PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 155, 549-553 (1977)

Lipid and Myelin Abnormalities of Brain in the Crinkled Mouse (39848)

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The effects of the mutant gene crinkled (*cr*) in mice have recently been shown to be partially prevented by dietary copper supplementation of the mother during gestation and lactation (1). Survival during Days 10–14 of postnatal life of offspring homozygous for the mutant gene was increased by maternal supplementation with copper. In addition, increased pigmentation and growth of the hair was seen in the high copper group. Since Days 10–16 of postnatal life in mice are critical for development of most areas of the brain (2), and since the importance of copper for myelination is well known (3), studies on brain lipid metabolism and morphology were indicated in the crinkled mutant. This paper reports studies on morphology and lipid content of the brain in crinkled and noncrinkled mice. Brain lipids were analyzed in 21-day-old mice since myelin formation has been found to be most active between 15 and 30 days after birth (4, 5). For comparison, brain lipid content was measured and histological examination was made in adult brains when myelin production was essentially complete (4).

Materials and methods. Mice of the B6C3F hybrid strain known heterozygous carriers of the mouse mutant gene crinkled (*cr*), were purchased from The Jackson Laboratory, Bar Harbor, Maine. They were fed a commercial pelleted stock diet (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) and were mated. At mating, some of the females were fed a purified diet (control) containing 6 ppm of copper (1). At Day 21, young were killed, and the gross morphology of crinkled and noncrinkled brains was compared. As a control for the possible effect of purified diet on lipid metabolism, other pregnant females and their litters were fed the stock diet until they were sampled for lipid analyses at 1 year or 17 months of age. The adult age studied was chosen randomly as the mutant colony was difficult to breed and attempts were made to utilize all mutants which survived and could be paired with controls. Female mice and their litters were observed frequently, and any behavioral changes were noted.

Lipid composition. The whole brain (21 day old mice) or right hemisphere (adult mice) from crinkled and noncrinkled mice was used for lipid analyses. To each sample, 7.0 ml of chloroform:methanol (2:1, v/v) were added, and the mixture was blended in a Lourdes homogenizer, filtered, and re-extracted once using 3.5 ml of the same solvent. The filtrates were centrifuged, and the second supernatant was combined with the first. The resultant extract was dried under N_2, redissolved, and filtered. This step was repeated until the solution was clear, providing evidence that no water was present. The filtrate was evaporated almost to dryness, and solvent was added to a final dilution of 3.0 ml [modification of Bligh and Dyer (6)].

Individual lipid classes were separated by two-dimensional thin-layer chromatography (tlc) on plates coated with silica gel H (Brinckmann Instruments, Cantague Rd., Westbury, N.Y.). Coated plates were washed by setting them in a glass tank containing a mixture of chloroform: methanol:water (65:35:4, v/v/v). When the solvent had migrated to the top of the plate, the plates were air dried and stored in a closed box containing dessicant. Before the lipid was applied the plates were activated at 120° for 30 min and were cooled at room temperature. Brain lipid extract (1.0–1.5 mg) was spotted with a microliter syringe on tlc plates under a stream of N_2. The plates were placed in a glass tank containing chloroform:methanol:28% ammonia (65:35:5, v/v/v). After the solvent had reached 14–15 cm on the plates, they were dried in a hood until all traces of ammonia were gone. They were then placed in a second solvent sys-

ISSN 0037-9727

tem (chloroform:acetone:methanol:acetic acid:water, 10:4:2:2:1, v/v/v/v/v) and developed to approximately the same distance. After exposure to iodine (5 min) the lipid spots were outlined. The iodine was evaporated from the plates which were dried for at least 2 hr before the gel spots containing lipid were removed. The separation and collection techniques that were used followed the procedure of Rouser *et al.* (7).

Phosphorus content in total phospholipid (adult samples) and individual phospholipids (phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol plus sphingomyelin) of brains from 21-day-old mice was analyzed by the methods of Eng and Noble (8); total cholesterol was measured according to Searcy and Bergquist (9). The cerebrosides and sulfatides were separated by tlc and were quantified according to the procedure of Drummond *et al.* (10). Cholesterol esters (adult brains) were separated from nonesterified cholesterol using tlc by a modification of the method of Hojnachi and Smith (11).

Morphological examination. Gross morphology of fresh brains from 21-day-old mice was examined during dissection and was scored as normal or abnormal before being homogenized for lipid analyses. These were not examined histologically since the entire brain was needed for lipid extraction. However, the larger adult brains, the right hemispheres of which were used for lipid analyses, were fixed in buffered formalin for histological examination. They were dehydrated in alcohol and chloroform and were infiltrated with paraffin under vacuum. Sagittal sections 10 μm thick were stained with Luxol fast blue G (12) to be viewed under the light microscope.

Results. Gross observations. Routine handling of the mice in the colony provided an opportunity to discover an abnormality of behavior. Many crinkled mice as well as known heterozygous carriers of *cr* were found to twirl in the air when picked up by their tails. Not all mice appeared to be affected, and the animals that were affected "twirled" with varying degrees of severity. This characteristic was not observed in other mice from the same colony. When brains from 21-day-old mice fed the purified control diet were dissected, 33% of the crinkled young had grossly malformed brains (Table I). Anomalies of the cerebellum included abnormal shape, cavitation, and reduction in size. In one case, the right side of the cerebellum was collapsed as if degenerated. An abnormal lateral fissure parallel to the median cerebral fissure was apparent in many of the samples.

Brain lipids. There were no statistical differences in brain weight between the crinkled and noncrinkled mice. Quantitation of 21-day-old mouse brain lipids showed no differences in cholesterol or cerebroside content (Table I). At this age there were no differences in individual phospholipids ($n = 3$): phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, or phosphatidyl inositol in combination with sphingomyelin. Sulfatides, however, were increased in 21-day-old crinkled mice compared to noncrinkled (Table I). In older 17-month-old mice, levels of cholesterol, sulfatides, and total phospholipids were not different from those of controls. However, cerebroside levels were significantly higher in crinkled than in noncrinkled mice. In addition, cholesterol esters were found in all adult samples of crinkled brain, but none were detectable in either of the two noncrinkled brain samples assayed on the same plate. Cholesterol esters were also identified in brains of 1-year-old crinkled mice, but were absent in brains of noncrinkled mice of the same age.

Morphological observations. Sagittal sections of 17-month-old noncrinkled mice showed normal organization and regular myelin staining. In one animal, the myelin in a few spots of the brain stem had a reticular appearance. In the same sample, there were one or two holes in the myelin tracts going to the stem in each section, but there appeared to be no fading of the myelin stain. Nuclei in the granular layer of the cerebellum and elsewhere were distinct, and nucleoli were evident (Fig. 1a).

When brain sections of *cr/cr* mice were viewed under low power, a striking difference from brain tissue of noncrinkled animals was evident. In myelinated areas, especially noticeable in the cerebellum, holes were present which seemed to be the result of swollen myelin sheaths (Fig. 1b). The

TABLE I. LIPID CONTENT OF BRAIN TISSUE FROM CRINKLED (*cr/cr*) AND NONCRINKLED (+/?) MICE.

Experimental group	Brain weight[a] (g)	Brain lipids μmole/g wet weight				Grossly malformed brains[b] (%)
		Phospholipids (total)	Cholesterol	Cerebrosides	Sulfatides	
21 days old						
+/?	0.38 ± 0.04(7)[c]	–	33.76 ± 0.90(5)	6.34 ± 0.63(8)	2.09 ± 0.19(7)	0 (0/16)
cr/cr	0.35 ± 0.01(8)	–	35.28 ± 0.82(5)	5.88 ± 0.62(8)	3.42 ± 0.57(6)*	33 (6/18)
Adult (17 months)						
+/?	0.40 ± 0.02(2)	79.49 ± 10.68	54.17 ± 0.14	8.73 ± 0.47	4.25 ± 0.55	
cr/cr	0.35 ± 0.05(4)	76.95 ± 9.80	66.43 ± 7.52	14.73 ± 0.59**	4.18 ± 0.14	

[a] Wet weight ± standard error of the mean.
[b] Twenty-one-day-old litters fed purified control diet.
[c] Number of samples in parentheses.
* Significantly different from noncrinkled, $P < 0.05$ (Student's t test).
** Significantly different from noncrinkled, $P < 0.01$ (Student's t test).

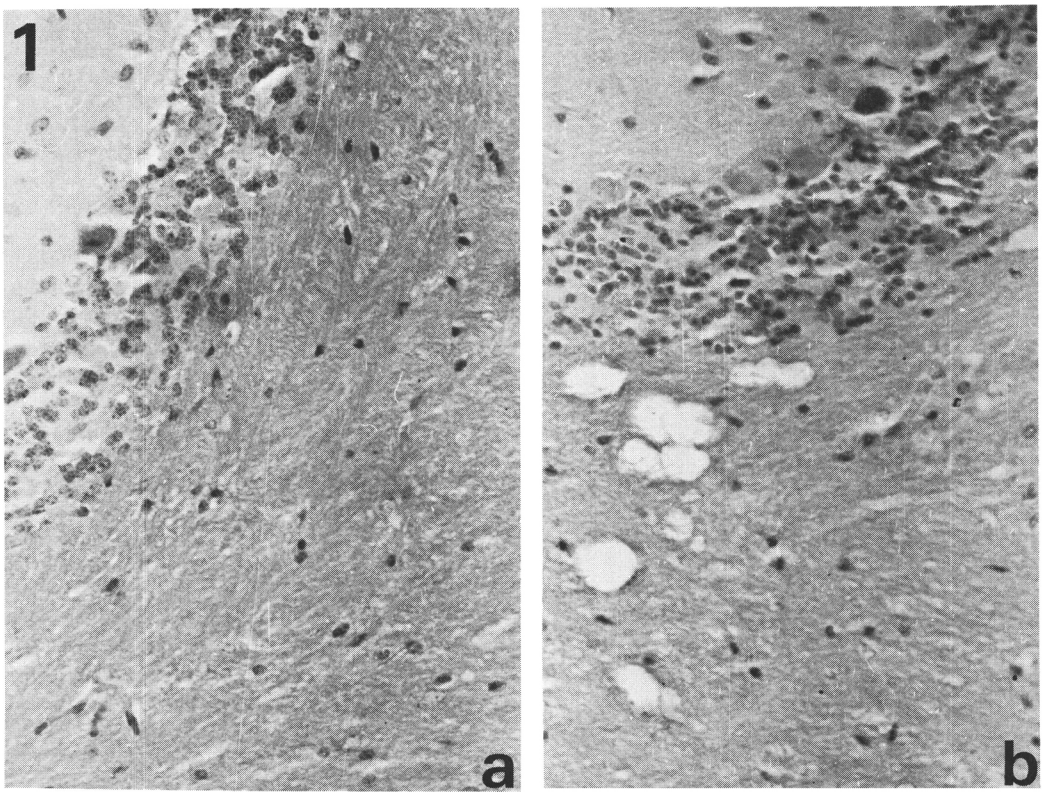

FIG. 1. (a) Photomicrograph at the medial region of a dorsal cerebellar folia from a noncrinkled mouse. × 264. (b) This photomicrograph shows the same area in a crinkled mouse. Note the large spongy areas and evidence of less organization in the mutant. × 264.

disarranged myelin appeared in patches, not diffusely. The rarefaction in myelinated areas was seen in the brains of all crinkled mice, although it was found more extensively in some samples than in others. The cerebellum was the most noticeable area of the brain affected, but, upon closer examination, disorganization was also found in myelin tracts in other parts of the brain.

In addition to these differences, myelin staining was also different in mutant mice and their nonmutant controls. Where spongy areas were found in myelin tracts of the brain in crinkled mice, the myelin stain was much paler than in noncrinkled mice. In the brains of two of the crinkled mice, there appeared to be possible pyknosis and loss of some Purkinje cells. All layers of the cere-

bellar cortex in crinkled mice were disorganized when compared to those of noncrinkled mice.

Discussion. The data presented here are the first to indicate that crinkled mutant mice have an abnormality of brain lipid metabolism, brain structure, and possibly brain function. There is evidence of a demyelinating process in brains of adult crinkled mice. Cholesterol esters, which are found only in trace amounts (13) or are essentially nonexistent in mature brain, were seen in all the brains of adult crinkled mice. They were not found in any control samples by the methods used. When cholesterol esters are present in adults, it is indicative of active demyelination (14). The pathology arises when myelin is broken down and segments of the sheath are engulfed by macrophages. Cholesterol is one myelin constituent that cannot be degraded to smaller units by macrophage lysosomal enzymes. It is esterified and remains in the phagocytes for some time (14).

Gross disturbances in the anatomy of crinkled brains was a clue to possible biochemical lesions and the possible causal factor for the observed "twirling" of the animals. Anomalies in brain cytoarchitecture, especially of the cerebellum, have been found to be the specific disturbance related to the abnormal behavior seen in the mutant mouse reeler (15). However, the "twirling" was also found in pallid mice (16). The presence of a few spongy and reticular areas of myelin in the brain from a noncrinkled animal point to the possibility that aging may have an effect on myelin, as the animals used for histological study were old adults. However, this explanation of degeneration in crinkled mice cannot account for the large differences seen histologically and biochemically between crinkled and noncrinkled brain samples.

In comparing lipid analyses between young and adult mice the situation appeared to reverse itself. Sulfatides, which were increased in 21-day-old crinkled mice, were normal in older mice, but cerebrosides were increased. These findings could be explained by abnormalities in cerebroside metabolism affected by developmental changes. This is the case in the quaking mutant which shows greater changes in lipid content with age (5). One explanation involves sulfotransferase, the enzyme converting cerebrosides to sulfatides, which appears functional in crinkled brains. As myelin is formed in the developing brain, it is paralleled by cerebroside synthesis. An abnormally high level of cerebrosides could be converted by sulfotransferase to sulfatides (17). As the rate of cerebroside biosynthesis declines along with myelination in adult brains (18), sulfatides, found to be increased in young crinkled brain, continue to be synthesized and, if produced in abnormally large amounts during development, can be degraded to cerebroside by cerebroside sulfate sulfatase (18). Subsequently, sulfatides could be expected to return to normal levels while cerebrosides accumulate.

Similar increases in cerebrosides in Gaucher's disease have been attributed to an increase in glucocerebrosides (19). Glucocerebrosides not normally found in the brain accumulate in such lipid storage diseases. Another pathological condition in which cerebrosides are sometimes found to be increased is globoid cell leukodystrophy (Krabbe's disease). Data from these cases suggest that myelin loss is due to primary demyelination (20). However, the observation of cholesterol esters in brain samples of adult crinkled mice is consistent with a condition in which abnormal myelination is secondary to another disturbance, i.e., axonal degeneration (18). The patchy nature of the myelin swelling in crinkled mice also supports the view of secondary effects on myelin in *crinkled* in contrast to the mouse mutant *quaking,* for example, which exhibits extensive and abnormal primary myelination (21).

The mouse mutant *crinkled,* shown previously to have some relationship with copper metabolism (1), has thus also been found to exhibit a disturbance in myelin structure in the adult and altered lipid content of the brain. Studies on myelin formation at earlier periods of development will be valuable in comparing myelin structure in crinkled mice with abnormal myelin produced by alterations in dietary copper. Thus, it may be possible to determine the site where a gene

and a nutrient interact, as well as to provide an additional animal model for the study of brain myelination.

Summary. Lipid composition and histology of brain samples from crinkled and noncrinkled mice fed a normal diet were studied. Sulfatides in young and cerebrosides in adults were found to be increased in brains from crinkled mice when compared to their noncrinkled controls. Cholesterol esters, not present at all in the controls, were found in all crinkled brains analyzed. Histological examination of brain showed abnormalities in myelin structures of varying degrees of severity in all brains from crinkled mice. The observation of cholesterol esters in the brains of crinkled mutants supports the view that the myelin disruption found in these mice is secondary to axonal degeneration.

This work was supported in part by Grant No. HD-02355 from the National Institute of Child Health and Human Development.

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Received July 30, 1976. P.S.E.B.M. 1977, Vol. 155.