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Toxicity of Organic Mercury Compounds. III.[1] Uptake and Retention of Mercury in Several Organs of Mice by Long Term Exposure of Alkoxyethylmercury Compounds

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Uptake and accumulation of alkoxyethylmercury compounds in several organs of mice, in comparison with alkylmercury compounds, were studied. Diets containing various organic mercury compounds were continuously fed to mice, and organic mercury in the organs was extracted with dithizone-carbon tetrachloride and determined by gas chromatography.

Histological studies in mice poisoned by methoxyethylmercury chloride showed a prominent damage of the kidney, and proliferation of glial cells and atrophy of nervous cells in the cerebrum.

In all of the compounds administered, the organic mercury was found in the liver and kidney. The ratio of the mercury contents in blood to plasma was higher in ethylmercury chloride than in alkoxyethylmercury compounds.

Ethylmercury chloride was highly incorpolated into the brain, while alkoxyethylmercury compounds were at much slower rates. The mercury contents in the brain at onset of the neural symptoms were much lower in methoxyethylmercury than in ethylmercury. In the brain after administration of alkoxyethylmercury compounds, it appeared to be present as an inorganic mercury for the most part, in contrast with significant amounts of the organic mercury in the case of the alkylmercury. It may be presumed that manifestation of the symptoms after exposure of organic mercury compounds is not merely related to mercury levels and not always in need of organic forms in the brain.

Introduction

The clinical signs and pathological findings caused by methylmercury compounds in animal experiments were known to be similar to Minamata disease manifested in human.[3–7] At the same time, the symptoms in cats, calves, and mice poisoned by ethylmercury compounds were similar to those in methylmercury compounds.[8–10] Further, as reported by Sebe, *et al.*,[11] alkylmercury compounds having short carbon chains (C_1—C_3) bring about the specific neurotoxicity and the signs of poisoning in rats, which are consisted of weight loss, ataxia, and clossing of the hindlegs. Saito, *et al.*[10] reported the dolphin kick convulsion as a criterion for experimental Minamata disease in mice.

In the preceding paper authors reported the manifestation of the dolphin kick convulsion occurred in mice which were fed continuously with diets containing methoxyethylmercury

1) Part II: M. Yonaha, T. Nakamura, and S. Ishikura, *Eisei Kagaku*, **18**, 248 (1972).
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3) T. Takeuchi and K. Morikawa, *Psychiat. Neurol. Jap.*, **62**, 1850 (1960).
4) T. Takeuchi and H. Matsumoto, *Acta Neuropathol.*, **2**, 40 (1962).
5) T. Takeuchi, H. Deguchi, M. Sonoda, and F. Kai, *Kumamoto Igk. Z.*, **40**, 1003 (1966).
6) T. Takeuchi, G. Koya, H. Deguchi, M. Sonoda, and F. Kai, *Kumamoto Igk. Z.*, **40**, 1016 (1966).
7) T. Miyakawa and M. Deshimaru, *Acta Neuropathol.*, **14**, 126 (1969).
8) N. Morikawa, *Kumamoto Med. J.*, **14**, 71 (1961).
9) J. Oliver and N. Platonow, *Am. J. Vet. Res.*, **21**, 906 (1960).
10) M. Saito, T. Osono, J. Watanabe, T. Yamamoto, M. Takeuchi, Y. Ohyagi, and H. Katsunuma, *Jap. J. Exp. Med.*, **31**, 277 (1961).
11) E. Sebe, Y. Ituno, S. Matumoto, T. Matuoka, and M. Akahoshi, *Kumamoto Igk. Z.*, **35**, 1219 (1961).

chloride[12] and ethoxyethylmercury chloride.[1] Lehotzky, *et al.*[13] described that rats intraperitoneally exposed to methoxyethylmercury chloride had shown evidence of impaired weight gain, renal damage, and nervous symptoms (ataxia, tremor, and palsy). It is recognized, however, that the longer administration period and the higher dose of alkoxyethylmercury compounds were required to produce the symptoms compared with ethylmercury compound.

The toxicity and the behavior of alkylmercury compounds in animal body has been studied in connection with the chemical structure by many investigators,[14–16] but there is little information on those of alkoxyethylmercury compounds. It appeared of interest to investigate the behavior of alkoxyethylmercury compounds in the body. The purpose of the present investigation was to collect more data concerning the uptake and retention of mercury in organs of mice after continuous administration of alkoxyethylmercury compounds, and to know a relationship between the toxicity and structure of alkoxyethylmercury compounds in comparison with alkylmercury compounds.

Materials and Method

The following mercury compounds were employed; ethylmercury chloride (EMC), butylmercury chloride (BMC), methoxyethylmercury chloride (MEMC), ethoxyethylmercury chloride (EEMC), and *n*-propoxyethylmercury chloride (*n*-PEMC). The mercury compounds were obtained as described previously.[1] Pluverulant diets (Oriental yeast Co.) mixed with organic mercury compounds were fed continuously to male mice of dd strain. The animals had free access to water. The following doses were used in the experiments.

EMC	60,	100	
BMC	100		
MEMC	250,	500	(ppm, as mercury in diets)
EEMC	250,	500	
n-PEMC	250		

At the time indicated were killed the animals and the following organs were taken for determination of total or organic mercury content; blood, liver, kidney, and brain. The brain, liver, and kidney were fixed in formalin, and prepared and stained by hematoxilin-eosin at the Biotest Kenkyusho for histopathologic examination.

Determination of Mercury in the Organs——Total mercury was determined as described in the preceding experiment.[1] The extraction of organic mercury from the organs were made by a modification of the previous method.[17] Namely, 6 ml of 0.05% dithizone in carbon tetrachloride was added to an aliquot of the blood or the 10% homogenate of the organs, shaken for 15 min, and centrifuged. The organic layer was subjected to alumina column chromatography (column, i.d. 5 mm). The organic mercury dithizonates obtained after removal of the solvent were dissolved in benzene and determined by gas chromatography. The calibration curves were made up using the dithizonate of each authentic organomercury compound. Shimazu GC-1B equipped with an electron capture detector was operated under conditions as follows; column 160 cm or 90 cm × 4 mm (glass column), column packing 4% DEGS-Cerite 545 (AW, HMDS) 60—80 mesh, column temperature 155°, detector oven temperature 180°, injection temperature 185°, carrier gas N_2 (40—50 ml/min). The relative retention times of dithizonates of ethylmercury, butylmercury, methoxyethylmercury, ethoxyethylmercury, *n*-propoxyethylmercury were 1.74, 2.34, 4.46, 3.97, and 4.73, respectively. The absolute retention time of methyl mercury dithizonate was 0.92 min by use of 160 cm column. The unknown peak from the dithizone reagent appeared 10.15, but it did not interfere the determination of organic mercury dithizonates. A suitable sample size for injection was in the range of 2×10^{-10}—10^{-9} g. Injection of large amounts caused the decomposition of mercury dithizonates or the disturbance of operation.

12) S. Ishikura, N. Inoue, and M. Yonaha, *Eisei Kagaku*, **17**, 33 (1971).
13) K. Lehotzky and S. Bordas, *Med. Lavoro*, **59**, 241 (1968).
14) K. Nose, *Jap. J. Hyg.*, **24**, 359 (1969).
15) T. Suzuki, T. Miyama, and H. Katsunuma, *Jap. J. Exp. Med.*, **33**, 277 (1963).
16) Y. Takeda, T. Kunugi, O. Hoshino, and T. Ukita, *Toxicol. Appl. Pharmacol.*, **13**, 156 (1968).
17) S. Ishikura, M. Yonaha, I. Sunaga, Y. Watanabe, N. Kikuchi, and R. Takeshita, *Eisei Kagaku*, **18**, 281 (1972).

Results and Discussion

Gas chromatographic determination of organic mercury compounds which were transferred from specimens into an organic solvent in acidic conditions was described by Westöö,[18] Kitamura, *et al.*,[19] and Sumino.[20] But these method are not suitable for the determination of an alkoxyethylmercury, since it is labile in such an acidic condition. Therefore, the extraction as dithizonates under neutral condition was applied to the determination of alkoxyethylmercury in organs. Mercury dithizonates thus obtained were isolated by means of alumina column chromatography and determined by gas chromatography.[21] Recovery of MEMC as organic mercury from tissues and excreta is shown in Table I.

After a long term administration of various mercury compounds to mice, distribution and chemical form of mercury in the organs were investigated.

TABLE I. Recovery of Organic Mercury from Biological Samples

Organ and Excreta	Amount of sample (g)	Addition[a] (μg Hg)	Recovery (%)
Brain	0.15	10	84.3
	0.15	10	87.9
Liver	0.50	10	98.2
	0.50	10	69.7
Kidney	0.15	10	81.9
	0.15	10	87.5
Urine	1.5 (ml)	10	99.8
Feces	1.00	10	82.2
	1.50	10	106.6

a) Methoxyethylmercury chloride was added to each sample.

Histological Studies

The organs of mice which showed the neural symptoms after the administration of MEMC for 14—16 days (500 ppm) were subjected to histological examination.

Brain (10 Mice)——In the cerebrum, proliferation of glial cells was present in 8 cases, and in two of the eight, atrophy of nerve cells was observed. Noted changes were not found in the cerebellum.

Liver (3 Mice)——In 2 cases, slight opaqueness of hepatic cells was observed and in one of the two, slight activation of Kupffer's cell was observed.

Kidney (3 Mice)——In 2 cases, slight opaqueness of tubular epithelial cells and cellular infiltration in the perivascular spaces were observed. In one of the two, protein-like substance was deposited in Bowmann's capusule and in the tubuli, and in the remaining one in the medulla as well. In the former, swelling of small artery accompanied with vacuolisation and exfoliation of renal tubular epithelium were further observed.

Total and Organic Mercury Contents in Liver and Kidney

The results of the determination of total and organic mercury contents in liver and kidney were given in Table II, where manifestation of symptoms were observed in mice which were administered with EMC (60, 100 ppm), MEMC (500 ppm). The total mercury contents in the liver were remarkably high in groups of EMC (60, 100 ppm) and MEMC (500 ppm). The

18) G. Westöö, *Acta Chem. Scand.*, **22**, 2276 (1968).
19) S. Kitamura, T. Tsukamoto, K. Hayakawa, and T. Shibata, *Med. Bio.*, **72**, 274 (1966).
20) K. Sumino, *Kobe J. Med. Sci.*, **14**, 115, 131 (1968).
21) G. O'G Tatton and P.J. Wagstaffe, *J. Chromatog.*, **51**, 283 (1970).

high accumulation of EMC and the increase of accumulation by an increasing dose of MEMC were consistent with an earlier study reported already.[12]

The organic mercury contents in the kidney were higher than those in the liver in all the cases except MEMC (500 ppm). The difference between total and organic mercury contents was greater in the liver than in the kidney.

Table II. Total and Organic Mercury Contents in the Liver and Kidney of Mice Administered with Alkylmercury or Alkoxyethylmercury Compounds

Mercury compound dose (ppm)	Period of administration (day)	μg/g tissue			
		Liver		Kidney	
		Total	Organic	Total	Organic
Control (0)	—	ND	ND	0.2	ND
EMC (100)	8	135.5	43.0	85.5	80.9
EMC (60)	13	134.4	38.2	54.9	43.2
BMC (100)	18	46.0	12.0	42.1	35.2
MEMC (500)	10	158.8	13.3	75.0	31.6
MEMC (250)	25	48.7	17.6	59.5	8.6
EEMC (500)	21	48.1	18.9	88.9	60.0
n-PEMC (250)	21	11.8	ND	40.5	11.4

Mercury contents are expressed in amounts of total mercury, and respective unchanged organic mercury chloride. Each value represents the mean of samples from two mice. ND: not detected

Uptake and Distribution of Mercury in Blood

The total mercury contents in the blood is shown in Table III. A constant increase of the mercury content in the blood as well as in the plasma after the administration of EMC was observed throughout the experimental period. On the contrary, in cases of alkoxyethylmercury compounds, the mercury contents in the blood and plasma were kept in relatively low level. The contents of mercury observed in the blood after the administration were varied among alkoxyethylmercury compounds; MEMC, EEMC, and *n*-PEMC in the decreasing order and also in the plasma likewise. The rapid elimination of mercury from the blood and plasma was observed after the removal of MEMC from diet. The ratio of mercury contents in blood to plasma became approximately 3.5 by EMC treatment, whereas about 2 or less by alkoxyethylmercury compounds.

The blood isolated from mice continuously administered with MEMC and EEMC were further fractionated into stroma-free hemolyzate, stroma, and plasma to investigate the distribution of mercury in these fractions. Almost similar patterns were shown in all fractions for both compounds. About 50% or more of mercury were found in stroma-free hemolyzate, around 30—40% in plasma, and the rest in stroma. These results indicate that alkoxyethylmercury compounds did not show so selective affinity towards the stroma-free hemolyzate as recognized on ethylmercury chloride.[16]

Uptake and Distribution of Mercury in the Brain

Generally, alkylmercury compounds are retained in higher concentrations in the brain than phenylmercury and inorganic mercury compounds.[16,22] Uptake of mercury into the brain of mice administered continuously with the various mercury compounds is depicted in Fig. 1. EMC was rapidly taken up into the brain, while alkoxyethylmercury compounds were at much slower rates. Manifestation of the specific neural symptoms was first observed in mice of MEMC-group at 11th—14th day (500 ppm) and 18th—24th day (250 ppm), and 9th—11th day (60 ppm) in EMC-group. Therefore, from Fig. 1, the total mercury

22) U. Ulfvarson, *Int. Arch. Gewerbepath.*, **19**, 412 (1962).

Table III. Concentration of Mercury in the Blood and Plasma of Mice Administered with Alkylmercury or Alkoxyethylmercury Compounds

Mercury compound dose (ppm)	Period of administration (day)	Blood (μg/g tissue)	Plasma (μg/g tissue)	Blood / Plasma
EMC (60)	4	19.97±2.16	5.95±0.91	3.36
	8	30.52±0.99	8.56±0.87	3.56
	12	41.15±5.90	11.26±0.78	3.66
MEMC (500)	4	9.13±1.61	5.18±1.20	1.77
	8	9.95±1.37	5.27±0.20	1.87
	12	12.34±2.05	6.33±0.82	1.95
	16	11.17±4.49	8.35±1.79	1.34
	a) 16	3.78±0.45	1.84±0.33	1.70
EEMC (500)	10	7.72±1.27	4.39±0.24	1.76
	15	6.42±1.40	2.91±0.98	2.21
	20	7.90±2.71	3.34±1.25	2.37
	25	7.16±0.66	3.14±2.28	2.28
n-PEMC (250)	10	5.06±0.48	2.49±0.22	2.03
	15	5.89±0.52	2.19±0.14	2.69
	20	5.18±0.88	2.52±0.55	2.06
	25	5.49±0.70	2.74±0.30	2.00

Each value represents the mean±SE of samples from four mice.
a) Determined after 4 days since the administration of the compound had been discontinued.

Table IV. Distribution of Mercury in the Blood Components of Mice Administered with MEMC and EEMC (500 ppm)

Experimental No.	Period of administration (day)	Distribution of mercury (%)		
		Plasma	Stroma free hemolyzate	Stroma
MEMC[a] M4	12	36.6	59.2	4.1
MEMC[a] M5		37.2	55.7	7.1
MEMC[a] M6		28.2	67.3	4.5
MEMC[a] M7		33.7	61.9	4.4
EEMC E21	25	40.6	41.4	18.0
EEMC E22		39.8	50.4	9.8
EEMC E23		34.5	54.9	10.6
EEMC[b] E24		45.4	45.4	9.2

a) specific neural symptoms including dophin kick convulsion.
b) slightly neural symptoms.
Distribution of mercury in each fraction is expressed in percent of the total mercury.

contents in the brain at onset of symptoms after administration of MEMC and EMC were about 6—7 μgHg/g brain and 21—24 μgHg/g brain, respectively.

The results show that the mercury contents in the brain of methoxyethylmercury poisoned mice were much lower than those seen in ethylmercury poisoned mice, although the higher dose of MEMC is required than EMC to produce the specific neural symptoms. On the other hand, after exposure of alkoxyethylmercury compounds, the accumulation of mercury in the brain was dose-dependent, but there were no prominent differences in the rate of uptake of mercury and mercury levels among alkoxyethylmercury compounds. This may be resulted from the fact that alkoxyethylmercury compounds are difficult to pass through the blood-brain barrier than alkylmercury compounds.

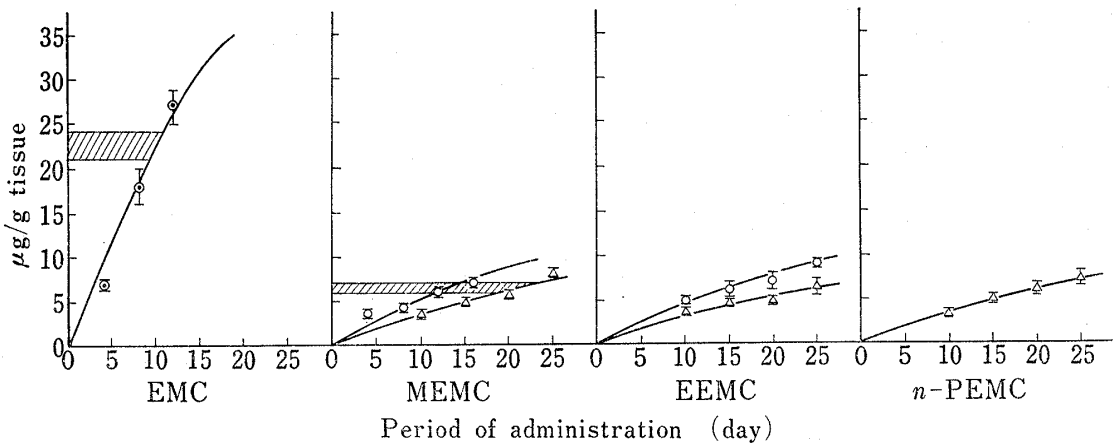

Fig. 1. Accumulation of Mercury in the Brain of Mice Administered with Alkylmercury and Alkoxyethyl Mercury Compounds

Data for each point represent the mean of samples from four mice, and bars represent standard errors. —⊙— :60 ppm, —△— :250 ppm, —○— :500 ppm
▨ :mercury levels at onset of symptoms

Mercury in the brain after administration of various mercury compounds was analyzed to know the proximate chemical form in tissue, and the results are presented in Table V.

In the brain of mice which were administered with alkylmercury compounds, a significant part of mercury was accumulated in an organic form, being in agreement with the results in rats reported by Nose.[14] Whereas, in the case of alkoxyethylmercury compounds, organic mercury was the minor of the total mercury recovered from brain. It reveals that the most part of alkoxyethylmercury compounds administered seemed to be present in brain as an inorganic mercury. It remained unidentified whether alkoxyethylmercury compounds, which were slowly incorporated into the brain, transformed there to an inorganic mercury, or the inorganic mercury, which were once produced from alkoxyethylmercury compounds in other organs, was slowly accumulated in the brain.

Table V. Total and Organic Mercury Contents in the Brain of Mice Administered with Alkylmercury of Alkoxyethylmercury Compounds

Mercury compound dose (ppm)	Period of administration (day)	Total (μg/g tissue)	Organic (μg/g tissue)
EMC (60)	13	16.93	10.07
BMC (100)	18	13.96	11.00
MEMC (250)	25	9.27±2.22	0.27
EEMC (500)	25	11.44±1.05	0.34
n-PEMC (250)	25	8.19±1.14	0.53

Mercury contents are expressed in amounts of total mercury, and unchanged organic mercury chloride. Each value represents the mean of samples from two mice in alkylmercury, and the mean ± SE of samples from three mice in alkoxyethylmercury.

Mercury in the brain was also analyzed by the method of Westöö,[18] since it would be expected that alkoxyethylmercury compounds were degraded in the body to an inorganic mercury, and subsequently methylated. However, no significant amount of methylmercury was detected.

The brain of mice fed MEMC or EEMC was fractionated into acid-soluble, lipid, nucleic acid and protein fractions according to the method of Schneider, *et al.*,[23] and the distribution

23) W.C. Schneider, *J. Biol. Chem.*, **161**, 293 (1945).

of mercury in these fractions was determined (Table VI). Similar results were obtained for the two compounds employed. About 60% of mercury accumulated in the brain was found in the protein fraction, about 30% in the acid-soluble fraction, only a few in the lipid and nucleic acid fractions. It is possible that the mercury in the brain of mice exposed against alkoxyethylmercury for a long term was almost present as a protein-bound inorganic mercury.

Table VI. Distribution of Mercury in the Brain of Mice Administered with MEMC and EEMC (500 ppm)

Experimental No.	Period of administration (day)	Distribution of mercury (%)			
		Acid soluble fraction	Lipid fraction	Nucleic acid fraction	Protein fraction
MEMC[a] M4	12	32.5	4.6	4.6	58.4
MEMC[a] M5		27.5	3.9	2.8	65.9
MEMC[a] M6		27.4	5.6	3.0	64.1
MEMC[a] M7		33.9	5.5	3.0	57.6
EEMC E2	25	32.1	3.1	2.2	62.5
EEMC[a] E3		35.8	2.6	1.9	59.7
EEMC E4		34.0	3.6	2.1	60.3
EEMC[b] E9		32.5	2.2	2.8	62.5

a) specific neural symptoms including dolphin kick convulsion.
b) slightly neural symptoms.
Distribution of mercury in each fraction is expressed in percent of the total mercury.

The brain mercury levels found after administration of methylmercury compounds are higher than those seen after administration of inorganic mercury and phenylmercury compounds.[16,22] Pathologically, noticeable changes are also found in the brain of methylmercury poisoned animals.[3-7] Saito, *et al.*[10] speculated that dolphin kick convulsion, the clinical signs of organic mercury poisoning in mice, was brought about from the loss of the inhibitory function of the higher centers. In the present experiments, the differences of residual levels and chemical forms of mercury incorporated into the brain of mice after the exposure of EMC and MEMC were prominent. But there were not obvious differences among every alkoxyethylmercury compound on concentration of mercury and chemical forms found in the brain, although manifestation of the neural symptoms was dependent upon the carbon-chain length of alkoxy group.

It is presumed that manifestation of the symptoms by organic mercury compounds is not merely related to mercury levels and also not in need of organic mercury as chemical form in the brain. Therefore, these observations may reveal that some of the clinical signs in animals caused by organic mercury compounds are not solely resulted from their effect on the brain.

On the other hand, the pathological evidence in methylmercury poisoned rats provided that peripheral nerves were primarily affected either the primary damage of the peripheral sensory fibres[24,25] or the primary cell bodies in dorsal root ganglia, with secondary deterioration of their fibres,[26-28] and central nervous system changes followed those in the peripheral nervous system. In addition to the noticeable changes in the posterior nerve root fibres, Miyakawa, *et al.*[29] assumed that some of the anterior nerve root fibres may be involved, since pathological

24) T. Miyakawa, M. Deshimaru, S. Mumiyoshi, A. Teraoka, N. Udo, E. Hattori, and S. Tatetsu, *Acta Neuropathol.*, **15**, 45 (1970).
25) L.W. Chang and H.A. Hartmann, *Acta Neuropathol.*, **20**, 316 (1972).
26) J.B. Cavanagh and F.C.K. Chen, *Acta Neuropathol.*, **19**, 208 (1971).
27) S.P. Herman, R. Klein, F.A. Talleyand, and M.R. Krigman, *Lab. Invest.*, **28**, 104 (1973).
28) G.G. Somjen, S.P. Herman, R. Klein, and M.R. Krigman, *J. Pharmacol. Exp. Ther.*, **186**, 579 (1973).
29) T. Miyakawa, M. Deshimaru, S. Sumiyoshi, A. Teraoka, and S. Tatetsu, *Acta Neuropathol.*, **17**, 80 (1971).

changes in methylmercury poisoned rats were present in part of the muscle. Herman, *et al.*[27] suggested that the clinical signs such as ataxic gate and the clossing phenomenon in methylmercury poisoned rats are manifestations of peripheral sensory neuropathy, not central nervous system lesions. From this point of view, contribution of organic mercury compounds administered to the peripheral nerves of mice may be involved.

Kasuya[30] reported the relationship between the structures of mercurial compounds and their toxicity on nervous tissue, dorsal root ganglia in culture. It can be considered that the different susceptibility of such nervous tissues to the structures of organic mercury compounds may play a role in poisoning by organic mercury compounds.

30) M. Kasuya, *Toxicol. Appl. Pharmacol.*, **23**, 136 (1972).