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BIOTRANSFORMATION OF METHYLMERCURY SALTS IN THE RAT STUDIED BY SPECIFIC DETERMINATION OF INORGANIC MERCURY*

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Abstract—Methylmercury chloride undergoes biotransformation in the rat resulting in the release of inorganic mercury. The reaction was followed by specific determination of inorganic mercury using an isotope exchange method. Small but significant amounts of mercury were detected in brain tissue during the first 10 days after a single injection of methylmercury chloride. Inorganic mercury accounted for the highest proportion of total mercury in the excretory organs and in feces. By comparing the present results with previously published reports using other analytical methods, the release of inorganic mercury was found to be the major biotransformation pathway available for methylmercury salts in rats.

The role of this biotransformation pathway is discussed with reference to the biochemical mode of action and the pharmacokinetics of these very hazardous compounds.

THE MAJORITY of organo-mercurial fungicides contain mercury linked covalently to a carbon atom.[1] Cleavage of this bond leading to the release of inorganic mercury is a possible biotransformation pathway. In order to follow this reaction in tissues, Miller *et al.*,[2–4] Gage,[5] and Platonow[6] measured total mercury and, by selective extraction in benzene, or chloroform, the intact organo-mercurial (i.e. organic mercury). It was assumed that the fraction of the total mercury not extractable was inorganic mercury. Takeda *et al.*[7] extracted all mercury in erythrocytes as dithizone complexes and determined the different forms after chromatographic separation. Recently, the cleavage of the carbon–mercury bond has been studied using ^{14}C- and ^{203}Hg-labeled methylmercury compounds,[8] and Daniel and Gage[9] used ^{14}C labeling combined with chemical determination of total mercury.

These methods have led to controversial results about the release of inorganic methylmercury salts *in vivo*. Further studies on the biotransformation of methylmercury chloride by a new independent method might resolve this difficulty, and at the same time supply information on the biochemical mode of action and the pharmacokinetics of these very hazardous compounds.

The method described in this report specifically measures inorganic mercury in the presence of organo-mercurial compounds. It is thus complementary to that of Miller *et al.*,[2–4] and of Gage,[5] and it was of interest to see if this method gave the same results.

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METHODS

Theoretical principles. This method utilizes three properties of mercury and its compounds: (1) Aqueous solutions of mercuric salts are capable of undergoing isotopic exchange with mercury vapor in contact with the solution; (2) After the isotope has exchanged into the vapor state, it is now volatile and can diffuse from one liquid phase into another; (3) Mercury bound covalently to a carbon atom in organic mercurial compounds does not undergo isotopic exchange or does so much more slowly than inorganic mercury.[10]

The isotope exchange reaction was first used to follow the biotransformation of an organo-mercurial chlormerodrin.[11] Cysteine added to alkaline tissue homogenate accelerated the rate of exchange between inorganic mercury in tissue and the vapor phase. However, cysteine caused some decomposition of chlormerodrin during the exchange period and a correlation had to be made for this. However, a correlation should not be necessary in the case of methylmercury compounds since Weiner *et al.*[12] have shown that the alkylmercurials are stable in the presence of cysteine. Consequently, when mixtures of methylmercury compounds and inorganic mercury salts, each labeled with the ^{203}Hg isotope, are exposed to mercury vapor, the exchangeable fraction should be equal to the fraction of inorganic mercury in the sample.

Procedures. Conway microdiffusion units were used because they were designed to give rapid diffusion from one liquid phase to another.[13] The inner well contained 0·1 ml of metallic mercury in a small dish making it easily removable for counting. The outer well contained the tissue homogenate or biological fluid to be analyzed in 0·05 M cysteine hydrochloride and 0·5 N sodium hydroxide. To get complete exchange of inorganic mercury present, a final volume of about 1·0 ml was found suitable for a standard exchange time of 4 hr for biological samples, and 2 hr for buffer samples at 40°. The composition and volume of various biological preparations added to the outer well of the Conway unit are given in Table 1. After the exchange was completed the

TABLE 1. CONTENTS IN DIFFUSION UNITS FOR DIFFERENT TISSUES*

Tissue	Contents
Brain, liver, kidney, spleen	0·5 ml of 40% homogenate
Red blood cells, whole blood	0·5 ml of 40% dilution in water
Plasma	0·2 ml directly
Urine	1·0 ml directly
Bile	0·5 to 1·0 ml directly
Intestinal cells, pancreas	1·0 ml of 10% suspension in buffer
Intestinal content, feces	0·5 ml of 20% suspension in buffer

*All volumes were corrected to 1·0 or 1·1 ml. All chambers contained cysteine hydrochloride, 0·05 M, and sodium hydroxide, 0·5 N, final concentrations.

pellet was removed and counted directly. A correction factor was determined for the self-absorption in the pellet in order to compare the pellet count with the activity originally added in the chamber. A standard 0·1-ml pellet of metallic mercury was placed in a solution of radioactive mercuric chloride of known activity for about 6 hr. During this time, isotopic exchange took place between the pellet and the mercuric ion. The pellet was then washed and counted. The correction factor was calculated from the relationship

$$p\lambda + S_t + W + g = S_0$$

where p, S_t, W, g and S_0 are respectively the observed counts in the mercury pellets the solution at the end of the exchange, the washings from the pellets, the glassware and the solution before the exchange was started. The average value of λ was 2·46 (S. D. = 0·04).

Methylmercury chloride labeled with the ^{203}Hg isotope was obtained using the exchange method described by Cross and Pinean.[14] Friberg applied this method to obtain labeled methylmercury hydroxide using labeled mercuric oxide and methylmercury hydroxide.[15] Methylmercury hydroxide was not commercially available, and, as earlier described modifications of the method seemed to give the labeled mercurial with an unknown amount of inorganic contamination, a new modification was worked out. Radioactive mercury was supplied as mercuric nitrate, specific activity approximately 1 mc/mg Hg, from Tracerlab, Waltham, Mass. Mercuric chloride was added as carrier and the exchange between the inorganic isotope and methylmercury chloride was run in 6 N hydrochloric acid. The strong acid was used because of the limited solubility of methylmercury chloride in water. Usually about 2 mc was added to a total volume of 20 ml containing 5 mM mercury as each mercury compound. The exchange was run at room temperature for 4 to 7 days. Methylmercury chloride was then extracted in 40 ml of benzene, and the benzene extract was washed twice with 40 ml of 6 N hydrochloric acid. The washed extract was evaporated to dryness under reduced pressure at 40°, and the methylmercury chloride finally dissolved in 5 mM sodium carbonate for injections or storage. The labeled compound was stable for weeks in this solution at room temperature. About 0·25 mc and 2·5 m-moles of mercury as methylmercury chloride were recovered in the final isotope. The specific activity and chemical purity of the methylmercury chloride was determined by polarography in a Britten Robinson buffer, pH 8·0, with mercury as a reference electrode. The tests were run in a 10-ml solution containing 0·1 M potassium chloride using 0·1 ml of 2% gelatin as suppressor. The half-wave potential of the first reduction step was found to be 0·47 V, identical to a standard solution.[16] Single samples of the washed benzene extract were analyzed by gas chromatography by the method of Westöö.[17] The retention time was found to be identical to a standard sample, and all radioactivity captured by the fraction collector corresponded to the ion capture detector peak. The isotope was also tested by thin-layer chromatography on Eastman chromagram sheet 6062 alumina developed in 1-butanol–95% ethyl alcohol–58% ammonium hydroxide, 8:1:3, for 4 to 6 hr.[18] On spraying with 4-4′-bis(dimethylamino)-thiobenzophenone, methylmercury chloride is seen as a violet spot.[17] The methylmercury chloride and a standard sample showed identical R_f values (0·24), and all radioactivity recovered was found corresponding to the methylmercury chloride spot.

Female rats weighing 150–200 g (Sprague–Dawley) were used. Methylmercury chloride was injected intravenously in the exposed great saphenous vein under a slight ether narcosis. Bile was collected by cannulating the common bile duct under barbiturate narcosis. Organs were homogenized in cold phosphate buffer, pH 7·4, using a teflon-glass homogenizer.

RESULTS

The isotopic exchange from liver homogenates containing labeled mercuric chloride added *in vitro* takes place at a rate determined by the concentration of mercury (Fig. 1). Concentrations below 10^{-5}M allow complete exchange in 4 hr. Higher con-

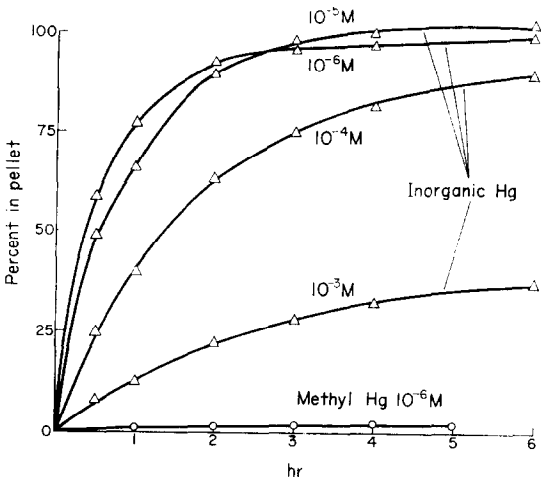

FIG. 1. Relative rates of mercury exchange of different concentrations of mercuric chloride and of methylmercury chloride. The mercury salts were added *in vitro* to the standard liver homogenate diffusion system.

TABLE 2. EXCHANGE OF MIXTURES OF METHYLMERCURY CHLORIDE AND MERCURIC CHLORIDE*

Inorganic added (%)	No. of samples	Inorganic found (%) Mean	Range
100	10	100	85–108
54	4	55	51– 56
1	6	3	2– 4
0	5	2	1– 3

*Methylmercury chloride and mercuric chloride were added *in vitro* to liver homogenate in the standard exchange system.

centrations require longer periods. When radioactive methylmercury chloride was added to the homogenate, a small exchange took place, at a rate of approximately 0·5 per cent/hr. As a result of these observations, homogenates were always sufficiently diluted to give concentrations of inorganic mercury less than 10^{-5}M and the exchange run stopped after 4 hr. Under these circumstances, the radioactivity found in the mercury pellet at 4 hr should be equal to the inorganic mercury plus a small number of counts equal to approximately 2 per cent of the radioactivity in the organo-mercurial. This conclusion is confirmed by the results shown in Table 2. The recovery of exchangeable counts averaged 100 per cent from labeled $HgCl_2$ alone, 55 per cent from a mixture containing 54% $HgCl_2$ and 46% methylmercury chloride, 3 per cent when 1% $HgCl_2$ was present and 2 per cent from methylmercury chloride alone.

The small fraction of exchangeable radioactivity from methylmercury chloride may be because of inorganic mercury as an impurity, or to the slow release of radioactivity from the organo-mercurial itself. The data in Fig. 2 indicate that one batch of freshly prepared methylmercury chloride released mercury at a steady rate. The rate of exchange from the other batch exhibited a rapid component complete within 2 hr, followed

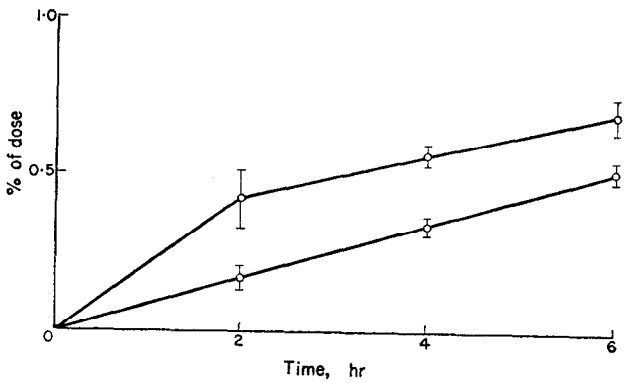

FIG. 2. Relative rates of mercury exchange from two different preparations of labelled methylmercury chloride from buffer without homogenate.

TABLE 3. EXCHANGE OF MERCURY FROM BUFFER WITH NO HOMOGENATE

No. of samples	No. of tests per sample	Compound added	Exchange ± S.D.* (%)
14	1	MeHgCl	0·39 ± 0·02
1*	4	MeHgCl	0·25 ± 0·05
9	1	$HgCl_2$	99·2 ± 2·8

*Exchange time 2 hr.
†Tested over a period of 1 week.

by a steady release. This rapid component could well be inorganic mercury, since in buffer systems as distinct from homogenates, exchange with inorganic mercury is complete within 2 hr (Table 3). Thus, the quantity of radioactivity recovered from labeled methylmercury chloride dissolved in buffer in 2 hr represents the maximum possible inorganic impurity. Measured in this way, the inorganic impurity in some 14 batches of freshly prepared methylmercury chloride averaged 0·4 per cent (Table 3). A batch allowed to stand 1 week after preparation had an average impurity of 0·3 per cent. As indicated by the curves of Fig. 2, these are maximum figures and the actual inorganic impurity is probably much smaller.

Further experiments were carried out to see if results similar to those obtained with liver homogenates could also be obtained with other tissues and biological fluids. The recovery of exchangeable isotope from 14 different liver homogenates containing labeled $HgCl_2$ averaged 100 per cent (S. E. = 2). The biological fluids and homogenates of the tissues listed in Table 1 gave recoveries not significantly different than the 100 per cent value obtained for liver.

Some differences, however, were seen when exchangeable mercury was measured after the addition *in vitro* of labeled methylmercury chloride (Table 4). Values for liver and kidney homogenates were close to 2 per cent. However, the exchangeable radioactivity recovered from suspensions of red cells was significantly higher (4 per cent) than the value obtained for liver and kidney homogenates. Plasma, urine and bile samples gave values significantly lower, approximately 1 per cent. Since the

labeled methylmercury chloride contains less than 0·4 per cent inorganic impurity, it is clear that most of the counts recovered from the homogenates must have come from the methylmercury chloride itself. All the data *in vivo* quoted in this report were corrected for this small exchange *in vitro*.

Some inorganic mercury is present in all animal tissues.[19] If isotope exchange could take place *in vivo* between this "endogenous" mercury and the injected methylmercury chloride, the results would give a misleadingly high figure for the rate of biotransformation. To check this possibility, the rate of biotransformation of methylmercury chloride in untreated animals was compared with the rate in animals injected with unlabeled $HgCl_2$. The results are shown in Table 5. Methylmercury chloride alone

TABLE 4. EXCHANGE OF MERCURY AFTER ADDING METHYLMERCURY CHLORIDE *IN VITRO* TO STANDARD EXCHANGE SYSTEMS

Organ	No. of tests	Exchange (%)	Range
Liver	5	2·3	1·2 – 3·0
Kidney	5	2·3	2·0 – 2·6
Red blood cells	5	4·1	4·0 – 4·2
Brain	5	1·6	1·5 – 1·7
Intestinal cells	5	1·8	1·2 – 2·1
Plasma	5	0·9	0·6 – 1·0
Feces	5	1·7	1·4 – 2·1
Urine	5	0·9	0·7 – 1·4
Bile	6	1·2	1·1 – 1·7

TABLE 5. KIDNEY CONTENT OF MERCURY AFTER INJECTION OF MERCURIC CHLORIDE AND METHYLMERCURY CHLORIDE COMPARED TO METHYLMERCURY CHLORIDE ALONE*

Injections	No. of rats	Total Hg (μg/g)	Inorganic Hg (μg/g)
$^{203}HgCl_2$	5		1·30†
$Me^{203}HgCl$	5	4·61 ± 0·10	1·30 ± 0·04
$Me^{203}HgCl$† $HgCl_2$	5	4·65 ± 0·10	1·21 ± 0·03

*Values given are mean ± S.E.M.
†Average of three rats killed day 5 and two rats day 11.

gave a level of inorganic mercury of 1·3 μg Hg/g wet wt. of kidney tissue. Ideally, a dose giving approximately the "endogenous" levels of inorganic mercury in kidney should be used. A higher dose would give more obvious results if exchange takes place, and a dose giving approximately the same tissue levels of inorganic mercury as in the methylmercury chloride animals was chosen. This was found using test doses of labeled mercuric chloride. Finally, animals were pretreated with the same dose of unlabeled mercuric chloride followed 5 days later by labeled methylmercury chloride. Theoretically, if complete isotope exchange *in vivo* had taken place at the time the animals were killed, the tissue level of inorganic mercury would appear to be from 2·0 to 2·6 μg/g dependent on the exchange taking place completely outside the kidney, or exclusively in the kidney respectively. In fact, only 1·21 μg Hg/g was found, a result not significantly different than obtained in untreated animals.

A study was made of the biotransformation of methylmercury chloride in rats (Table 6). Approximately 1 week after a single injection of 0·5 mg Hg/g as methylmercury chloride, 39 per cent of the radioactivity in the kidneys was present as inorganic mercury. The proportion of inorganic mercury in liver, plasma and feces averaged 11, 22 and 49 per cent respectively. The brain, however, contained only about 3% inorganic mercury. Data reported by Gage[5] for rats given methylmercury chloride are quoted in Table 6 for comparison. The agreement between 49·4 ± 5·2 per cent inorganic in feces found by Gage, and 48·8 ± 4 per cent determined by the isotope exchange method, is excellent. Both Gage's measurements and ours were made on rats following a single injection, Gage using the subcutaneous route of injection as

TABLE 6. INORGANIC MERCURY IN ORGANS AND IN FECES AFTER INJECTION OF METHYLMERCURY SALT

Organ	Isotope exchange			Gage[6]		
	Dose* (mg Hg/kg)	Time (days)	Inorganic ± S.E.M. (%)	Dose† (mg Hg/kg)	Time (days)	Inorganic ± S.E.M. (%)
Kidney	0·5	4–6	39·3 ± 2·1	1·2	7	30·7
Liver	1·0	3	11·4 ± 1·6	1·2	7	18·5
Plasma	1·0	1–10	21·5 ± 2·1	1·2	42	27·3 ±
Feces	1·0	1–10	48·8 ± 4·0	1·0	1–14	49·4 ± 5·2
Brain	1·0	1–10	2·8 ± 0·8			

*Single doses.
†Doses given three times per week throughout the experiment. The data on feces are after a single dose.

contrasted to intravenous doses in our experiment. Values for liver, 11 and 19 per cent; for kidney, 39 and 31 per cent; and for plasma 21 and 27 per cent, show reasonable agreement, considering that Gage gave multiple doses of methylmercury chloride as compared to our single dose. Only organs containing more than about 10% inorganic mercury can be compared as the extraction method gives too variable results for smaller amounts.[5]

DISCUSSION

Our results agree with Gage's findings that methylmercury chloride, after injection into rats, is slowly converted to inorganic mercury. This slow rate of metabolism creates stringent demands on the analytical method. Depending upon the time after injection, at the organ or tissue selected for study, inorganic mercury is deposited in the presence of a large excess of the organo-mercurial. The selectivity of the analytical method is thus a crucial factor. Furthermore, the presence of a small inorganic impurity in the injected compound may seriously compromise the experiment. Consequently, we have devoted much of this paper to a full description and validation of the technical procedures.

Reports have appeared in the recent literature[8,20] referring to studies on the biotransformation of methylmercury salts using the "double label" technique. The organo-mercurial is labeled with both ^{14}C and ^{203}Hg isotope. The ratio of ^{14}C to ^{203}Hg activity is measured in the animal tissue. Any departure of the ratio from unity (i.e. the ratio in the injected solution) indicates that some cleavage of the carbon–Hg bond

must have taken place. However, the converse is not necessarily true. For example, the ratio would remain unchanged despite biotransformation if the ^{14}C and ^{203}Hg metabolites remained in the tissues. This may account for the conclusion based on "double label" techniques that all the mercury in brain, following injection of methylmercury salts, is present as the unchanged mercurial and for the more general conclusion that methylmercury compounds are stable in the body.[8] Such conclusions are at variance with our finding (Table 6) that a small proportion of mercury in brain is in the inorganic form, and the findings of Gage,[5] Platanow[6] and of this report that substantial proportions of mercury in liver, kidney, and feces have undergone biotransformation.

The benzene extraction method and the present isotope-exchange technique possess the advantage of isolating at least 1 component of the biotransformation reaction. The extraction method is suitable for studying the overall breakdown of the organomercurial since it measures the rate of disappearance of the parent molecule. The isotope exchange technique is selective for 1 biotransformation pathway; viz., the release of inorganic mercury from the parent molecule. Accepting Gage's assumption that his extraction method specifically extracts the unchanged organo-mercurial,[5] the fact that these two methods give similar results indicates that the cleavage of the carbon mercury bond is the major biotransformation pathway available to methylmercury compounds.

The term "biotransformation pathway," as used above, refers only to those reactions leading to a chemical modification of the methylmercury radical, like for example, the cleavage of the carbon mercury bond or to a substitution of the hydrogens of the methyl group by other radicals or substituents. Biotransformation does not include the variety of complex salts that form between the methylmercury radical and various tissue ligands such as sulfhydryl and imidazole groups.[21] For example, organomercurials have been shown to be excreted in urine of dogs as the cysteine complex.[12]

Small but significant amounts of inorganic mercury were detected in brain tissue in the first 10 days after injection of methylmercury chloride (Table 6). Since the brain is the target organ for the alkyl-mercurials,[1] these results indicate that we cannot neglect the possibility that inorganic mercury may be the toxic agent. Studies on the biotransformation of mercurial diuretics in kidney tissue indicate that the release of inorganic mercury may be responsible for the renal effects.[11] However, in the case of methylmercury salts, more detailed studies will be necessary to establish a causal relationship between inorganic mercury and brain damage.

The high proportion of inorganic mercury observed in excretory organs and feces suggests that biotransformation may play an important role in the excretion of mercury after exposure to methylmercury compounds. The extreme hazard arising from exposure to the short-chain alkyl mercurials is related to the slow excretion rate and the accumulation of mercury in the brain.[8] Inorganic mercury may be the molecular form of mercury most readily excreted. A further understanding of the biotransformation of these organo–mercurials becomes important in understanding the toxicity of these compounds.

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