Changes in fatty acid composition of myelin cerebrosides after treatment of the developing rat with methylmercury chloride and diethylmercury

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Abstract Suckling rats were exposed to methylmercury chloride or diethylmercury in order to induce chronic sublethal intoxication during the period of active myelination. Doses of 5 mg Hg/kg body weight were injected every second day from 5–25 days of age. The rats were killed at 27–28 days of age, and the brains contained about 1 μg Hg/g wet weight. No changes in brain weight, myelin content of proteins or phospholipids were found, whereas the cholesterol and galactolipid levels were slightly reduced. The most significant change observed was a decrease in the ratio between α-hydroxy fatty acid and the nonsubstituted fatty acid in the myelin cerebrosides. The biochemical changes were less pronounced in the animals given diethylmercury than in animals receiving methylmercury.—**Grundt, I. K., E. Stensland, and T. L. M. Syversen.** Changes in fatty acid composition of myelin cerebrosides after treatment of the developing rat with methylmercury chloride and diethylmercury. *J. Lipid Res.* 1980. **21:** 162–168.

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Mammalian organisms are known to be highly susceptible to the toxic effects of several alkylmetal compounds. Among these methylmercury (MeHg) is well known as an environmental poison. Several reports of MeHg intoxication of epidemic proportions have been connected to industrial waste (1, 2) and use of fungicide-treated grains as food (2). The first report of organic mercury compound toxicity was due to exposure to diethylmercury (Et_2Hg) (3).

Alkylmercurials pose a serious problem because of the ready absorption from the gastrointestinal tract and excretion. Because methylmercury is a small hydrophobic molecule, it is readily absorbed by the brain. Although other organs may accumulate more organic mercury than does the brain, it is in the nervous system that the first and most severe symptoms appear (4).

Chronic maternal exposure to MeHg during gestation is known to introduce irreversible damage to the brain of the fetus (5). Morphological changes in myelin and demyelination have been demonstrated in "Fetal Minamata Disease" (5, 6). It appears that MeHg is readily transferred across the placental barrier, and that the concentration of Hg in the brain of the fetus exceeds that of the pregnant mother (7). This may explain the susceptibility of the fetus to MeHg poisoning.

The mechanism of the toxic effects, particularly on the molecular level, is not well understood. The interference by methylmercury with enzymes containing SH-groups has been proposed (8) and has been discussed by others (9). Which enzyme systems in the brain are first affected by the poison, what biochemical changes are first induced, and how these are related to functional disturbances remain unclear.

We have studied the effect of alkyl-Hg compounds during the time of myelin formation in rats. Chronic sublethal intoxication was induced by giving subtoxic doses of MeHgCl or Et_2Hg to rat pups beginning at 5 days of age. Myelin composition was studied with particular emphasis on the fatty acid composition of both nonsubstituted fatty acids (NFA) and α-hydroxy fatty acids (HFA) of the myelin cerebrosides.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, the chemicals were of the highest obtainable purity from Merck, Darmstadt, West Germany.

Materials

Nine litters of newborn Wistar SPF rats were divided into three groups. One group was treated with methylmercury chloride (K & K Laboratories, Inc., Plainview, NY), the second group was treated with diethyl-

Abbreviations: MeHgCl, methylmercury chloride; Et_2Hg, diethylmercury; NFA, nonsubstituted fatty acids; HFA, α-hydroxy fatty acids, GLC, gas–liquid chromatography.

mercury (Koch-Light, Colnbrook, Bucks, England), and the third group was the reference group. The mercury compounds were dissolved in peanut oil (1 mg Hg/ml oil) and the dosage given was 5 mg Hg/kg body weight. The solutions were injected every second day, beginning at 5 days of age. Eleven injections were given. In order to avoid injury to the animals, the first four injections were given subcutaneously, the remaining injections were given intraperitoneally. The rats in the reference group were injected with peanut oil. The animals were decapitated on the second or third day after the eleventh injection. The brains were immediately removed and weighed. Five brains from each litter were pooled, washed and homogenized in ice-cold 0.32 M sucrose containing 1 mM EDTA (1 g tissue/10 ml sucrose). A Potter-Elvehjem homogenizer (3000 rev/min, 3 up and down movements of the piston) was used. Aliquots of the homogenate were taken for protein and enzyme determinations; myelin was isolated from the remaining homogenate.

Isolation of myelin

The isolation procedure was based on methods described earlier by Cuzner and Davison (10) and Banik and Davison (11). The 10% (w/v) homogenate was spun in a Sorvall Super centrifuge (rotor SS-1) for 10 min at 1000 g_{max} at 4°C. The supernatant was removed, and the pellet (nuclei and debris) was washed once in 0.32 M sucrose. The supernatant from the first step and the washing solution were combined and spun at 13,500 g_{av} for 15 min in a Beckman L 2-50 centrifuge with a type 40 rotor to give a crude mitochondrial fraction containing the bulk of myelin.

Myelin was also isolated from the pellet containing nuclei and cell debris. The pellet was suspended in 0.32 M sucrose and 1.2 M sucrose was added to give a final concentration of 0.8 M sucrose. After centrifugation at 53,500 g_{av} for 60 min in a SW-41 rotor, the myelin was collected by using a slice cutter.

The crude mitochondrial fraction and the myelin isolated from the nuclei fraction were combined and homogenized in 0.32 M sucrose; 1.2 M sucrose was added to give a suspension of 0.8 M sucrose, and the homogenate was spun at 53,500 g_{av} for 60 min in a SW-41 rotor as above. The myelin from this centrifugation was collected in ice-cold water and left for 60 min at 4°C, then spun for 15 min at 60,000 g_{av} in a 40 rotor. The pellet was suspended in 0.32 M sucrose and spun at 53,500 g_{av} for 60 min in a SW-41 rotor. The purified myelin was washed three times by suspension in ice-cold water and centrifugation at 60,000 g_{av} for 15 min in a 40 rotor. The last pellet was finally suspended in 10 ml of distilled water.

Purity of myelin

Contamination by microsomes and nerve-ending particles was estimated by measuring the activity of glucose-6-phosphatase (EC 3.1.3.9) (12) and acetylcholinesterase (EC 3.1.1.7) (13).

Determination of protein

Protein in the whole brain homogenates and in the myelin suspension was dissolved in 0.05% (w/v) sodium deoxycholate and determined according to the method of Lowry *et al.* (14). Bovine serum albumin was used as a standard.

Lipid analyses

Myelin lipids were extracted with 19 vol of chloroform–methanol 2:1 (15). After 1 hr at room temperature, proteins were removed by filtration (Schwartzband, Selecta, West Germany). The lipid extract was partitioned by adding 0.2 vol of water, mixing, and centrifuging at 15,000 rpm for 10 min. The lower phase was mixed with 0.2 vol of tripotassium citrate in theoretical upper phase (chloroform–methanol–0.1 M tripotassium citrate in water 3:48:47) to split proteolipids (16). Finally the lower phase was washed twice with pure theoretical upper phase (chloroform–methanol–water 3:48:47). The washed lipid extract was then dried in a rotary evaporator under reduced pressure at 30°C, after adding 100 ml of benzene (17). To dry the residue completely, 100 ml of benzene was added twice and evaporated. Lipids were dissolved in chloroform–methanol 2:1, the extract was filtered into a graduated test tube, the filter was washed, and the extract was adjusted to 10 ml of chloroform–methanol 2:1. Aliquots were then removed for lipid analyses. Lipid phosphorus was determined by a modified method of Bartlett (18) directly and after separation of individual phospholipids on TLC in a solvent system containing chloroform–methanol–acetic acid–water 25:15:4:2 (19). Cholesterol was determined according to Klungsøyr, Haukenes, and Closs (20) and lipid galactose was determined by gas–liquid chromatography of the trimethylsilyl derivatives of methylgalactosides (21, 22), using mannitol as the internal standard. The content of galactolipids was calculated from three peaks of methyl galactosides and from the mannitol peak. Peak areas were measured by planimetry and corrected for the difference in response for methyl galactosides and mannitol.

Isolation of cerebrosides

Galactolipids were isolated from the lipid extract on a silicic acid column (Unisil 100–200 mesh, Clark-

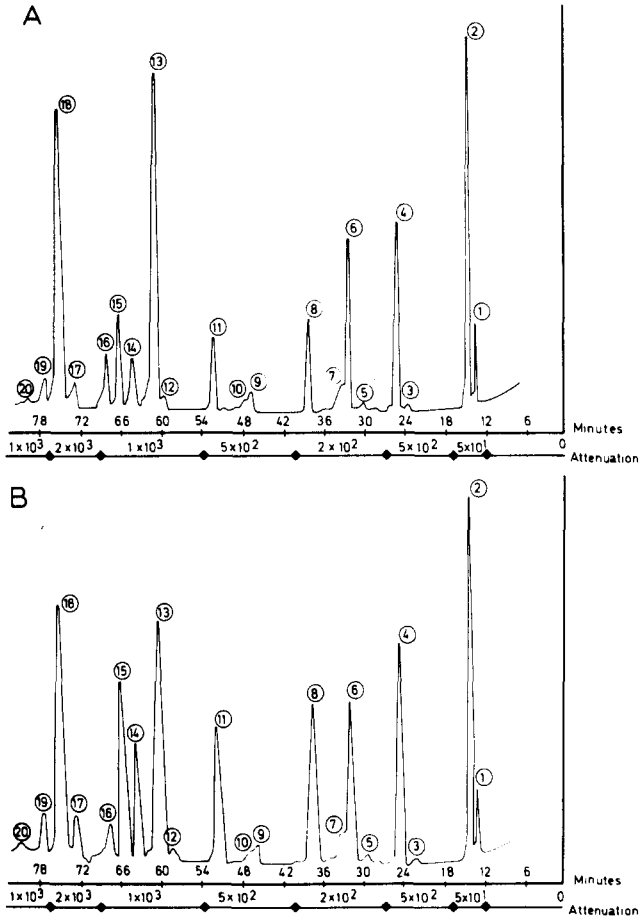

Fig. 1. Chromatograms of myelin cerebroside fatty acids from: A. Reference rats and B. MeHg-treated rats. The peaks in the chromatograms represent the following fatty acids:

1 = C16 :1,	2 = C16 :0,	3 = C18 :1,	4 = C18 :0,
5 = C19 :1,	6 = C18h:1,	7 = C18h:0,	8 = C20 :0,
9 = C20h:1,	10 = C20h:0,	11 = C22 :0,	12 = C23 :1,
13 = C22h:0,	14 = C24 :1,	15 = C24 :0,	16 = C23h:0,
17 = C24h:1,	18 = C24h:0,	19 = C25 :0,	20 = C26 :1.

son Chemical Co., Williamsport, PA) according to a modified method of Rouser et al., (23). A 6 × 600 mm glass column was loaded with 5 g of Unisil in chloroform and washed with 100 ml of chloroform. Eight ml of the lipid extract was dried in a stream of nitrogen, dissolved in 5 ml of chloroform, mixed with about 0.1 g of Unisil, and poured into the column. The neutral lipids were eluted from the column with 200 ml of chloroform and discarded. The galactolipids were then eluted with 700 ml of acetone. The acetone fraction, containing cerebrosides, sulfatides and traces of cholesterol, was dried in an evaporator under reduced pressure. Lipids were dissolved in 20 ml of chloroform–methanol 2:1. The solvent was removed and the lipids were finally dissolved in 2 ml of chloroform–methanol 2:1. The cerebrosides were isolated from sulfatides in the galactolipid extract by preparative thin-layer chromatography on 20 × 20 cm plates covered with a 0.5 mm thick layer of silica gel (Kieselgel G). The plates were developed twice for 15 cm in chloroform–methanol–water 144:25:2.8, and were dried for 15 min at 60°C between the runs (24). The fractions were visualized by brief exposure of the plates to iodine, which was quickly removed in a stream of nitrogen. The silica gel containing the cerebrosides, was scraped into test tubes fitted with Teflon-coated screw-cap stoppers (Sovirel, France).

Gas–liquid chromatographic analysis of fatty acids in the cerebrosides

For transmethylation, 2 ml of sulfuric acid–methanol 6:94 was added to the silica gel containing the cerebrosides (25). The tubes were filled with nitrogen, closed, and placed in a sandbath at 110°C overnight. The next day the tubes were cooled and 1 ml of water was added. The fatty acid methyl esters of the cerebrosides were extracted four times with 2-ml portions of hexane. The combined extracts were evaporated, the esters were redissolved in carbon disulfide, and analyzed in a gas-chromatograph (Model 104, Pye Unicam, Ltd., Cambridge, England). For simultaneous separation of NFA and HFA methyl esters, a column (⅛″ × 250 cm silanized steel) was packed with 3% (w/w) OV-1 on Gas-Chrom Q (100–120 mesh) (Applied Science Laboratories, Inc., State College, PA). Nitrogen (20 ml/min) was used as carrier gas. The column temperature was programmed from 160°C to 250°C at a rate of 1°C/min. Detector temperature was 300°C. Injection was made directly onto the column. Representative chromatograms are shown in **Fig. 1.**

The fatty acid esters were identified by isothermal gas–liquid chromatography at 180, 200, and 220°C, from direct and relative retention times of peaks in the chromatograms from samples and standards, and by logarithmic plotting of retention times versus number of carbon atoms in the molecules. For further identification of NFA and HFA in the chromatograms, NFA and HFA methyl esters in samples and in standard mixtures were separated on a silicic acid (Unisil) column (26), and fractions were analyzed separately by GLC. The standard mixtures GC Mix-H-A and GC Mix-H-C (Applied Science Laboratories, Inc.) used as a reference contained methyl esters of NFA and HFA with 14–18 carbon atoms and with 20–24 carbons, respectively. For identification of unsaturated acids, samples were run independently on a non-polar OV-1 column at 180 and 220°C, and on a polar column of 10% (w/w) EGSS-x (Applied Science Laboratories, Inc.) on Diatomite C 100–120 mesh (Pye Unicam, Ltd.), at 180 and 200°C. The retention times

of peaks in the samples were compared to those obtained with the reference mixtures, GC Mix-H-A, GC Mix-H-C, NHI mix-F (The Hormel Institute, Austin, MN) and with a mixture of C 16:0 and C 16:1. After these preliminary experiments, the temperature-programmed run on the OV-1 column was developed (Fig. 1). The conditions permitted all major fatty acids of myelin cerebrosides to be separated on one column. Fatty acids, which in the samples represented more than 1% of total fatty acids, and with 16–25 carbon atoms, NFA, HFA, saturated and mono-unsaturated, were eluted from the column with retention times which made it possible to calculate peak areas with a standard error <10% (Fig. 1). Accuracy in triangulation was tested with standard mixture NFI mix-F, which contained the acids C 14:0–C 24:0. The results agreed with the stated composition data with a relative error less than 4% for major components (>10% of total mixture) and less than 10% for minor components (<10% of total mixture). Detector response was measured with the aid of mixture GC Mix-H-A and Mix-H-C, and was for HFA only 44% of that for NFA. This measurement of relative response, was performed for each new column, and was constant with a variation of ±3%. It was not possible to find an internal standard that did not overlap with other peaks in the chromatogram. Therefore all results of fatty acid levels in the present study are given as percents of weight of the total cerebroside fatty acids. The ratios HFA/NFA and C24 HFA/C24 NFA were calculated in each chromatogram from the sums of these groups of fatty acids.

Determination of mercury in brain

The mercury content of the brain homogenate and of myelin was measured as previously described (27). The brain Hg content of Et_2Hg-treated animals was measured by electron activation at the Institute for Atomic Energy, Kjeller, Norway. The Hg content in the brains of MeHgCl-treated animals was measured in a γ-counter (scale and timer made by Philips, Holland; crystal and detector made by LDL, England) after giving $Me^{205}HgCl$ to the rats.

RESULTS

The rats were 27–28 days old when they were killed, and weighed 50–60 g. The weights of the brains were 1.3–1.4 g. There were no differences in body or brain weights in the three groups.

Analyses of marker enzymes indicated a high degree of purity of the myelin preparations. The protein content in the myelin, the protein content in the total brain, the total phospholipid content, and the content of individual phospholipids in the myelin, were all the same and unchanged in all groups of animals. The concentrations of cholesterol and galactolipids were lower in myelin from MeHg-treated animals compared to reference animals ($0.02 > P > 0.05$). In the Et_2Hg group the same changes, but less pronounced, were observed. The decrease in cholesterol level was 12% in the MeHg group and 11% in the Et_2Hg group; this represented decreases from 8.3 ± 0.1 to 7.3 ± 0.5 and 7.4 ± 0.5 μmol/g wet weight, respectively. The decrease of galactolipid was 14% in the MeHg group (2.92 ± 0.03 to 2.5 ± 0.2 μmol/g wet weight) and 8% in the Et_2Hg group (2.92 ± 0.03 to 2.7 ± 0.2 μmol/g wet weight).

The mercury content of the brains (μg Hg/g wet weight), when measured in the total homogenate, was 1.62 ± 0.31 for the MeHg group, and 1.57 ± 0.27 for the Et_2Hg group. These values were considerably less than 10 μg Hg/g wet weight, which was found to be the lowest toxic brain concentration or the lowest concentration registered to give clinical manifestation (4). Data on Hg levels in myelin, showed about equal distribution in the two experimental groups: 0.07 ± 0.02 μg/g Hg in the MeHg group and 0.10 ± 0.02 in the Et_2Hg group.

Composition of cerebroside fatty acids

Typical GLC chromatograms of cerebroside fatty acids in myelin from reference and MeHgCl-treated rats are shown in Fig. 1. The composition of acids in the reference group resembles that previously reported by Hoshi, Williams, and Kishimoto (28). The composition of NFA and HFA in the reference and treated groups was almost similar. The only significant difference between reference and treated animals was found in the proportion of HFA in the myelin cerebrosides; in particular, the relatively short peaks of acids C22h:0 and C24h:0 (peaks nr. 13 and 17, Fig. 1) in the chromatograms of the Hg-treated samples emphasize this difference. The ratio HFA/NFA was calculated to be 3.2 in reference animals and 1.4 in MeHg-treated animals. The ratio C24 HFA/NFA was 4.1 in reference animals and 1.7 in MeHg-treated rats, as shown in **Table 1.**

DISCUSSION

Only myelin from MeHg-intoxicated animals showed significant variation from normal myelin composition. Few investigations of the toxicity of dialkylmercury compounds have been published (3, 4, 27, 29, 30). It has been proposed that dialkylmercury

TABLE 1. Composition of cerebroside fatty acids from myelin of rats treated with MeHgCl and Et_2Hg

Normal Fatty Acids	Reference	MeHgCl	Et_2Hg	Hydroxy Fatty Acids	Reference	MeHgCl	Et_2Hg
16:0	0.4 ± 0.1	0.8 ± 0.1					
18:0	3.0 ± 0.2	7.0 ± 0.3	3.6 ± 0.2	18 h:0	0.8 ± 0.1	0.6 ± 0.1	
20:0	1.2 ± 0.1	2.0 ± 0.1	1.3 ± 0.1	18 h:1	0.8 ± 0.1	1.9 ± 0.2	1.2 ± 0.1
22:0	3.1 ± 0.1	5.3 ± 0.1	3.6 ± 0.1	20 h:0	1.1 ± 0.2	0.6 ± 0.1	1.0 ± 0.1
22:1	0.4 ± 0.1	0.5 ± 0.1		22 h:0	19.8 ± 1.5	14.7 ± 1.1	15.6 ± 1.0
23:1	0.8 ± 0.1	1.3 ± 0.2	0.8 ± 0.1	23 h:0	4.6 ± 0.5	2.0 ± 0.2	3.9 ± 0.4
24:0	8.2 ± 0.9	12.5 ± 1.0	12.5 ± 0.8	24 h:0	43.2 ± 3.1	32.9 ± 2.7	41.6 ± 3.0
24:1	3.8 ± 0.3	9.6 ± 0.5	4.2 ± 0.3	24 h:1	5.8 ± 0.6	5.7 ± 0.6	8.2 ± 0.5
25:0	3.0 ± 0.2	2.6 ± 0.2	2.4 ± 0.2				
Total	23.9 ± 4.2	41.6 ± 9.3	28.5 ± 2.8	Total	76.1 ± 4.2	58.4 ± 9.3	71.5 ± 2.8
Ratio: HFA/NFA	3.2 ± 0.4	1.4 ± 0.3	2.5 ± 0.2	Ratio: C24HFA/C24NFA	4.1 ± 0.5	1.7 ± 0.4	3.0 ± 0.3

Cerebrosides were prepared from purified myelin. Galactolipids were first isolated on a Unisil column and sulfatides were removed by TLC. The fatty acids were analyzed by gas–liquid chromatography as described in detail in the text. Results represent the mean of triplicate determinations of each of three preparations, and are expressed as percentage by weight ± standard deviation of the total cerebroside fatty acids in each animal group.

compounds are less toxic than monoalkylmercury compounds because they are not accumulated in the brain to as great an extent as monoalkylmercury compounds (29). The levels of Hg measured in the brains of Et_2Hg-intoxicated animals were, however, about the same as the amount of the Hg in the MeHg rats.

Loss of body weight has been reported to precede neurological disturbances due to exposure to MeHg (31). A normal body weight, a normal behavior, and the low level of Hg in the brains (about 1 μg Hg/brain) of experimental animals, emphasize that we were looking for early indicators of toxicity, introduced by subclinical levels of the poison.

Low levels of cholesterol and cerebrosides in myelin, as we found in exposed animals, have frequently been used as markers for demyelination (32, 33). The only significant change observed in this study, a decreased proportion of HFA in myelin cerebrosides, has been related to myelin deficiency (34). The α-OH groups in HFA of myelin cerebrosides are located in a close-packed structural region of the membrane. This region is characterized by lateral interactions mainly through hydrogen bonding. It is obvious that variations in structure and composition of the lipids will determine the extent of these polar lateral interactions and thus the stability and fluidity of the membrane (35). In myelin of mutant mice with a general retardation of myelin maturation, low levels of myelin cholesterol and cerebrosides, with a decreased elongation of fatty acids (36) and inhibition of fatty acid desaturation (37) have been observed. A decreased hydroxylation of long-chain fatty acids was also found in mutant mice (34). Our material differs from that in the mutant mice because no decreased elongation or inhibition of fatty acid desaturation was observed. Kark, Menon, and Kishikawa (38) reported that no significant reduction in body weight, brain weight, or protein content in the brain was observed in rats exposed to low levels of MeHg during development. However, synthesis of protein was greatly decreased, although lipid synthesis in general was less affected. Such results prompt the suggestion that alterations in fatty acid composition of myelin galactolipids by low levels of MeHg in the brain may be induced through a selective interference with activities of enzymes involved in galactolipid synthesis.

The observed decrease in HFA of cerebrosides accounts for a decrease of approximately 18% galactolipid. The decreased level of total galactolipids (cerebrosides and sulfatides) was 16%. If MeHg specifically inhibits the synthesis of HFA-cerebrosides, the observed decrease in HFA-cerebrosides was apparently partly compensated for by increased synthesis of NFA-cerebrosides. It has been proposed that the synthesis of HFA-cerebrosides in brain is regulated by the hydroxylation of long-chain fatty acids (39). It is also known that the enzyme UDP-galactose: ceramide galactosyltransferase catalyzes the synthesis of HFA-cerebrosides (40). Less is known about the synthesis of NFA-cerebrosides in the brain (41–43), and the mechanism involved in the regulation of the levels of HFA- and NFA-cerebrosides in the brain is not known.

In view of the above, besides the fact that low levels of MeHg-intoxication produce dysmyelination in the developing rat, it is of interest to note that the interference of MeHg with the galactolipid deposition

in myelin may provide a new experimental model in the study of galactolipid synthesis in relation to demyelinating disease.

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