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DISTRIBUTION OF MERCURY IN ENZYMATICALLY CHARACTERIZED SUBCELLULAR FRACTIONS FROM THE DEVELOPING RAT BRAIN AFTER INJECTIONS OF METHYLMERCURIC CHLORIDE AND DIETHYLMERCURY

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Abstract—Wistar rats were given CH_3HgCl (^{203}Hg-labeled) and $(CH_3CH_2)_2Hg$ in peanut oil intraperitoneally (5·0 mg/kg) every second day from 5 until 27 days of age. A reference group was given identical injections of oil only. The rats appeared normal throughout the experimental period. The brains of the three groups were subjected to subcellular fractionation and myelin, nerve-ending particles, mitochondria and microsomes were isolated. Protein, total mercury, inorganic mercury, succinic dehydrogenase and acetylcholinesterase were measured in the fractions. The two groups showed an equal mercury content in the brain and the subcellular distribution of mercury showed a correlation with the protein content. The succinic dehydrogenase activity of the mitochondrial fraction was considerably decreased in both mercury groups compared with the reference group. The myelin fraction contained 4 times as much inorganic mercury relative to total mercury compared with other fractions after CH_3HgCl injections.

AMONG the mercurials the short chain alkyl mercury compounds are outstanding in their ability to accumulate in the central nervous system and to produce neurological symptoms. The typical clinical picture of alkyl mercury poisoning has been described both in man and the rat.[1-3] The pathological changes are fairly well established both in man[1,4,5] and in rats.[6-8] The intracellular distribution of MeHg has been investigated by subcellular fractionation in nervous tissue[9] and in liver.[10] Since Yoshino's paper[9] appeared there have been improvements in the subcellular fractionation techniques of rat brain[11] and these have been used in the present study. Yoshino[9,12] used a very large single dose, 85 mg MeHg/kg, administered to adult rats. In the present study a repeated small dose was used on very young animals to elucidate any effect of MeHg on the development of the rat brain. Norseth and Clarkson[13] have shown that only a small amount of Hg^{2+} is present in brain after MeHg exposure. In the present study the subcellular distribution of this Hg^{2+} was investigated in the animals given MeHgCl.

Although the reaction of mercury compounds with sulfhydryl groups has been well established,[14] nothing is known about the nature of the specific groups involved in the neurotoxic action of MeHg. A reduced protein synthesis has been postulated by

Abbreviations used: MeHg, methylmercury; EtHgEt, diethylmercury; AChE, acetylcholine esterase; SDH, succinic dehydrogenase.

Yoshino *et al.*[12] as the primary cause of neurological symptoms. Based on this postulate and the demyelinating effect of MeHg[15–17] it was assumed that the brain during development would be very sensitive to MeHg. In the rat brain the cell growth is very high from days 0 to 10, while myelination is very active between the days 10 to 20. Thereafter the myelination slowly decreases until the rat is about 100 days old.[18] In the present investigation the rats were injected with MeHg and EtHgEt from the 3rd day until the 27th day after birth.

METHODS

The ^{203}Hg-labeled MeHgCl was prepared from ^{203}Hg-labeled $MeHgNO_3$ obtained from AB Atomenergi, Studsvik, Sweden. The exchange of anion was done by shaking the $MeHgNO_3$ with 5 ml 6N HCl for 5 min and then leaving it for 30 min. This was repeated three times. The MeHgCl formed was then extracted with 2 × 30 ml of benzene and the extracts were combined. The extract was washed with 2 × 10 ml 6N HCl, from which the benzene phase was drawn off and evaporated at 30° in a stream of nitrogen. The residue was dissolved in 50 ml peanut oil. The specific activity of the MeHgCl was determined by gas chromatography and a scintillation counter equipped with a NaI well crystal (scaler and timer made by Philips, Holland; crystal and detector made by IDL, England). The specific activity was 45885 dis/min per Hg. Non-radioactive EtHgEt was obtained from Koch-Light, England.

Nine groups of 5 Wistar rats were used: three groups were given MeHgCl, three were given EtHgEt and three groups were used for estimating the reference or normal values. The rats were given 5·0 mg/kg of the mercury compound dissolved in peanut oil every second day from 5 until 27 days of age (total of 12 injections). The reference rats were given peanut oil only. The first five injections were given subcutaneously, the others intraperitoneally.

The rats were killed by decapitation on the 28th day after birth and subcellular fractions prepared as described by Cuzner and Davison.[11] For this procedure a Beckman Spinco L2-50 ultracentrifuge with Type 40 and SW 41Ti rotors was used. The morphology of the fractions was checked by electron microscopy.[19]

In the rats given ^{203}Hg-labeled MeHgCl the mercury content was measured in 1·0-ml samples in the γ-counter and compared with 1·0-ml standards containing known amounts of radioactive mercury thereby excluding any geometrical errors in the crystal. In two groups of the rats given EtHgEt the mercury was measured by electron activation at Institutt for Atomenergi, Kjeller, Norway. For the analysis of organic and inorganic mercury extraction with dithizon according to Takeda and Ukita was employed.[20] Excess dithizon was removed by chromatography on alumina (Merck, grade II-III). In order to obtain complete separation of organic and inorganic mercury-dithizon complexes the fractions containing radioactivity were pooled and chromatographed on thin-layer silica gel as described by Östlund.[21] The fractions were transferred from the plate into counting vials by means of a sharp razor blade. Recovery in the chromatographic procedure was better than 80 per cent and identical for organic and inorganic mercury. It was impossible to resolve the mercury extract from the nuclear fraction into organic and inorganic mercury.

In the homogenate and subcellular fractions acetylcholinesterase (acetylcholine acetyl-hydrolase, EC.3.1.1.7) was assayed according to Ellman *et al.*[22] using acetyl-

thiocholine as substrate and 5,5′-dithiobis-(2-nitrobenzoic acid) as colour reagent. For the assay of succinic dehydrogenase (succinate: (acceptor) oxidoreductase, EC.1.3.99.1) the method of Laatsch *et al.* was used.[23] In this method the formation of colour is based on the reduction of INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride). All substrates and colour reagents were purchased from Sigma Chem. Co., St. Louis, U.S.A. Protein was measured according to Lowry *et al.*[24]

TABLE 1. BODY AND BRAIN WEIGHTS AFTER METHYLMERCURIC CHLORIDE AND DIETHYLMERCURY ADMINISTRATION

	Reference	MeHgCl	EtHgEt
Body wt (g), 5 days	10·0 ±0·9	11·8 ±0·9	12·0 ±0·5
Body wt (g), 28 days	52 ±4	62 ±5	71 ±6
Braun wt (g), 28 days	1·33 ±0·03	1·38 ±0·03	1·34 ±0·1
Body wt: Brain wt ratio	38·7	44·7	53·0

The values are expressed as mean ± S.D. for 15 animals in each group.

RESULTS

From Table 1 it is evident that there was no retardation of growth during the exposure period. The mercury groups tended to have a higher body weight initially. This difference persisted during the experimental period, and I have no obvious explanation for this. The animals were kept under same litter type and received the same food and treatment. The brain weights were also fairly constant in the different groups while the ratio of body weight to brain weight showed an increase which was particularly marked for the EtHgEt-group.

Each animal received a mean total dose of 1·5 mg Hg, and the mercury content of the homogenates (Table 2) was somewhat smaller than the value reported for adult rats.[25] The total mercury content in the brain was the same for the MeHgCl and EtHgEt groups. According to Östlund's experiments[21] with dimethylmercury in mice 60 per cent of an intravenous dose was exhaled as intact dimethylmercury

TABLE 2. MERCURY, PROTEIN AND ENZYME ACTIVITY IN TOTAL BRAIN HOMOGENATE AFTER METHYLMERCURIC CHLORIDE AND DIETHYLMERCURY ADMINISTRATION

		Reference	MeHgCl	EtHgEt
Total mercury	(μg Hg)		1·6 ± 0·3	1·6 ± 0·3
Protein (mg)		80 ± 8	91 ± 9	90 ± 6
Acetylcholine esterase	(μmole/hr)	721 ± 91	701 ± 85	689 ± 32
Succinic dehydrogenase	(μmole/hr)	53 ± 1	77 ± 12	85 ± 19

The values represent mean ± S.D. measured in brain homogenate in three parallel experiments in each of the three groups. All values are calculated per gram tissue wet weight.

TABLE 3. PROTEIN AND MERCURY DISTRIBUTION IN BRAIN AFTER METHYLMERCURIC CHLORIDE AND DIETHYLMERCURY ADMINISTRATION

	Mercury			Protein	
	MeHgCl	EtHgEt	Reference	MeHgCl	EtHgEt
Nuclei	11·1 ± 1·9	15·3 ± 1·9	10·2 ± 2·1	9·1 ± 1·9	8·4 ± 0·8
Myelin	4·3 ± 1·2	6·4 ± 1·3	6·7 ± 1·1	5·9 ± 0·8	8·0 ± 1·0
Nerve-endings	19·1 ± 3·1	17·2 ± 2·6	19·2 ± 5·8	19·1 ± 4·6	20·9 ± 1·9
Mitochondria	9·3 ± 1·2	10·8 ± 3·2	10·6 ± 2·4	10·2 ± 2·1	9·9 ± 0·8
Microsomes	13·6 ± 0·6	12·7 ± 2·5	15·0 ± 1·0	12·9 ± 2·8	15·5 ± 0·8
Supernatant	25·3 ± 0·6	19·8 ± 3·2	18·8 ± 4·5	16·9 ± 2·3	19·9 ± 2·1
Recovery	83 ± 16	82 ± 14	81 ± 8	74 ± 8	83 ± 6

All values are calculated as percent content in the fraction relative to the homogenate value. Each value represents the mean ± S.D. of three parallel experiments in each group.

within 2 hr after injection. This resulted in a lower mercury content in the brain after dimethylmercury than after MeHgOH injections. Diethyl- and dimethylmercury have a similar volatility, and one would therefore have expected the results in the present report to be comparable with those of Östlund. With an intravenous injection the compound can readily equilibrate with air in the lungs. In the present experiment, however, the dose was given intraperitoneally, and thus a likely explanation would be that the diethylmercury was transformed to ethylmercury before it reached the blood. Although there are differences in the total protein content of the brains in the different groups (Table 2), these differences become insignificant when the standard deviation is considered. The same holds for AChE-activity while the SDH-activity is almost significantly increased ($0{\cdot}01 < P < 0{\cdot}05$ in both groups).

When computing data for the subcellular distribution (Tables 3–5) I have not corrected for the reduced yield in the fractionation procedure. With such corrections, which are frequently seen in the literature, one estimates the loss and correspondingly corrects all values. However, loss of material probably occurs at specific steps in the procedure and these are not known with certainty. Therefore a general correction throughout the data is a mere manipulation of data and not a true correction of inadequate technique.

The subcellular distribution of mercury and protein (Table 3) should be compared with the enzyme distribution. In the separation procedure employed the nuclear

TABLE 4. DISTRIBUTION OF TOTAL AND INORGANIC MERCURY IN SUBCELLULAR FRACTIONS AFTER 5 mg/kg INJECTIONS OF MeHgCl

	Total mercury (μg/g wet wt)		% Inorganic Hg of total Hg in fraction	
	Mean	Range	Mean	Range
Myelin	0·07	0·05–0·09	18·7	16·7–20·2
Nerve-endings	0·31	0·25–0·35	5·7	2·7–7·4
Mitochondria	0·15	0·13–0·17	3·4	2·0–5·2
Microsomes	0·22	0·20–0·23	4·9	4·2–5·9
Supernatant	0·41	0·39–0·45	4·5	1·6–6·5

The results have not been corrected for loss during subcellular fractionation, but loss in the chromatography of mercury compounds has been accounted for. The values represents the mean of three groups.

TABLE 5. EFFECT OF METHYLMERCURIC CHLORIDE AND DIETHYLMERCURY ON ENZYME ACTIVITY DISTRIBUTION IN THE BRAIN

	Acetylcholinesterase		
	Reference	MeHgCl	EtHgEt
Nuclei	4·0 ± 0·4	4·8 ± 0·8	4·4 ± 1·2
Myelin	2·7 ± 0·7	3·4 ± 1·0	3·7 ± 0·4
Nerve-endings	18·9 ± 1·4	27·0 ± 4·4	23·0 ± 0·6
Mitochondria	6·0 ± 2·2	6·1 ± 1·3	4·6 ± 0·5
Micromes	37·1 ± 13·8	28·9 ± 1·6	29·8 ± 1·5
Supernatant	12·8 ± 3·5	14·3 ± 5·9	15·5 ± 1·2
Recovery	81·5 ± 10	84·5 ± 10	81·0 ± 3·8
	Succinic dehydrogenase		
	Reference	MeHgCl	EtHgEt
Nuclei	4·5 ± 0·6	4·0 ± 1·0	3·2 ± 1·1
Myelin	1·0 ± 0·1	1·3 ± 0·7	1·1 ± 0·2
Nerve-endings	29·4 ± 8·2	26·6 ± 2·7	29·8 ± 3·9
Mitochondria	36·8 ± 6·4	24·3 ± 2·7	19·9 ± 1·8
Microsomes	4·5 ± 0·4	4·5 ± 0·6	5·5 ± 0·5
Supernatant	1·3 ± 0·02	0·3 ± 0·03	1·5 ± 0·4
Recovery	77·5 ± 1·5	61·0 ± 9·3	61·0 ± 13·6

All values are calculated as percent content in the fraction relative to the homogenate value. Each value represents the mean ± S.D. of three parallel experiments in each group.

fraction contains, in addition to nuclear material, erythrocytes and some myelin.[9] The relatively high mercury content of this fraction does not represent the mercury content of the morphological cell nucleus. The distribution showed that mercury was predominantly bound to the more highly membraneous fractions and these fractions contain the bulk of brain protein. This confirms the results of Yoshino *et al.*[9] that virtually all mercury is bound to proteins. One should therefore take into account also the protein content of the fractions (Fig. 1). This is well illustrated by the myelin fraction where the absolute value showed that myelin contained only 4–6 per cent of the total brain mercury. The relative specific distribution showed that on a protein basis the mercury content in myelin was comparable with the other fractions. The

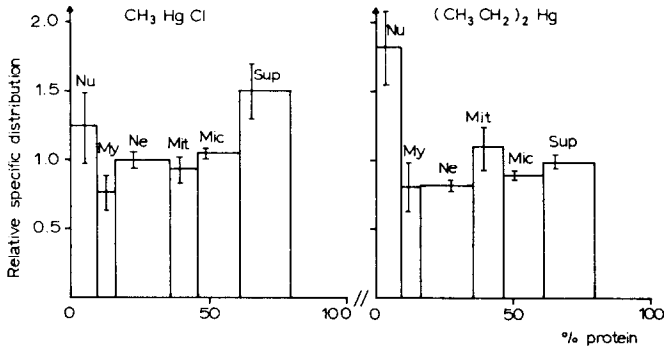

FIG. 1. Relative specific distribution of mercury in subcellular fractions (calculated as % Hg in fraction/% protein in fraction relative to the homogenate). Horizontal bars indicate S.D. in the MeHg-group and the two values obtained in the EtHgEt-group. Nu, nuclear; My, myelin; Ne, nerve-ending particles; Mit, mitochondria; Mic, microsomes; Sup, supernatant.

nerve-ending particle fractions of both mercury groups had a high absolute mercury content (Table 3) while the relative values (Fig. 1) were comparable to those of the other fractions. From Fig. 1 it is further seen that MeHg tended to concentrate in the supernatant while EtHgEt showed the highest value in the mitochondrial fraction. The EtHgEt-treated animals had an exceptionally high value in the nuclear fraction, but this probably represents contamination by erythrocytes and debris. Matched values of mercury and protein are plotted in Fig. 2 and straight lines have been fitted to the data by the method of least mean square. The correlation coefficient

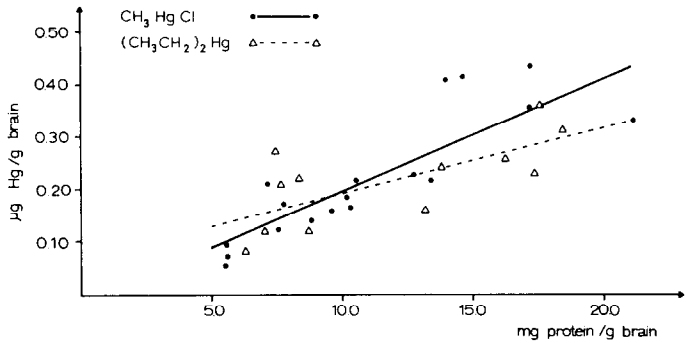

FIG. 2. Correlation between mercury and protein in the subcellular fractions. Each point represents one fraction. The lines were calculated by the method of least mean squares.

is 0·85 for the MeHg-group and 0·88 for the EtHgEt-group. From Table 4 it may be seen that there was a preferential accumulation of inorganic mercury in the myelin fraction. The other fractions contained 2–6 per cent of the inorganic mercury, the content in the synaptosomal fraction being the highest. This may be due to the fractionation procedure as the synaptosomal fraction also is apt to contain some myelin fragments. Even though the present results indicate a content of inorganic mercury in the bulk matter of brain somewhat higher than the 1–4 per cent reported by Norseth and Clarkson[13] we find the agreement with their results satisfactory. The mercury content of myelin was only 5 per cent of the content in total brain, and any elevation of inorganic mercury in such a small fraction would not be detectable by whole-organ measurement.

Two enzymes were assayed both to have a day-to-day check on the fractionation procedure and to detect any effect of the two mercury compounds on these enzymes. AChE-activity was found both in the nerve-ending particle and the microsomal fraction. The activity in the nerve-ending particle fraction is believed to come from the outer part of the synaptosomes[26] while the activity of the microsomal fraction comes from the axolemma[27] and the plasma membrane.[28] There was a slight decrease in the AChE-activity of the homogenate (Table 2) and from Fig. 3 one can clearly see a change in the distribution of AChE-activity. There was an increase in the AChE-activity of the nerve-ending particle fraction and the supernatant while the microsomal fraction showed a decrease (Table 5). The sum of the AChE-activity of these fractions decreased which substantiates the decrease in activity of the homogenate. The other changes in the activity and distribution of AChE was minor (Table 5 and Fig. 3). SDH-activity is found only in mitochondria[29] and is thereby a very good marker for mitochondria. The recovery of this enzyme activity was far

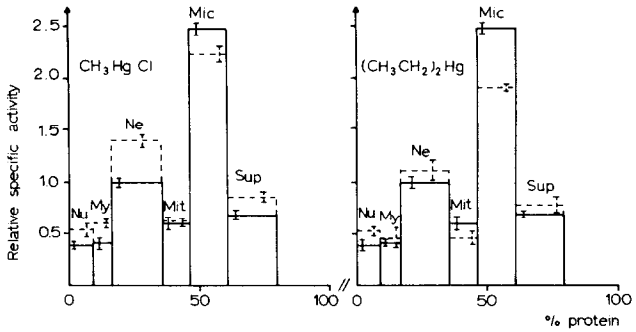

FIG. 3. Relative specific distribution of acetylcholinesterase. Solid lines, reference group; broken lines, mercury treated groups. Each value is the mean of 3 experiments. Horizontal bars give the S.D. Calculation and abbreviations as in Fig. 1.

lower than for any other measured parameter (Tables 3 and 5). This loss of activity during fractionation was probably due to exposing the mitochondria to 1·2 M sucrose.[30] The SDH-activity was, as expected, mainly found in the mitochondrial fraction (Fig. 4). The activity found in the nerve-ending particle fraction was probably due partly to contamination and partly SDH-activity from the nerve-ending particles themselves.[29] The decrease of SDH relative specific activity (Fig. 4) was 31 per cent for the MeHg-group and 42 per cent for the EtHgEt-group.

DISCUSSION

Previous studies have shown a correlation between the regional distribution of mercury and pathological lesions in nervous tissue.[9,31] The same correlation could be true at the intracellular level, and one might expect biochemical effects of mercury in the organelles where it is deposited. On the other hand, mercury might bind to both physiologically "sensitive" and "insensitive" ligands[32] and the toxic effects of mercury could be induced by only a minor amount of the mercury bound to cell components.

The results reported here indicate a fairly uniform distribution of mercury among the fractions. On this basis the various intracellular organelles would be equally

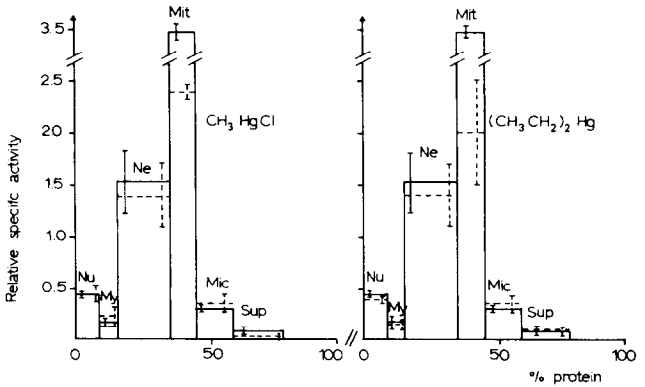

FIG. 4. Relative specific distribution of succinic dehydrogenase. For legend, see Fig. 3.

exposed to the effects of mercury. The question then is whether this distribution represents the actual situation in the intact cell. It is evident that the purity of the fractions is important, and high purity is particulary difficult to achieve with brain tissue. With the method employed here both the nuclear and nerve-ending particle fractions are particularly liable to be contaminated. The nuclear fraction is certainly contaminated by unbroken cells and red blood cells which contribute to the relatively high amounts of mercury in this fraction. Chang and Hartmann[31] with their histological approach to intracellular distribution of mercury were unable to find any mercury in the nucleus. The nerve-ending particle fraction is isolated at the very place in the gradient where the crude mitochondrial fraction is applied before centrifugation. Any material which does not move during centrifugation is thereby isolated in this fraction.

The other factor which can change the "real" distribution of mercury is redistribution of mercury during the subcellular fractionation. Norseth[33] concluded that such redistribution of mercury is very unlikely to take place when preparing subcellular fractions from liver. In principle there is no difference between liver and brain subcellular fractionation, and it seems justifiable to assume that there is no or insignificant redistribution of mercury during subcellular fractionation of brain tissue. Of special interest is the finding that myelin contains relatively more Hg^{2+} than the other fractions. MeHg poisoning is accompanied by vacuolization and fragmentation of myelin both *in vitro*[17] and *in vivo*.[15] These morphological changes in myelin of adult rats appear much faster[15] than what is believed to be the turn-over time for myelin proteins.[34] Thus, these changes cannot be explained as a result of MeHg-induced inhibition of protein synthesis. Both Yoshino *et al.*[12] and Cavanagh and Chen[35] have reported a reduced incorporation of radioactive amino acids into protein in MeHg treated animals both with and without symptoms. Other biochemical changes associated with MeHg intoxication are reduced RNA synthesis[36,37] and reduced SDH-activity.[12] Myelin consists of tightly packed membranes and it is important to distinguish between MeHg and Hg^{2+} when considering molecular interactions. The divalent Hg^{2+} has the possibility to cross-link two protein molecules and to establish intramolecular cross-links with a concomitant change in protein configuration. It might well be that there are different mechanisms of action for MeHg in myelin and the nerve cell body. The interaction of MeHg with both protein synthesis[12,35] and RNA synthesis[36,37] points to protein synthesis as a target for MeHg toxicity in the neuronal cell body. In the present experiment the period of exposure was deliberately chosen to be the time when the brain undergoes maximal development. From the results in Table 1 there seems to be no serious retardation of brain growth which one would expect if protein synthesis was the key target of MeHg. There was, however, a change in the body : brain weight ratio which could indicate that brain development is affected to a limited extent only. On the other hand, one might expect that the brain has its maximum ability of protein synthesis in this period, and that impairment of some of this ability does not affect over-all brain growth. The pathological effects of MeHg are restricted to very limited areas, and one would not expect therefore to pick up changes confined only to these areas when using the whole brain for measurement. It would seem profitable to concentrate future efforts on the isolation of material from areas which show pathological changes after MeHg-treatment in order to learn more about the primary effects of MeHg neurotoxicity.

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