Lipid class analysis of the central nervous system of myelin-deficient Wistar rats

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Abstract Brains and spinal cords of myelin-deficient (md) and littermate control rats were analyzed serially for myelin lipids during the period from 13 to 32 days of age. The glycolipids of md rat brains were severely reduced and remained so during the period of study; brain cholesterol and phospholipids increased moderately but never reached the values for control brains. Deficiency of all three lipid classes was marked in the spinal cord and did not change with age. Among the glycolipids of md rats, deficiency was more severe in cerebrosides than sulfatides. The pronounced reduction of cerebrosides in the early stages of myelination suggests that abnormal synthesis of these glycolipids may be the most important biochemical anomaly responsible for myelin deficiency.—**Csiza, C. K.** Lipid class analysis of the central nervous system of myelin-deficient Wistar rats. *J. Lipid Res.* 1982. **23:** 720–725.

Supplementary key words lipid class • cholesterol • glycolipid • cerebroside • sulfatide • phospholipids

An X-chromosome-linked recessive mutation in the Wistar rat has been previously characterized (1). The defect in the mutant is expressed by lack of myelin formation in the central nervous system, while the peripheral nervous system is unaffected (1, 2). Affected hemizygous males develop tremors at 12 to 14 days of age. Generalized seizures occur 17 to 21 days postnatally, and animals usually die within 30 days of birth. The mean age at death for 100 affected pups was 24.7 ± 6.0 (mean ± S.D.) days.

This communication reports lipid alterations in the brain and spinal cord of md rats.

MATERIALS AND METHODS

Chloroform, ascorbic acid, and ammonium molybdate-4-hydrate were of analytical grade and purchased from J. T. Baker, Co., Phillipsburg, NJ. Methanol, hexane (glass-distilled), and diethyl ether were ACS grade and purchased from M. C. B. Reagents, Cincinnati, OH. Cholesterol and phosphorus standard solutions and D(+)-galactose (crystalline) were purchased from Sigma Chemical Co., St. Louis, MO. Orcinol and sulfuric and acetic acids were reagent grade; hydrogen peroxide (30%), ferrous sulfate (crystalline), potassium citrate, and sodium sulfite were ACS certified and obtained from Fisher Scientific Co., Rochester, NY. Precoated silica gel 60 thin-layer chromatography glass plates without fluorescent indicator were purchased from VWR Scientific Co., San Francisco, CA. Standard TLC mixtures 2 and 3 were purchased from Applied Science Labs, State College, PA.

Eleven- to 32-day-old myelin-deficient rats and control littermates were anesthetized with ether and killed by exsanguination. The brains and spinal cords were removed and, if not used immediately for experimentation, were flooded with nitrogen gas and frozen at −80°C.

Fresh or thawed brains and spinal cords were homogenized in double-distilled water (1.6 and 2.0 ml, respectively, per brain or spinal cord) with Ten Brock tissue grinders. Total lipids were extracted (3) with 19 volumes of chloroform–methanol 2:1 (v/v). The mixture was kept in an atmosphere of nitrogen overnight at 4°C and then filtered. The filtrate was washed according to the methods of Folch, Lees, and Sloane Stanley (3) and Webster and Folch (4). The lipid extracts were dried under nitrogen and used for cholesterol, galactose, and phosphorus determinations and for TLC.

To determine the effect of mutation at the md locus on copper metabolism in the Wistar rat, brains from ten pairs of 25-day-old normal and md rats were examined by atomic absorption spectrometry (5).

Analyses of total lipid extract

Cholesterol in the lipid extract was measured by the Liebermann–Buchard color reaction as modified by Searcy and Burquiss (6). Glycolipid hexose was determined by the orcinol–sulfuric acid procedure of Sorensen and Hougaard, as modified by Hess and Lewin (7, 8).

Abbreviations: md, myelin-deficient; CNS, central nervous system; PNS, peripheral nervous system; TLC, thin-layer chromatography; C–M, chloroform–methanol; C/S, cerebroside/sulfatide ratio; solvent ratios are v/v.

TABLE 1. Values of body, brain, and spinal cord weights in control and md rats (mean ± S.D.)

Rats	Age	Body Weight	Brain Weight	Spinal Cord Weight
	days	*g*	*g*	*g*
Control	13	20.7 ± 0.6 (5)[a]	1.11 ± 0.02	0.11 ± 0.01
md	13	20.6 ± 1.7 (7)	1.11 ± 0.04	0.10 ± 0.01
Control	17	27.4 ± 5.2 (5)	1.27 ± 0.03[b]	0.15 ± 0.01
md	17	27.4 ± 2.2 (5)	1.26 ± 0.08[c]	0.13 ± 0.01
Control	22	33.4 ± 1.8 (8)	1.34 ± 0.08	0.17 ± 0.01
md	22	32.7 ± 3.1 (9)	1.32 ± 0.04	0.14 ± 0.01
Control	27	57.6 ± 2.9 (6)	1.41 ± 0.02	0.21 ± 0.01
md	27	43.2 ± 5.2 (5)	1.31 ± 0.08	0.16 ± 0.01
Control	32	84.1 ± 11.9 (4)	1.52 ± 0.04[d]	0.28 ± 0.02
md	32	46.6 ± 4.1 (4)	1.31 ± 0.01[e]	0.16 ± 0.01

[a] Number of animals in each group is shown in parentheses.
Water as percentage of wet weight was: [b], 83.5 ± 0.3 (6); [c], 84.2 ± 0.2 (4); [d], 80.0 ± 0.4 (6); [e], 82.2 ± 0.1 (3).

For total phosphorus determination, the samples and standards were first mineralized by heating with sulfuric acid and hydrogen peroxide (9, 10). Phosphorus was measured by the method of Ammon and Hinsberg as modified by Chen, Toribara, and Warner (9).

Separation of lipids

Individual lipid classes were separated by one-dimensional TLC on precoated silica gel 60 plates, which were activated at 110°C overnight prior to use (11–15). Lipids were identified by comparison to reference compounds run at the same time. The TLC plates were developed with one of the following solvent mixtures: a) C–M–water 14:6:1 for glycolipids (11); b) ethyl acetate–n-propanol–C–M–0.25% aqueous KCl 25:25:25:10:9 for phospholipids (modified from ref. 13 by substituting ethyl acetate for methyl acetate); and c) n-hexane–diethyl ether–glacial acetic acid 70:30:1 for cholesterol and cholesteryl ester (14, 15).

Bands were detected with iodine vapor. The iodine was allowed to evaporate before the bands were scraped from the glass plate into test tubes. Cerebrosides and sulfatides were eluted three times with developing solvent (5 ml each time) and assayed for hexose (7).

Phospholipids were assayed without prior elution from silica gel as described above. Cholesteryl esters were hydrolyzed with 1 M ethanolic KOH without elution from silica gel (15). The nonsaponifiable material was extracted into hexane. To avoid elution and repeated extraction of free cholesterol from silica gel, the cholesterol was treated identically to the cholesteryl ester fraction. Aliquots of the hexane phase were dried under nitrogen and assayed by the method of Searcy and Burquiss (6).

RESULTS

Growth rates (**Table 1**) of body and the central nervous system in md rats and normal littermates were indistinguishable during the first 3 weeks of life. As seizures became more frequent, the rate of growth slowed.

The analytical data on washed, total lipid extracts from brains are summarized in **Table 2**. The mean cholesterol content of normal, 13-day-old control rats was 7.02 mg/g wet weight, which increased to 11.98 mg/g at 32 days of age. For md littermates, the mean cholesterol concentration was 9% lower at 13 days and 25% lower at 32 days of age. The mean phosphorus content at 13 days was 1.02 and 0.97 mg/g in control and md rats, respectively. In both groups the phospholipids increased gradually but they did so at a slower rate in the

TABLE 2. Rat brains: composition of lipid extracts from control and md brains during development (mg/g wet weight, mean ± S.D.)

Rats	Age	Cholesterol	Decrease	Phospholipid P	Decrease	Glycolipid Hexose	Decrease	C/S[a]
	days		%		%		%	
Control	13	7.02 ± 0.90 (7)[b]		1.02 ± 0.12 (7)		0.36 ± 0.15 (7)		2.3
md	13	6.37 ± 0.61 (10)	9	0.97 ± 0.10 (10)	5	0.16 ± 0.07 (10)	55	0.9
Control	17	9.18 ± 0.77 (6)		1.36 ± 0.14 (6)		0.63 ± 0.07 (6)		2.6
md	17	7.92 ± 0.64 (9)	14	1.14 ± 0.10 (9)	16	0.32 ± 0.07 (9)	50	1.1
Control	22	9.09 ± 0.93 (8)		1.36 ± 0.12 (8)		1.33 ± 0.17 (8)		5.0
md	22	7.55 ± 0.84 (10)	17	1.11 ± 0.11 (10)	18	0.26 ± 0.15 (10)	81	1.0
Control	27	11.76 ± 0.64 (10)		1.61 ± 0.10 (10)		1.23 ± 0.41 (10)		5.7
md	27	8.95 ± 0.61 (9)	24	1.30 ± 0.10 (9)	19	0.21 ± 0.04 (7)	83	1.3
Control	32	11.98 ± 0.62 (4)		1.81 ± 0.10 (4)		1.49 ± 0.14 (4)		6.7
md	32	9.04 ± 0.92 (4)	25	1.45 ± 0.03 (4)	20	0.15 ± 0.03 (4)	90	1.8

[a] Cerebroside/sulfatide ratio after TLC separation.
[b] Number of animals in each group is shown in parentheses.

TABLE 3. Rat spinal cords: composition of lipid extracts from control and md spinal cords during development (mg/g wet weight, mean S.D.)

Rats	Age	Cholesterol	Decrease	Phospholipid P	Decrease	Glycolipid Hexose	Decrease	C/S[a]
	days		%		%		%	
Control	13	12.74 ± 1.94 (7)[b]		1.50 ± 0.18 (7)		1.73 ± 0.27 (7)		2.8
md	13	6.54 ± 0.84 (10)	57	1.08 ± 0.06 (10)	28	0.34 ± 0.09 (10)	80	1.2
Control	17	17.71 ± 1.02 (5)		1.66 ± 0.09 (5)		2.84 ± 0.30 (5)		3.8
md	17	8.03 ± 0.65 (8)	55	1.06 ± 0.13 (8)	36	0.66 ± 0.07 (8)	77	1.2
Control	22	21.65 ± 2.22 (8)		1.92 ± 0.12 (8)		3.97 ± 0.39 (8)		5.6
md	22	7.35 ± 0.83 (10)	66	1.09 ± 0.06 (9)	43	0.39 ± 0.11 (9)	90	1.3
Control	27	22.22 ± 1.06 (9)		2.12 ± 0.17 (9)		4.30 ± 0.40 (8)		5.3
md	27	8.27 ± 1.68 (9)	63	1.17 ± 0.14 (9)	45	0.51 ± 0.13 (9)	88	2.1
Control	32	23.17 ± 1.83 (6)		2.42 ± 0.09 (3)		4.35 ± 0.89 (6)		7.9
md	32	6.41 ± 1.00 (5)	72	1.00 ± 0.11 (5)	59	0.38 ± 0.16 (5)	91	1.9

[a] Cerebroside/sulfatide ratios after TLC separation.
[b] Number of animals in each group is shown in parentheses.

md rats, whose phospholipids were 20% lower at 32 days of age. The mean glycolipid hexose content was 0.36 mg/g in control rats and 0.16 mg/g in md rats. The glycolipids increased rapidly in normal rats, over fourfold by age 32 days, but remained essentially unchanged in md littermates. Thus, mutant brains showed a lower cholesterol and phospholipid content and cessation of glycolipid synthesis.

In spinal cord extracts from md rats all three major lipid constituents had reached their maximum concentration by 13 to 17 days of age, although they were significantly lower than controls, and remained essentially unchanged thereafter (**Table 3**). The most pronounced deficiency was in glycolipids, followed by cholesterol, and then phospholipids. In normal rats at 13 days of age the lipid content of the spinal cord approached or exceeded that of the brain at 32 days of age. Spinal cord concentrations then continued to increase during the experimental period.

In the md spinal cords, as in the brains, the cerebroside content remained unchanged, while the sulfatide hexose decreased slightly with age. The ratio of the mean cerebroside to sulfatide content (C/S) increased from 0.9 to 1.8 in md brains and from 1.2 to 1.9 in md spinal cords. In control rats, the C/S increased from 2.3 to 6.7 in the brains and from 2.8 to 7.9 in the spinal cords.

The decreases in cholesterol levels in md brains and spinal cords were similar when assays were done on Folch lower-phase lipid extracts or after TLC. The quantity of cholesteryl esters was very small (less than 0.5 mg/g tissue in brain and 1.0 mg/g in spinal cord) and was similar in both groups of rats. The percent distribution of individual phospholipids in the md brains and spinal cords was comparable to that of the control littermates.

In the present study brain copper concentrations were similar in md rats and littermate controls (1.72 ± 0.22 μg/g and 1.76 ± 0.17 μg/g, respectively) at 25 days of age.

DISCUSSION

The onset of myelination varies from species to species and occurs at different times in various CNS structures (16, 17). In the CNS, myelin is formed by the oligodendroglial cell as an outgrowth of the plasma membrane, which provides segments of myelin for several axons (18–20). Myelin deposition occurs on neuronal axons, first in the spinal cord and later in higher CNS centers (18). In the brain of normal rats myelination is most rapid from 10 to 21 days of age (18).

The major lipid constituents of myelin are cholesterol, phospholipids, and glycolipids (18–20). Glycolipids with a general structure of N-acylsphingosine-1-β hexoside are cerebrosides. Galactose-containing cerebrosides are myelin-specific (18–20). About 70% of the sulfatide content (cerebroside sulfate) is also located in the myelin (18); the rest is in the glial cells. Lipid extracts from the CNS, prior to the first appearance of myelin rings, are rich in cholesterol and phospholipids, low in cerebrosides, and similar to the composition of glial cell lipids (19). Incorporation of cerebrosides into the oligodendroglial cell membrane marks the deposition of myelin around the axons (19).

Previous studies of the lipid composition of the rat brain have indicated a wide variation in the quantities of glycolipids (21–26). The most likely causes of this disparity are differences in the methods used, variations among the strains of rats studied, and the possibility that

investigators were unsure of the animal's exact age at the time of sacrifice. Our values for glycolipids (Table 2) in normal rat brains fall within those reported in the literature. No such studies for the rat spinal cord have been published.

The relative accumulation of cerebrosides and sulfatides (C/S) in the normal brain during development has been studied by Wells and Dittmer (22) and Hauser (23). In the present study, in which the rats were 13 to 32 days of age, the C/S ratios were slightly lower at 13 and 17 days and higher at 32 days than those found by the above mentioned authors. The reasons for this discrepancy may reflect the methodology used, which allowed for greater loss of glycolipids, especially from the spinal cords and brains of older rats having the greatest lipid concentrations. The poor yields of sulfatide could explain our findings for C/S ratios.

Previous reports of total cholesterol and phospholipid contents of normal brain lipid extracts (22, 26, 27), as well as the percent distribution of individual phospholipids (21, 22), are in agreement with our observations.

During early postnatal development, analysis of lipids from the spinal cord and brain of the same rat permits a study of myelination in the CNS at different developmental stages (Tables 2 and 3). In normal rats, while cholesterol and phospholipids continue to increase with age, glycolipids accumulate at a much faster rate and concentrations of lipid constituents are much higher in the spinal cord than in the brain. This is not true for the md rat. From 13 to 32 days of age there is a smaller than normal increase in the cholesterol and phospholipid components of brain lipids and virtually no change in any of the three major components of the spinal cord lipids. Thus, maximum concentrations had been achieved by the time clinical signs of md were expressed (12–13 days). Oligodendrocytes of md rats failed to form compact myelin and contained electron-lucent vacuoles or homogenous lipid inclusions (2). These ultrastructural abnormalities were evident as early as 3–7 days postnatally. Axonal abnormalities were not observed prior to the occurrence of seizures at 17–20 days of age.

The enzyme systems and various cofactors concerned with myelin formation are susceptible to change during the period of myelination. In the rat postnatal hypothyroidism (28), dietary folate (29) or copper (30) deficiency, or malnutrition (27) significantly decreased brain weight and myelin formation without affecting its lipid composition. A defect in copper metabolism resulting in abnormal myelin structures has been observed in mice (31), lambs (32), and man (33). In the present study md rats were found to have normal amounts of brain copper.

Other mutations characterized by inadequate and abnormal myelin formation have been reported in several species (34–36). Mutations in mice resembling the md rat and studied most extensively (37–44) are jimpy (jp) (37) and myelin synthesis deficiency (jp^{msd}) (38), an allelic relative of jp (39). The defect in the jp and jp^{msd} mutations appears to be in oligodendroglial proliferation and maturation (38, 44). The few oligodendrocytes that escape the genetic block make nearly normal or normal myelin. Although the md rat has many similarities to the jimpy mouse mutant, it probably has a different molecular defect. The rat mutant does not form compact myelin; its CNS contains no macrophages with sudanophilic cystoplasmic droplets; and the glycolipid deficiency is more severe.

In humans a rare dysmyelinating condition, seen in the first few months of life, was observed in one family over four generations by Pelizaeus (5 cases, 1885) and Merzbacher (9 cases, 1910–1923) (45–47). In the original family the inheritance of the disease was X-chromosome-linked recessive, but later two of the affected siblings were females. Since the original descriptions, a number of cases with similar clinical and neuropathologic findings have been reported. Some of these cases occurred later in life and included the presence of sudanophilic myelin breakdown products. In 1970 Pelizaeus-Merzbacher disease was divided into two main groups: the congenital type with virtually no myelin (the classic Pelizaeus-Merzbacher or Seitelberger type) and the adult (Lowenberg-Hill) type. The defect of the md rat is analogous to the classic Pelizaeus-Merzbacher disease in man, not only in biochemical findings, but also in the method of its inheritance. Since the colony was established, all but five of the md pups have been males. Female rats with myelin deficiency were anatomically female with no evidence of hermaphroditism. The disease in females was clinically, histologically, electron-microscopically, and biochemically indistinguishable from that of male counterparts.

Affected females occurred once each in five breeding pairs in the first to the fifth litter and from the fourth to the seventh generation. These five carrier females produced a total of 216 young in 21 litters; 51 of 106 male and 5 of 110 female pups were myelin-deficient. As soon as the disease was recognized in a female pup, nearly all clinically normal littermates were saved to be used as breeders. The progeny of brother–sister matings and back-crossing of daughters to fathers resulted in no additional affected daughters. The colony is now in its 10th generation.

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