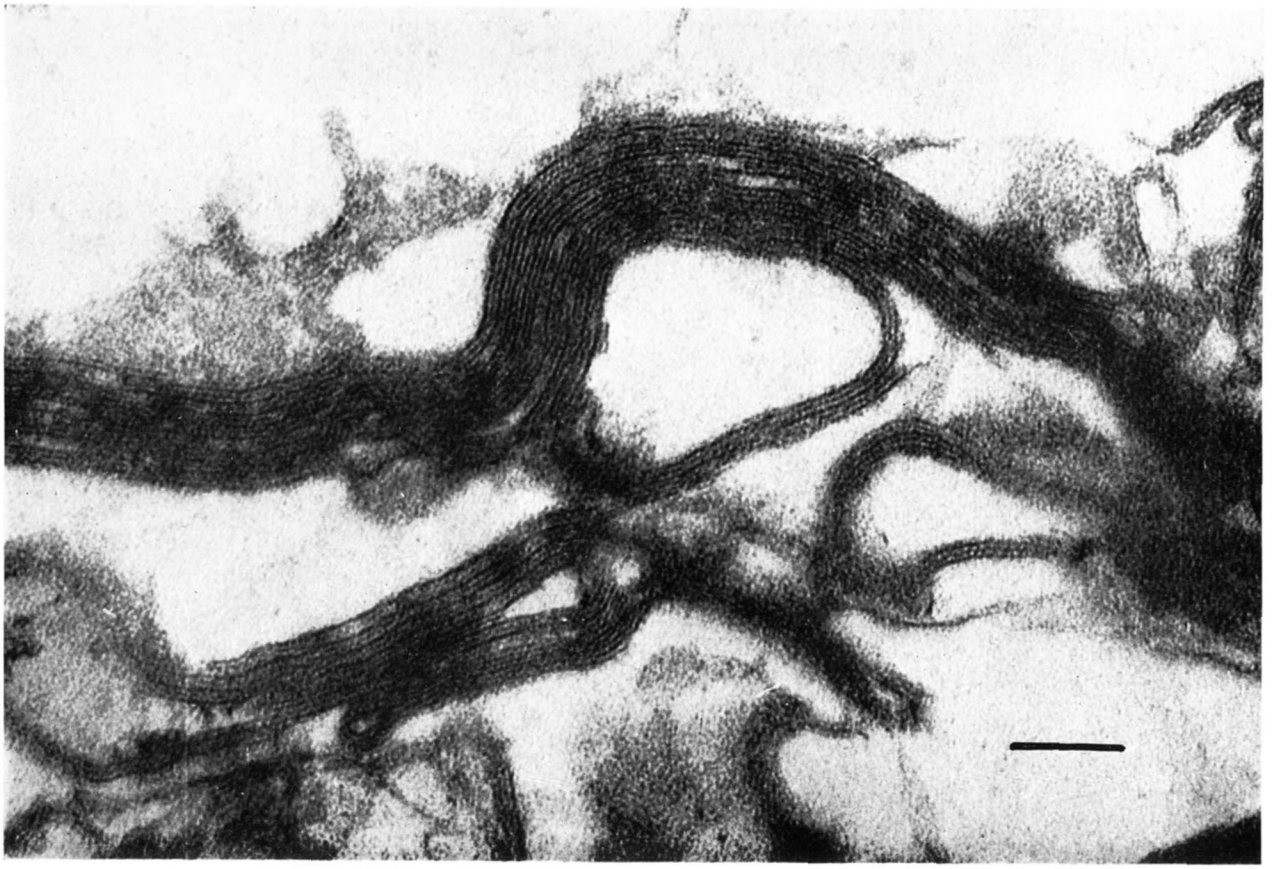

FIG. 1. An electron micrograph of the isolated myelin fraction. The normal lamellar structure of myelin is well preserved with alternating major and minor period lines clearly visible. The fraction contained occasional nonmyelin membranous fragments as contaminants. The fraction was fixed with Dalton's osmium tetraoxide solution, embedded in Araldite, and the sections were stained with uranyl acetate and lead citrate. The line indicates the scale of 0.1 μ.

no deficiency of the longer chain fatty acids. In both cerebroside and sulfatide, unsubstituted fatty acids with chain length longer than 20 carbons constituted 80% of total; α-hydroxy fatty acids with more than 20 carbons constituted 90–95% of the total. The only abnormality was the reversed ratio of $C_{24:0}$ to $C_{24:1}$ in cerebroside, resulting from a relative decrease of nervonic acid ($C_{24:1}$) and a relative increase of lignoceric acid ($C_{24:0}$).

DISCUSSION

It had often been postulated that in globoid cell leukodystrophy, chemically abnormal myelin, lacking in sulfatide as the result of cerebroside–sulfatide sulfotransferase deficiency, might be produced, and that such abnormal myelin is unstable and rapidly breaks down resulting in the severe myelin loss observed in this disorder. The initial aim of this study was to test this hypothesis. The result clearly indicated that, at least at the terminal stage of the disease, the remaining myelin has essentially normal lipid composition.

These data, therefore, appear to exclude the possibility that there is an abnormally rapid breakdown of once-formed myelin due to its abnormal lipid composition. In order for this hypothesis to be valid and consistent with the analytical data, we must postulate that almost all myelin formed in globoid cell leukodystrophy is chemically abnormal, but that myelin with normal lipid composition is also formed in a very minute amount. Then most of the myelin breaks down rapidly during the course of the disease, and at the terminal stage only that portion of myelin with normal lipid composition would remain. The data do not strictly exclude this possibility, but it seems quite unlikely in view of the generalized hereditary nature of the disease. Isolation and analysis of myelin from globoid cell leukodystrophy at an earlier stage should definitively answer this question. There is one previous report in the literature which appeared to support the above hypothesis; this concerned sphingolipids of myelin isolated from a patient with globoid cell leukodystrophy (20). The data indicated that the proportion of sulfatide is approximately half normal when expressed as

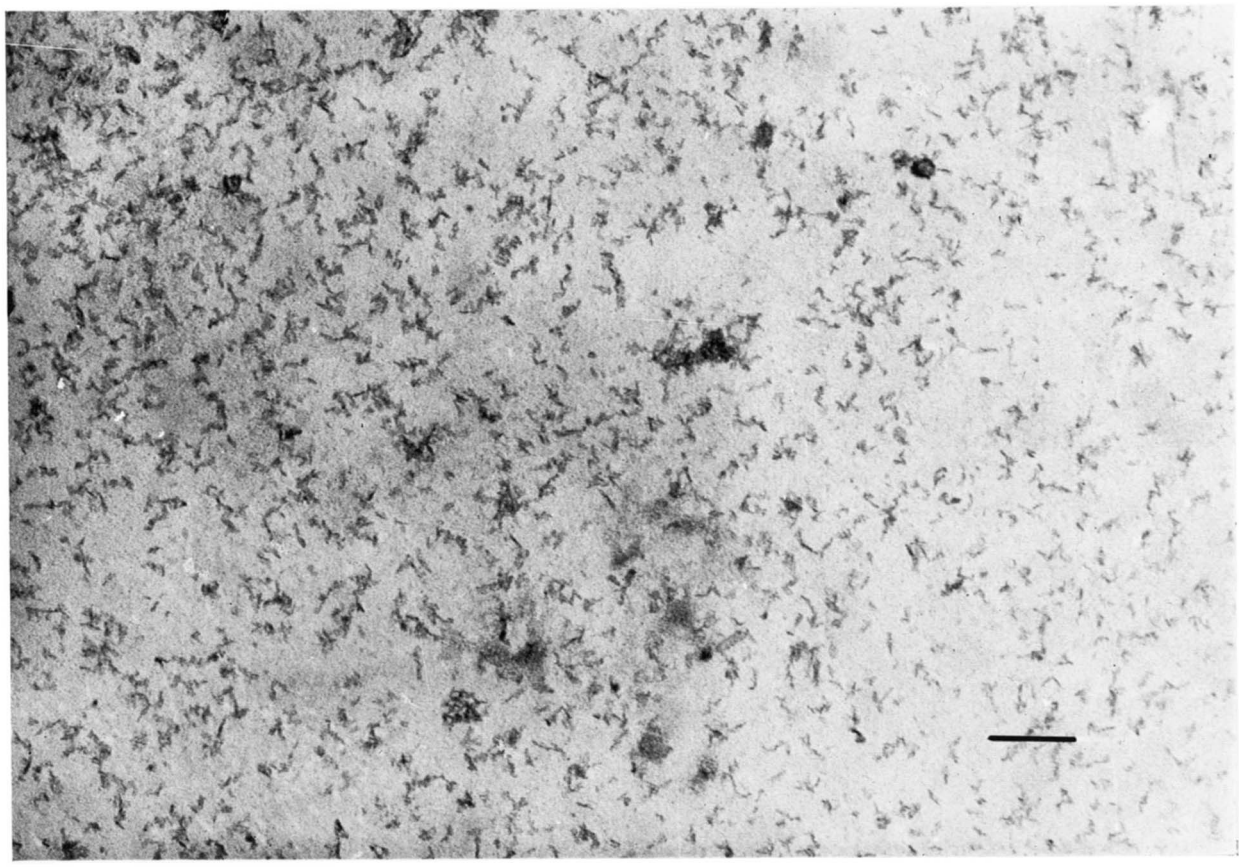

FIG. 2. An electron micrograph of the abnormal fraction lighter than myelin. The fraction was quite homogeneous and consisted of fine fragments of what appears to be the abnormal inclusions found *in situ* in globoid cells. Note the entirely different ultrastructural appearance of this fraction compared with the myelin fraction in Fig. 1. No myelin was recognized in this fraction. The technical details are the same as in Fig. 1. The line indicates the scale of 0.1 μ.

percentage of total sphingosine. However, since the data were not given for protein, total lipid, or sphingolipid content of the myelin fraction, it is not possible to relate the data to the actual amounts of individual sphingolipids in myelin. The data do not allow one to distinguish a fraction with abnormally low sulfatide and normal cerebroside from that with abnormally high cerebroside and normal sulfatide. The technique of myelin isolation used by Cumings, Thompson, and Goodwin (20) does not eliminate subcellular components that have buoyant density lighter than myelin. We found that the abnormal cytoplasmic inclusions abundantly present in globoid cells have a density slightly lighter than myelin and had to be carefully eliminated in order to obtain a clean myelin fraction. In our specimen, there was a larger amount of this abnormal lighter fraction than myelin, and this fraction contained a high proportion of cerebroside but practically no sulfatide.[2] This abnormal light fraction is likely to have been included in the myelin fraction of Cumings et al. (20) and may have given an erroneously high proportion of cerebroside.

On the other hand, our results are not necessarily incompatible with the theory that lack of sulfotransferase results in deficient sulfatide content of the tissue in globoid cell leukodystrophy. There may simply be an almost complete failure of myelin formation due to the unavailability of an important normal constituent, sulfatide. This assumption, however, is difficult to reconcile with the observation on the cellular level. If we assume a generalized nature of hereditary metabolic disturbance, and if availability of sulfatide is the limiting factor for myelin formation in Krabbe's disease, deficient myelin formation should be uniform on the cellular level, such as one myelin lamella on every axon. The actual morphological examination showed that myelin was completely absent in most areas of white matter, but, when found, it appeared to have the normal multilamellar structure. This morphological picture would indicate that, if the availability of sulfatide sets the upper limit of total myelination, the metabolic disturbance would not be gener-

[2] Eto, Y., and K. Suzuki. Unpublished observation.

TABLE 1 CHEMICAL COMPOSITION OF ISOLATED MYELIN

	Globoid Cell Leuko-dystrophy	Normal Control*
Yield, mg/10 g wet wt	3.8	1000
	%, dry weight	
Chloroform–methanol insoluble residue	25.7	12.4
Proteolipid protein	12.3	21.0
Total lipid	62.0	66.6
Cholesterol	12.2	15.6
Total phospholipid	30.3	30.1
Ethanolamine phospholipid	7.6	9.7
Lecithin	12.9	9.2
Sphingomyelin	5.0	5.1
Monophosphoinositide and serine phospholipid	4.2	5.8
Phosphatidalethanolamine	6.3	—
Total galactolipid	17.0	17.4
Cerebroside	12.4	13.6
Sulfatide	4.6	3.8

* Average of two myelin preparations from normal brains, ages 2.5 and 5.5 yr.

TABLE 2 COMPOSITION OF UNSUBSTITUTED FATTY ACIDS IN GLYCOLIPIDS OF MYELIN*

Fatty Acids	Globoid Cell Leukodystrophy		Normal Control†	
	Cerebroside	Sulfatide	Cerebroside	Sulfatide
14:0	tr.	0.5	1.9	0.9
15:0	0.4	0.5	—	—
16:0	2.2	6.7	9.3	4.0
17:0	0.4	tr.	—	—
18:0	12.9	8.4	14.6	10.0
18:1	0.5	3.8	6.6	2.0
19:0	0.9	tr.	—	—
20:0	3.3	0.9	1.2	1.7
21:0	0.6	0.5	—	—
22:0	5.7	4.0	4.3	5.7
22:1	tr.	tr.	1.0	2.3
23:0	3.4	2.1	1.2	1.0
23:1	tr.	tr.	0.6	0.3
24:0	34.0	21.3	15.2	24.0
24:1	13.8	24.0	27.4	31.0
25:0	8.9	9.1	2.0	1.8
25:1	1.6	3.3	3.6	2.6
26:0	4.3	4.5	1.6	1.7
26:1	7.0	10.3	7.3	8.5
Sum of 14:0–20:0	20.6	20.8	33.6	18.6
Sum of 21:0–26:1	79.3	79.1	64.2	78.9

* Expressed as percentage of the total unsubstituted fatty acids.
† 10 months old, from O'Brien and Sampson (19).

alized among all oligodendroglial cells, but that a very small number of oligodendroglial cells can produce sufficient sulfatide to form morphologically and chemically normal myelin sheath, whereas all other oligodendroglial cells are totally incapable of producing any sulfatide. Also there is a morphological study which showed more myelin in early lesions of globoid cell leukodystrophy compared with later stages, indicating that there might indeed be rapid breakdown of existing

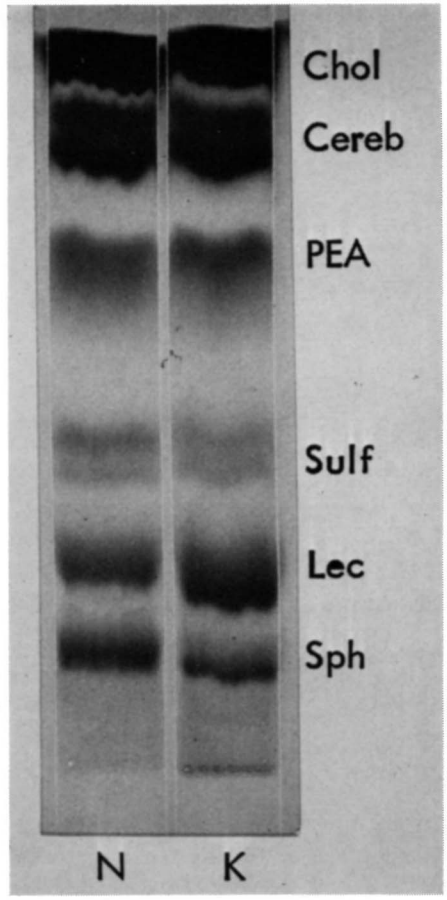

FIG. 3. A thin-layer chromatogram of total lipid of myelin. Approximately 0.5 mg of proteolipid protein-free lipid was spotted on a 250 μ thick Silica Gel G plate. The solvent system was chloroform–methanol–water 70:30:4 (v/v/v). Spots were visualized by sulfuric acid spray and charring. *N*, normal; *K*, Krabbe's disease; *Chol*, cholesterol; *Cereb*, cerebroside; *PEA*, ethanolamine phospholipids; *Sulf*, sulfatide; *Lec*, lecithin; *Sph*, sphingomyelin. In this solvent, serine phospholipids streak diffusely from the origin to above sphingomyelin. Note the virtually identical lipid patterns, particularly with regard to cerebroside and sulfatide. Lecithin is relatively increased in myelin of globoid cell leukodystrophy.

myelin during the course of the disease (21). A simple failure of myelination would not explain this observation.

It was this logical dilemma which prompted us to re-examine the previous concept of globoid cell leukodystrophy (lack of sulfatide as the result of deficient sulfotransferase) and to explore the defect in the catabolic pathway of galactocerebroside. We have demonstrated a profound deficiency of galactocerebroside β-galactosidase in the brain, liver, and spleen from three cases of globoid cell leukodystrophy (8). The deficiency of galactocerebroside β-galactosidase is more likely to be the primary enzymatic defect of this disorder. We proposed a hypothesis to explain the known features of globoid cell leukodystrophy on the basis of this deficiency (8). The following is a summary of this hypothesis. In immature

TABLE 3 COMPOSITION OF α-HYDROXY FATTY ACIDS IN GLYCOLIPIDS OF MYELIN*

Fatty Acids	Globoid Cell Leukodystrophy		Normal Control†	
	Cerebroside	Sulfatide	Cerebroside	Sulfatide
14h:0	tr.	tr.	1.1	0.2
16h:0	0.2	1.3	0.8	3.1
17h:0	0.2	tr.	—	—
18h:0	1.6	3.2	1.3	2.4
18h:1	tr.	tr.	tr.	1.1
19h:0	0.3	1.3	—	—
20h:0	1.0	2.8	4.5	3.6
21h:0	0.2	0.6	—	—
22h:0	8.7	6.2	9.5	1.1
23h:0	10.0	10.2	9.2	7.4
24h:0	51.3	39.6	49.2	42.7
24h:1	8.9	14.5	12.3	19.5
25h:0	6.1	6.8	4.4	3.6
25h:1	1.5	1.2	1.9	2.8
26h:0	4.3	7.6	1.2	3.2
26h:1	5.5	4.7	4.6	8.0
Sum of 14h:0–20h:0	3.3	8.6	7.7	10.4
Sum of 21h:0–26h:1	96.5	91.4	92.3	88.7

* Expressed as percentage of the total α-hydroxy fatty acids.
† 10 months old, from O'Brien and Sampson (19).

brains before myelination, cerebroside is practically absent, and therefore, lack of galactocerebroside β-galactosidase is of little consequence. As soon as myelination begins, myelin starts undergoing normal turnover, but cerebroside from catabolized myelin cannot be degraded due to the deficiency of the enzyme. Free galactocerebroside then specifically elicits the globoid cell reaction in the tissue (22). Mesodermal macrophages invade white matter, phagocytize excess cerebroside, and transform themselves to globoid cells. However, before myelination can proceed much further, the ever-increasing globoid cells become so overwhelming in size and number that death of oligodendroglial cells ensues. Since galactocerebroside is highly concentrated in myelin and oligodendroglia, there will be no further production of cerebroside after death of all oligodendroglial cells, thereby limiting the total amount of galactocerebroside that can accumulate in the brain of patients with globoid cell leukodystrophy.

On the basis of the above hypothesis, it may not be surprising to find myelin with normal lipid composition because the biosynthetic mechanism of myelin formation should be intact. However, the finding of the abnormally high sulfatide content in myelin in metachromatic leukodystrophy (4, 5) further complicates this picture. Both diseases are characterized by genetically determined deficiency of lysosomal hydrolytic enzymes, arylsulfatase A in metachromatic leukodystrophy, and galactocerebroside β-galactosidase in globoid cell leukodystrophy. In both diseases, the enzymatic mechanism of myelin synthesis should be normal. Then why is normal myelin formed in globoid cell leukodystrophy and myelin with excess sulfatide in metachromatic leukodystrophy? The availability of excess lipids to oligodendroglial cells seems to explain this difference. Sulfatide is present in the brain at a small concentration even before myelination (23). This indicates that sulfatide is less specifically localized in the myelin sheath and must also be an important constituent of other membranes. It is expected, therefore, that, in metachromatic leukodystrophy, there may already be some abnormal accumulation of sulfatide in the brain before myelination due to turnover of other sulfatide-containing membranes. Also in metachromatic leukodystrophy, sulfatide accumulation occurs primarily within oligodendroglial cells, which survive well into the terminal stage of the disease. Therefore, when the normal mechanism of myelin formation is operating in the brain of metachromatic leukodystrophy patients, the excess sulfatide is readily available to myelinating oligodendroglial cells and might be inadvertently incorporated into myelin. In globoid cell leukodystrophy, on the other hand, galactocerebroside is virtually absent until myelination begins (23), and the excess free cerebroside, produced from catabolized myelin as the result of galactocerebroside β-galactosidase deficiency, does not accumulate in oligodendroglia but is taken up by globoid cells. Therefore, at any stage of the disease, the excess cerebroside is not available to myelinating oligodendroglial cells, which then produce myelin with normal chemical composition. The above hypothesis does not take into account the possible low activity of sulfotransferase. Although probably secondary, it may already be low in the early stage of the disease. If so, how this additional enzymatic derangement, which is in the synthetic mechanism of myelin formation, fits into the over-all pathophysiology of globoid cell leukodystrophy will still remain to be explored.

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