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LIPIDS OF DEVELOPING BRAIN OF TWITCHER MOUSE

AN AUTHENTIC MURINE MODEL OF HUMAN KRABBE DISEASE

by HIDEKI IGISU, KOICHIRO SHIMOMURA, YASUO KISHIMOTO *and* KUNIHIKO SUZUKI

(*From the Saul R. Korey Department of Neurology, Department of Neuroscience and the Rose F. Kennedy Center for Research in Mental Retardation and Human Development, Albert Einstein College of Medicine, Bronx, NY 10461, USA, and the J. F. Kennedy Institute, Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*)

SUMMARY

Brains of the newly discovered neurological mutant of the mouse, twitcher, were analysed from birth to the terminal stage of the disease for major tissue constituents and the lipid composition. A genetic deficiency of galactosylceramidase is the underlying cause of this mutant. The affected mice failed to gain body weight after 20 days (30 per cent of normal at 42 days) but the brain weight was much less affected (85 per cent of normal at 42 days). The water content and the four major fractions, chloroform-methanol insoluble residue, chloroform-methanol soluble protein, upper phase solids and total lipid, were essentially unaltered except that the total lipid was reduced slightly towards the terminal stage. Among lipids, only galactolipids, galactosylceramide and sulphatide were abnormal in the twitcher brain. Galactosylceramide was decreased at 37 and 42 days whereas decrease in sulphatide occurred earlier from 25 days, resulting in an increased ratio of galactosylceramide to sulphatide. The analytical abnormalities found in the twitcher brain are qualitatively similar to and quantitatively milder than those in the brain of human patients with globoid cell leukodystrophy (Krabbe disease), of which the twitcher is an enzymatically authentic animal model. The secondary abnormalities observed in brains of human patients as the result of tissue devastation were generally not present in the twitcher brain. Mouse brains contain relatively little myelin. As the pathology in twitcher is greatest in the spinal cord and brainstem (Duchen *et al.*, 1980), the fact that the changes in twitcher brains are quantitatively milder than those of human patients may partly reflect the relative amount of myelin in the two species. While more detailed studies of some specific constituents, such as galactosylsphingosine, and of other tissues, such as the peripheral nervous system and the kidney, will have to be performed, the present results serve as the reference for comparison between the human and murine galactosylceramidase deficiency states and for future experiments with the twitcher mutant, which is an invaluable tool for studies of globoid cell leukodystrophy.

Requests for reprints to: Dr Kunihiko Suzuki, The Rose F. Kennedy Center, Albert Einstein College of Medicine, Bronx, NY 10461.

INTRODUCTION

Globoid cell leucodystrophy (GLD, Krabbe disease) in man is a rare genetic disorder caused by a deficiency of galactosylceramidase (EC 3.2.1.46) activity (Suzuki and Suzuki, 1970). Studies of human patients are severely restricted because of its rarity and for ethical reasons. Appropriate animal models can provide invaluable experimental tools for studies of pathobiology of the disease. Genetic GLD has been reported in three other relatively large mammalian species; cat (Johnson, 1970), sheep (Pritchard *et al.*, 1980), and dog (Fankhauser *et al.*, 1963; Fletcher *et al.*, 1966). Of these, no enzymatic confirmation was available for the feline disease. The sheep model did show profound deficiency of galactosylceramidase but, to our knowledge, no sheep colony with the disease has been established. The canine form of GLD occurs in pure breeds of West Highland and Cairn terriers as the result of genetic galactosylceramidase deficiency (Suzuki *et al.*, 1970). Since a colony of these dogs was maintained for research purposes, the canine model was used extensively for studies of genetic galactosylceramidase deficiency (*see* Suzuki and Suzuki, 1983, for review). However, the dog colony has recently been terminated (T. F. Fletcher, personal communication) and is therefore no longer available for research except for sporadic cases.

While the canine model alleviated some of the constraints associated with studies on human patients, its size, the limited number of litters, slow reproduction and the requirements for elaborate and expensive facilities for maintenance made it unsuitable for experiments that would require a large number of affected and/or heterozygous animals. The recently recognized murine mutant, twitcher, can overcome these disadvantages of the previously available animal models. The pathological alterations of the nervous system of affected mice are very similar to those of human patients (Duchen *et al.*, 1980) and the underlying genetic cause is also the deficiency of galactosylceramidase activity (Kobayashi *et al.*, 1980). On the other hand, even if caused by the same enzymatic defect, species differences are expected in the clinicopathological consequences and, therefore, detailed basic characterization is the prerequisite initial phase of the study in order to assure proper interpretation of the results of subsequent experiments. The present report describes in some detail the developmental changes in the lipid composition of twitcher brains.

MATERIALS AND METHODS

Animals

A colony of twitcher mice is maintained in the animal facility of this institution. The genetic status of each animal—affected, heterozygous and normal—is routinely determined within 10 days after birth by assaying clipped tails for galactosylceramidase activity (Kobayashi *et al.*, 1982). All animals were kept with their mothers and were weighed when they were killed. After 30 days, the standard laboratory animal chow was soaked in tap water and placed on the floor of the cage to facilitate feeding

of affected mice. Artificial forced feeding was not employed. Under these conditions, it was difficult to maintain affected mice beyond 45 days. At least 1 normal, 2 affected and 2 heterozygous animals were killed at each time point, selected at intervals from 1 day to 42 days. Mice were killed by decapitation. Blood was blotted with filter paper, the brain carefully removed free of cranial nerves, and stored frozen in an air-tight container until analysed. All structures above the foramen magnum, including the cerebellum, were included in the specimen.

Analytical Procedures

Analyses were carried out within three months of storage. Five and 3 brains were pooled for each sample for 1-day and 8-day-old mice, respectively. Individual brains were analysed separately for all other ages. Brains from affected, heterozygous and normal mice of the same age were processed simultaneously. Wet weight of the brain was measured in a preweighed Potter-Elvehjem glass homogenizer tube. The brain was then extracted with 19 vol. of chloroform-methanol (2:1, v/v) with 70 strokes of a motor-driven glass pestle.

The general principle of lipid analysis was essentially as described previously (Suzuki *et al.*, 1969), based on the method of Folch *et al.* (1957). The 'chloroform-methanol insoluble residue', 'upper phase solids', 'chloroform-methanol soluble protein', and 'total lipid' fractions were weighed after complete desiccation. The sum of these four major fractions was the dry weight of the tissue.

The 'upper phase solids' fraction was dissolved in water and dialysed against water for 40 h at 4° C with at least 3 changes of water. After total ganglioside in the dialysed upper phase was estimated by determination of the sialic acid content (Svennerholm, 1957; Miettinen and Takki-Luukkainen, 1959), it was dried and dissolved in chloroform-methanol (1:1, v/v) to a sialic acid concentration of 0.5 μg/μl. Ten μl of this solution was applied as a 7 mm streak on a Merck high-performance silica gel 60 thin-layer plate (EM Laboratories, Englewood Cliffs, NJ). The plate was developed in chloroform-methanol-water (55:45:10, v/v/v) containing 0.02 per cent $CaCl_2 \cdot 2H_2O$, and ganglioside visualized by spraying the resorcinol reagent and heating (Ando *et al.*, 1978). Quantitative determination of individual gangliosides was done with Shimadzu CS-910 TLC scanner at 580 nm in the linear scanning mode, and the peak areas were calculated with Shimadzu CR-1A integrator-recorder. The ganglioside nomenclature was based on the system of Svennerholm (1963).

The total lipid fraction was dissolved in a known volume of chloroform-methanol (2:1, v/v). For a general semiquantitative view of individual lipids, 100 μg of the total lipid was chromatographed on the Merck high-performance silica gel 60 thin-layer plate in a solvent system of chloroform-methanol-water (70:30:4, v/v/v) and the spots were visualized by 50 per cent sulphuric acid spray and heating. Total cholesterol (Searcy and Bergquist, 1960) and total lipid phosphorus (Marinetti *et al.*, 1959; Norton and Autilio, 1966) were determined. Total phospholipid was estimated with an assumption that the average molecular weight of brain phospholipids is 775. Major phospholipids were separated by unidimensional thin-layer chromatography on silica gel G plates (Analtech, Newark, DE) in chloroform-methanol-concentrated ammonium hydroxide (65:25:5, v/v/v) and were visualized in iodine vapour. Each spot was scraped and the phophorus content determined directly (Norton and Autilio, 1966). The molecular weights used to calculate amounts of individual phospholipids were as described previously (Suzuki *et al.*, 1969).

Quantitation of galactosylceramide and sulphatide was by high-performance liquid chromatography (Nonaka and Kishimoto, 1979) except for a minor modification in the desulphation procedure. Benzoylated product, after drying, was dissolved in 0.25 ml of 7 per cent trifluoroacetic acid in ethylacetate and heated at 60° C for 45 min. The reaction mixture was evaporated to dryness and purified by partition in acetonitrile-hexane-water. Details of this modified procedure for sulphatide determination will be reported elsewhere (K. Shimomura and Y. Kishimoto, personal communication).

RESULTS

Determination of the genetic status of individual mice was unequivocal on the basis of galactosylceramidase activity in clipped tail (Table 1). These results were consistently confirmed by either galactosylceramidase activity in the spinal cord or kidney at the time of sacrifice or by the clinical disease for those mice killed at twenty-five days or thereafter. Evaluation of the results clearly indicated that there is no difference in the body weight, brain weight and in any other analytical parameters determined in this study between the genetically normal and heterozygous mice. Therefore, the data from these two groups are pooled into a single 'control' group hereafter, unless otherwise specified. The number of control samples at each time point was 4 (20 brains) at 1 day, 4 (12 brains) at 8 days, 5 at 15 days and 4 for all other ages.

TABLE I. GALACTOSYLCERAMIDASE ACTIVITY IN CLIPPED TAILS OF MICE USED FOR BRAIN LIPID ANALYSIS

Genetic status	*Enzyme activity* (*nmol/h/mg protein* $\pm$ *SD*)
Normal	2.49 ± 0.45 (n = 28)
Heterozygote	1.30 ± 0.16 (n = 29)
Affected	0.29 ± 0.13 (n = 28)

Affected mice gained body weight normally until 21 days, after which time, however, they not only failed to gain weight further, but lost some weight towards the terminal stage (fig. 1). At 42 days the twitchers weighed less than 30 per cent of the control mice. Brain weight was much less affected than body weight. It tended to be slightly lower than controls at 33 days and was significantly lower at 37 days ($P < 0.004$) and 42 days ($P < 0.003$) (fig. 1). Even at the terminal stage, wet weight of the twitcher brain was 85 per cent of the control brain. While the proportion of the dry weight, relative to wet weight, increased during development, as expected, there were no statistically significant differences between the twitchers and the controls throughout the experimental period (fig. 1). This means that the dry weight per brain in the twitcher mice at the terminal stage is lower than the controls by the same percentage as the reduction in the wet weight. The developmental changes of the four major fractions in the twitcher brains generally followed the patterns of the control brains, on the basis of percentage of dry weight, except that there were small decreases in the total lipid at 37 days ($P < 0.11$) and 42 days ($P < 0.03$) (fig. 2).

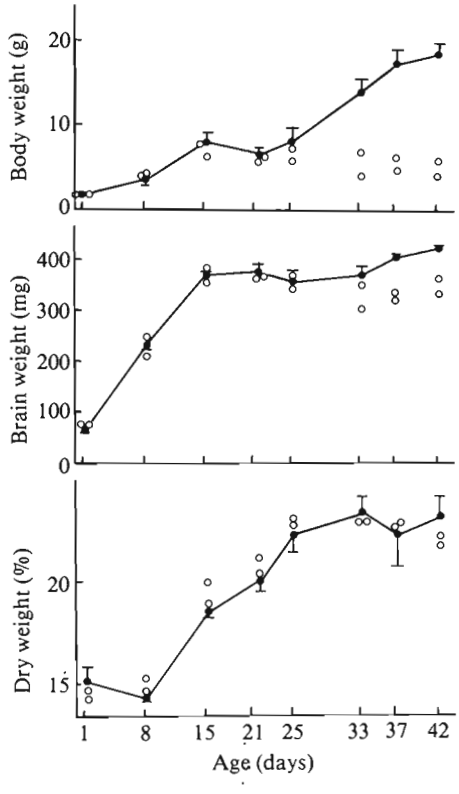

FIG. 1. The body weight, brain weight and dry weight of the brain of the twitcher mouse during development. The control values are given in closed circles with the standard deviations, while the values of the twitchers are given individually with open circles.

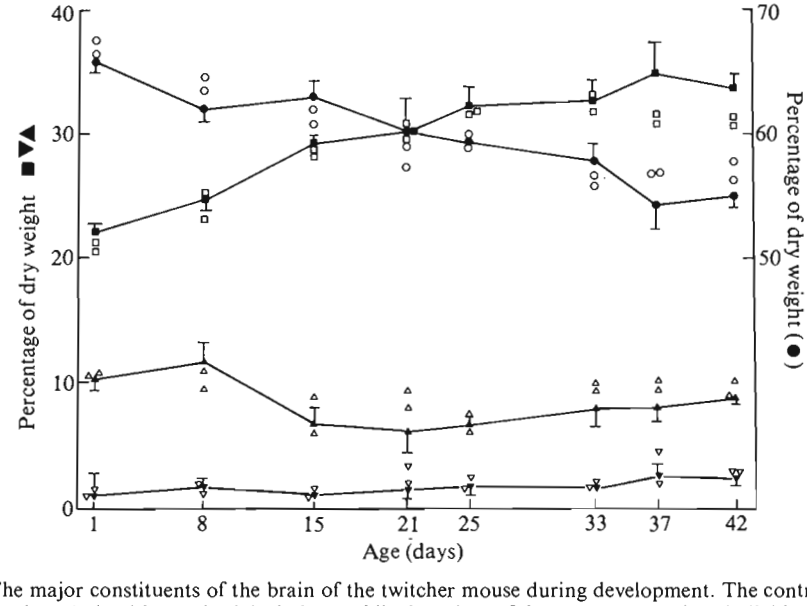

FIG. 2. The major constituents of the brain of the twitcher mouse during development. The control values are given in closed symbols with standard deviations, while the values of the twitchers are given individually with open symbols. Chloroform-methanol insoluble residue (● ○). Total lipid (■ □). Upper phase solids (▲ △). Chloroform-methanol soluble protein (△ ▲).

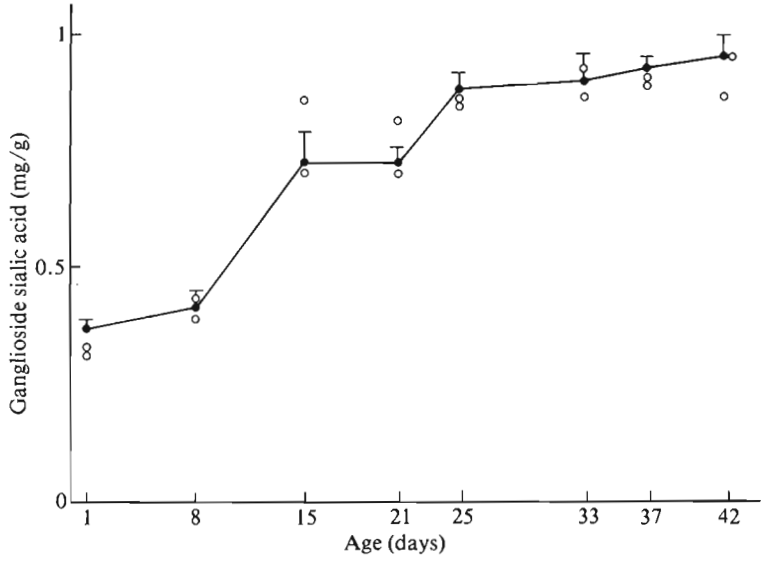

FIG. 3. The total sialic acid in the ganglioside fraction of the brain of the twitcher mouse during development. The control values are given in closed circles with standard deviations, while the values of the twitchers are given individually with open cicles.

The total sialic acid in the dialysed upper phase increased during development on the basis of unit brain weight (fig. 3). Since the increment in the wet weight of the brain was greatest around 10 days, the increase in total ganglioside sialic acid per brain was also steepest at this period. No significant differences were found, however, between the affected and control mice. Proportions of individual gangliosides likewise showed developmental changes but no differences between the twitchers and the controls (fig. 4). The four major gangliosides, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, and three minor gangliosides, G_{D2}, G_{D3} and G_{Q1b}, were quantitated separately throughout the development. Two minor spots with chromatographic mobility between G_{D1b} and G_{T1b} increased five-fold in proportion during development (G_{x1} and G_{x2}). Both were labile to mild alkaline treatment. Their exact structures were not determined.

Visual examination of thin-layer chromatograms of the total lipid fractions indicated that there were no discernible differences between the twitchers and the controls up to 33 days. At 37 days, the proportion of galactosylceramide appeared possibly lower and at 42 days clearly lower than the controls. The quantitative determinations essentially confirmed the visual impression (*see below*). The total cholesterol, total phospholipids and the individual major phospholipids in the twitcher brains followed the normal developmental changes (fig. 5).

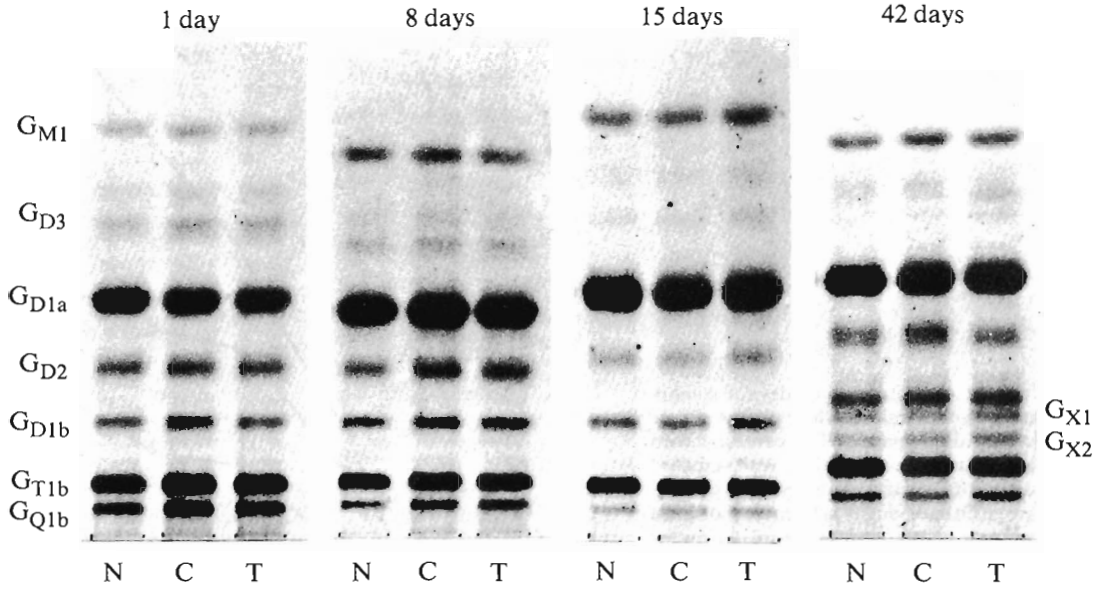

FIG. 4. Thin-layer chromatography of mouse brain gangliosides during development. The technical details were as described in the text. N = normal. C = carrier. T = twitcher. Since this figure is a composite of four thin-layer chromatograms, migrations of individual gangliosides are not necessarily identical.

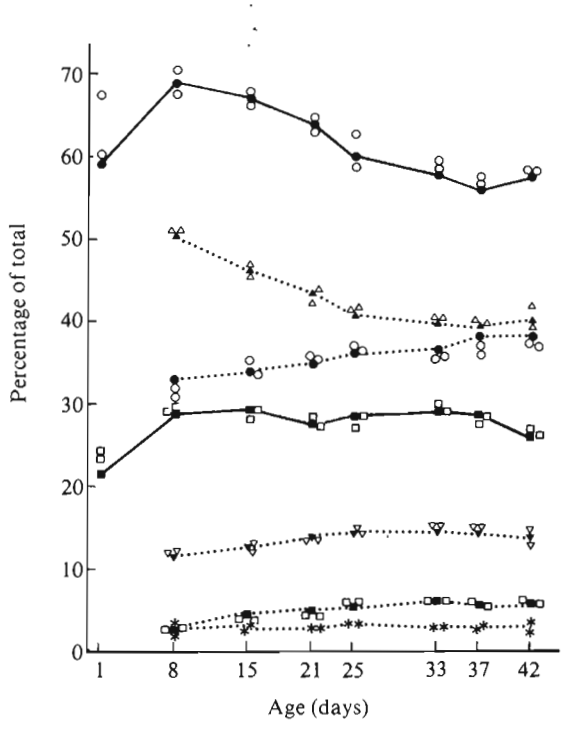

FIG. 5. Cholesterol and phospholipids in the brain of the twitcher mouse during development. The values for controls are in closed symbols and those for twitchers are in open symbols. Total phospholipids (●—○). Total cholesterol (■—□). Phosphatidylcholine (▲ - - - △). Ethanolamine phospholipids (● - - - - ○). Serine phospholipids (▼ - - - ▽). Sphingomyelin (■ - - - - □). Phosphatidylinositol (✱ - - - - ✱). (Only the values for the twitchers are given but the control values were identical with the twitcher values.) Total cholesterol and total phospholipids are expressed as a percentage of total lipid, while the individual phospholipids are given as a percentage of total phospholipid. In order to avoid cluttering the graphs, standard deviations for the controls are omitted. They were always within ± 5 per cent or less of the means.

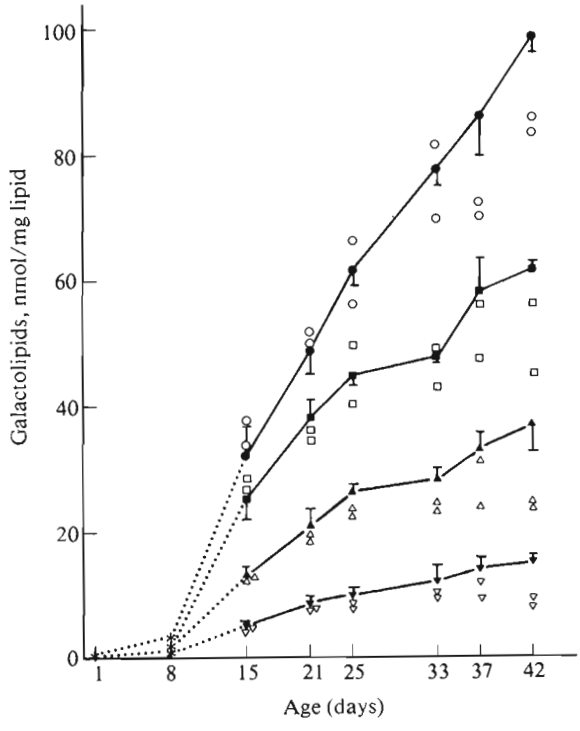

FIG. 6. Galactosylceramides and sulphatides in the brain of the twitcher mouse during development. The control means are given in closed symbols with standard deviations while the values for twitchers are given individually in open symbols. The values for 1 day and 8 days were too low for the scale of this graph and they are given in Table 2. The dotted lines indicate approximations for these time points. HFA-gal-cer (● ○). NFA-gal-cer (■ □). NFA-sulphatide (▲ △). HFA-sulphatide (▼ ▽).

Both galactosylceramide and sulphatide increased drastically after 8 days, concomitant with the period of active myelination in both the control and the twitcher mice (fig. 6, Table 2). At 1 day, α-hydroxy fatty acid-containing galactosylceramide (HFA-gal-cer) in the twitcher brain appeared higher than normal, while nonhydroxy fatty acid-containing galactosylceramide (NFA-gel-cer) and both types of sulphatides (HFA-sulphatide and NFA-sulphatide) were normal (Table 2). Since the amounts were extremely small at 1 day and thus the signal-to-noise ratio poor, this apparent difference should be interpreted with caution. Both

TABLE 2. BRAIN GALACTOLIPIDS IN EARLY DEVELOPMENT

	1-day old		*8-day old*	
	Control ($n = 4$)	*Twitcher* ($n = 2$)	*Control* ($n = 4$)	*Twitcher* ($n = 2$)
*Galactolipid**	(*nmol/mg lipid*)			
NFA-gal-cer	0.046 ± 0.020	0.06, trace	1.40 ± 0.40	1.78, 1.16
HFA-gal-cer	0.370 ± 0.060	0.80, 1.06	3.34 ± 0.56	5.00, 3.88
NFA-sulphatide	0.62 ± 0.26	0.60, 0.42	2.48 ± 0.58	2.72, 2.14
HFA-sulphatide	0.26 ± 0.08	0.30, 0.38	1.02 ± 0.34	1.20, 0.82

* NFA = nonhydroxy fatty acid. HFA = α-hydroxy fatty acid. The control values are given as the means ± SD, while individual values are given for the twitcher samples. Five brains were pooled for a sample at 1 day and three brains for a sample at 8 days.

HFA- and NFA-gal-cer, however, were normal in the twitcher brain from 8 to 33 days. At 37 and 42 days, HFA-gel-cer in twitchers was significantly lower than the controls ($P < 0.05$ at 37 days and $P < 0.002$ at 42 days). NFA-gal-cer was significantly lower only at 42 days ($P < 0.02$). On the other hand, both types of sulphatides, particularly HFA-sulphatide, were decreased earlier than galactosylceramides; at 25 days ($P < 0.02$ for NFA-sulphatide and $P < 0.005$ for HFA-sulphatide), at 33 days ($P < 0.003$ for NFA-sulphatide and $P < 0.002$ for HFA-sulphatide) and at 42 days ($P < 0.03$ for NFA-sulphatide and $P < 0.005$ for HFA-sulphatide).

DISCUSSION

In human globoid cell leucodystrophy (GLD) it is practically impossible to examine the evolution of the disease process from the early stages because available specimens are inevitably from the terminal stage. Even in the canine form of the disease, it is difficult to obtain a large number of specimens. Thus there has been no study in man or in animal models with GLD concerning the changes of the lipid composition of the brain throughout development. On the other hand, there are many analytical studies of brain lipids in human patients at the terminal stage available in the literature (*see* Suzuki and Suzuki, 1983, for review), the most extensive series being that by Vanier and Svennerholm (1974*a*, *b*, 1975). The twitcher mouse offers a unique opportunity to follow the biochemical evolution of the disease process and also to compare the findings at the terminal stage with those of the human disease. Identification of the genetic status immediately after birth on clipped tails permits reliable sampling well in advance of the clinical onset of the disease.

The major and perhaps the most surprising finding in the present series of the survey analysis of brain lipids in twitcher mice is that the composition of the brain lipids remains remarkably close to normal even at the advanced stage of the disease. A dramatic decrease in the total lipid and an increase in water content occur in the brain of human patients, more severely in the white matter but also in the gray matter (*see* Eto and Suzuki, 1971; Vanier and Svennerholm, 1974*a*). Only a slight decrease in total lipid and practically no change in the water content were observed in the twitcher even at the terminal stage. The associated reduction in cholesterol, the relative decrease in ethanolamine phospholipids and the relative increase in phosphatidylcholine found consistently in human patients were not apparent. Only the galactolipids, galactosylceramide and sulphatide were significantly decreased in the twitcher brains in later stages of the disease but the degree of the decrease was much milder than that in human patients. For example, the data of Vanier and Svennerholm (1974*a*) on 19 cases of human patients, ages 7 to 32 months, showed galactosylceramide levels from 40 per cent of normal in the youngest specimen to well below 10 per cent in others. In contrast, galactosylceramide in the 42-day twitcher brain was 80 to 85 per cent of the controls and sulphatide 60 to 65 per cent of the controls on the basis of wet weight of the brain. Similarly, there were

essentially no alterations in the total amount or in the proportions of ganglioside in the twitcher brain at any stage in contrast to the finding of Vanier and Svennerholm (1975). In human patient brains they found reductions of G_{D1a} and G_{M1} and relative increases of G_{D2} and G_{D3}.

These findings are consistent with the pathological findings of twitcher brain. Although clinical manifestations become apparent around 20 to 23 days, myelination in the early stages is essentially normal with thinning of the myelin sheath around the axons of larger diameter detectable at 20 and 30 days only by extensive morphometric analysis (Nagara *et al.*, 1982). Globoid cell infiltration is also moderate up to this stage, although these cells appear in small numbers from very early stages. In human GLD it is generally accepted that myelination is never normal and 'numerous' globoid cells have been observed in the spinal cord of an affected fetus aborted at 22-week gestation (Ellis *et al.*, 1973). Human patients are cared for much more intensively and thus survive to later stages than mice. Much more extensive alterations are expected in brains of human patients. Nevertheless, human GLD appears to affect the CNS much more severely from early stages of the disease. The species differences, such as this, make it imperative to characterize the twitcher mutant in detail pathologically and biochemically before experimental results can be interpreted with confidence.

Since the fundamental genetic defect is in galactosylceramidase activity and since the most profound abnormalities in the brain of human patients occur in galactolipids, special attention must be focused on the galactolipids. One of the most unusual features of human GLD is the lack of abnormal accumulation of galactosylceramide despite the block in the catabolic pathway. Although the level was not as low as those seen in human cases, galactosylceramide also does not accumulate abnormally in the twitcher brain. The most consistent finding in human GLD is the increase in the galactosylceramide to sulphatide ratio (Vanier and Svennerholm, 1974*a*; Suzuki and Suzuki, 1983). It is interpreted to mean that the low sulphatide level is more representative of the myelin loss, while the relative preservation of galactosylceramide is indicative of its presence within the globoid cells as a undegraded material due to the genetic defect. The same phenomenon occurs in the twitcher, most likely for the same reason. The sulphatide level was significantly lower than that in the controls already at 25 and 33 days when the galactosylceramide level was still normal.

In the present series of studies designed for a broad analysis of brain lipids, it was not possible to examine other minor glycolipids. The white matter of human patients contains abnormal amounts of more complex glycoplipids, lactosylceramide, galactosyl-galactosyl-glucosylceramide, globoside and the blood group substance H (Eto and Suzuki, 1971; Vanier and Svennerholm, 1975; Svennerholm *et al.*, 1980). They are interpreted as being localized in the globoid cells which are of mesodermal origin. Analysis of these glycolipids requires much larger samples than a single mouse brain and therefore pooling of several brains. Another important galactolipid related to the pathogenesis of GLD is galactosylsphingosine

(psychosine). It is also a natural substrate of galactosylceramidase (Miyatake and Suzuki, 1972*a*, 1974) and thus cannot be degraded in GLD (Miyatake and Suzuki, 1972*b*). The 'psychosine hypothesis' was developed as the biochemical pathogenetic mechanism of GLD based on the highly cytotoxic nature of galactosylsphingosine (Suzuki and Suzuki, 1983). In the human disease, strong support for the hypothesis was provided by Svennerholm *et al.* (1980) who showed at least 10- to 100-fold increases of galactosylsphingosine in the white matter of five patients over the essentially unmeasureable normal level. The possibility of its presence in twitcher brains would be of interest. The analytical technique of Svennerholm *et al.* (1980) requires more than 5 g of white matter. A more sensitive method therefore must be developed before galactosylsphingosine analysis can be attempted on twitcher brains.

Attention should also be directed to tissues other than the CNS. In the human disease, the PNS is moderately but consistently involved, with segmental demyelination manifested by reduced conduction velocity (Suzuki and Suzuki, 1983). From the available information, the PNS in the twitcher mice is affected earlier and more severely than the CNS, suggesting another important point of species differences (Duchen *et al.*, 1980; Kinuko Suzuki, personal communication). In the kidney of human patients, there is no accumulation of galactosylceramide despite the normal histology and function and the catabolic block (Suzuki, 1971). This finding prompted a speculation for a regulatory reduction of galactosylceramide synthesis as a possible explanation for the lack of abnormal accumulation. Our recent metabolic study appeared to exclude this possibility (Kodama *et al.*, 1982) but the level of galactosylceramide in the twitcher kidney needs to be ascertained for a more conclusive interpretation.

The analytical studies discussed above which all require larger amounts of tissues or more sensitive methods than the initial survey analysis presented in this report are planned for the near future.

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