STUDIES ON THE BINDING OF METHYLMERCURY BY THIONEIN

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SUMMARY

The 113,000 x g supernatant fraction from liver and kidney of rats and Japanese quail fed toxic levels of methylmercury for prolonged periods was fractionated on Sephadex G-75. Only a few percent of the total Hg was accounted for in the fractions corresponding to metallothionein. Methylmercury, in contrast to Hg^{++} and Cd^{++}, was found to have a low affinity for the metal-free form of metallothionein isolated from horse kidney. Metallothionein appears to have no significant role in the detoxification or body distribution of methylmercury except to scavenge Hg^{++} formed by the slow biotransformation of methylmercury in the body.

Since the mysterious Minamata disease was identified as methylmercury intoxification from contaminated fish, mercury compounds, especially methylmercury, have drawn much scientific attention. Jakubowski et al. found that injected $^{203}Hg^{++}$ was bound to a low molecular weight protein (MW = 11,000), which represented the chief fraction of mercury in the liver and kidney of the rat (1). Later, these investigators reported that this low molecular weight protein was identical to metallothionein (2, 3). Ellis and Fang also found that a substantial fraction of mercury in rat kidney was bound to protein fractions corresponding to metallothionein after exposure of animals to mercuric acetate and phenylmercuric acetate (4), and apparently ethylmercury salts as well (5). Since metallothionein, a sulfhydryl-rich protein, was first isolated from horse kidney by Margoshes and Vallee (6) and characterized by Kagi and Vallee (7), a role for this protein in detoxification of heavy metals such as cadmium and mercury has been frequently implied (1, 8, 9, 10). Methylmercury has a high affinity for sulfhydryl groups, although not as high as that of Hg^{++} (11, 12). It is therefore possible that thionein, the metal-free form of metallothionein, may be also involved in binding methylmercury in the body. To investigate this, we studied the binding of methylmercury to

thionein in vivo, after long-term feeding of methylmercury, and in vitro with purified thionein.

MATERIALS AND METHODS

Methylmercury binding in vivo. Japanese quail were fed a practical diet containing 17% of tuna (13) plus supplemental Hg added as CH_3HgOH for 18-47 weeks. The tuna supplied about 0.5 ppm of Hg and the CH_3HgOH supplement brought the total Hg to 2.5, 7.5, or 10 ppm. Rats were fed a semipurified diet (13) containing 2 or 10 ppm of Hg as CH_3HgOH for 29 weeks, with or without 0.5 ppm of Se as sodium selenite. Immediately after sacrificing the animals, livers and kidneys were removed, pooled and stored at -20°C. To measure mercury content in protein fractions corresponding to metallothionein, pooled livers or kidneys were homogenized in a Potter-Elvehjem homogenizer with 4 volumes of buffer (0.001M Tris-0.05M KCl, pH 8.6). Homogenates were then centrifuged at 113,000 x g for 60 minutes and a 10-ml portion of the supernatants so obtained was chromatographed on a Sephadex G-75 column (2.5 x 95 cm) which was preequilibrated and eluted with buffer. After chromatography, mercury was assayed in single fractions or pooled peaks according to Munns and Holland (14). The Sephadex column was calibrated with bovine serum albumin, ovalbumin and cytochrome c.

Methylmercury binding in vitro. Metallothionein was isolated from the soluble fraction of horse kidney cortex and purified by Sephadex G-75 chromatography, followed by DEAE-cellulose chromatography. This partially purified protein was then treated with 0.2M 2-mercaptoethanol plus 8M urea at pH 8.5. After 4 hours at room temperature, the mixture was adjusted to pH 2 and the metal-free thionein obtained by chromatography on a Sephadex G-75 column (2.5 x 43 cm) which was preequilibrated and eluted with 0.01N HCl, pH 2. Protein was measured by Lowry's method (15) and thiol content with Ellman's reagent (16). The thionein contained 6.8 μmole -SH/10,000 μg of protein. Although this value is lower than the value of 26 μmole -SH/10,000 μg protein (dry weight) reported by Kagi and Vallee (7), the purity was judged adequate for the purpose of comparing relative binding by different mercurials.

RESULTS AND DISCUSSION

The total mercury content and the percentage of mercury present in the soluble fraction of liver and kidney are shown in Table I. Approximately 50-70% of the total mercury was present in the soluble fraction. Dietary mercury level did not appreciably alter the percentage of mercury in the soluble fraction. Selenium, which decreases methylmercury toxicity (13), consistently decreased the uptake of mercury in the soluble fraction. The results of further separation of the soluble fraction of quail liver on Sephadex G-75 are shown in Figure 1. The mercury content of individual fractions closely paralleled the A_{280}, with most of the mercury eluting at the void volume (peak I), and at the position of hemoglobin (peak II), plus an additional peak in the low molecular weight area (peak IV). Surprisingly, no mercury peak was observed in the region (peak III) corresponding to metallothionein, i.e., ^{109}Cd peak with $V_e/V_o = 2.04$. Similar results were obtained in a fraction-by-fraction analysis of kidney soluble fraction from the same birds. Additional studies were made of the mercury content in pooled peaks (pooled as indicated in Figure 1), for tissues from rats or quail fed various levels of methylmercury (Table I). In all cases, when toxic or near toxic levels of methylmercury were fed, only a few percent of the mercury in the soluble fraction was associated with the 9,600 molecular weight fraction. Only when lower levels of mercury were fed, and only in rat kidney, was a significant part (17%) of the mercury found in this peak.

It is known that Hg^{++} binds strongly to thionein, and is slowly formed from CH_3HgOH in the body. Mercuric ion is liberated rapidly from certain organomercurials such as phenylmercury. Since it is not known if CH_3HgOH itself can bind to thionein, in vitro studies with thionein purified from horse kidney were carried out. Titration of thionein with Cd^{++} produced a well defined spectral change in the 250 nm region (Figure 2) similar to that reported by others (7). A spectral change was also observed with CH_3HgOH, although of lesser magnitude and at longer wavelengths (Figure 2). More

Table I. Mercury in tissues and soluble fraction and its distribution within soluble fraction after feeding methylmercury.

Tissue	Dietary Hg		Hg in fresh tissue	% Hg in soluble fraction	Hg distribution within soluble fraction[1]				
	dietary level	feeding period			peak I	peak II (hemoglobin)	peak III (thionein)	peak IV (low MW)	total recovery
	ppm	weeks	µg/g	%	%	%	%	%	%
Quail liver	2.5	18	8.9	71.2	25.7	20.0	3.6	15.0	64.3
Quail kidney	2.5	18	16.5	44.5	25.1	16.0	4.0	12.0	57.8
Quail liver	7.5	18	36.6	62.4	19.5	30.2	2.5	13.6	65.8
Quail kidney	7.5	18	40.4	50.8	15.2	18.8	2.1	14.6	50.7
Rat liver	2	29	8.2	67.7	18.1	17.5	1.2	24.0	60.8
Rat liver	2(+Se)	29	5.4	44.4	21.9	24.7	0.0	21.9	68.5
Rat kidney	2	29	42.0	61.7	15.2	17.3	14.3	11.2	58.0
Rat kidney	2(+Se)	29	25.5	56.3	20.7	17.1	17.4	10.8	66.0
Rat liver	10	29	24.4	62.5	14.3	17.9	1.5	24.6	58.3
Rat liver	10(+Se)	29	44.4	52.0	15.8	15.5	1.0	27.4	59.7
Rat kidney	10	29	38.3	73.5	14.3	17.3	7.7	13.5	52.8
Rat kidney	10(+Se)	29	66.1	52.1	18.6	12.2	3.0	14.2	48.0

[1] see Figure 1 for position of peaks.

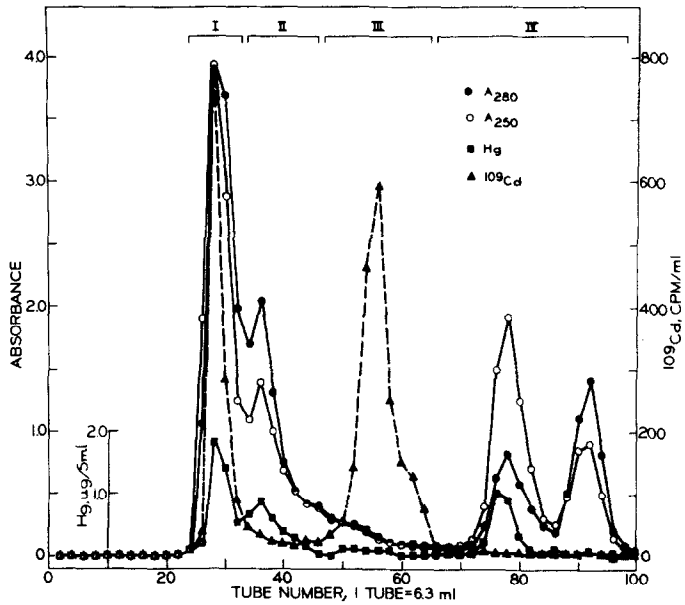

Figure 1. The liver soluble fraction (10 ml) from quail fed 10 ppm Hg as CH_3HgOH for 47 weeks was chromatographed on a Sephadex G-75 column (2.5 x 95 cm) at 4°. In this experiment, a trace amount of $^{109}Cd^{++}$ (0.06 µgm) was also added to the soluble fraction prior to chromatography to help locate metallothionein, presumably the Cd-binding protein at V_e/V_o = 2.04 corresponding to a molecular weight of 9620.

importantly, the concentration of metal required to give half the maximum spectral change was 1.8×10^{-5} M for Cd^{++} and 5.5×10^{-5} M for CH_3HgOH. These results indicate that CH_3HgOH has a lower affinity than Cd^{++} for thionein, whereas Hg^{++} has a higher affinity (7). This conclusion is further supported by studies in which pure thionein treated with these metals was subjected to Sephadex G-25 chromatography (Table II). Thionein (228 µgm) passed through the column after treatment with excess Cd^{++} retained 37 nmoles of Cd^{++}. The same amount of thionein bound 22 nmoles of Hg^{++}, despite the fact that Sephadex G-25 has a high affinity for Hg^{++} (recovery off the column = 3.4%). In contrast to Cd^{++} and Hg^{++}, only 0.2 nmole of CH_3HgOH was eluted with the thionein.

From these studies *in vivo* and *in vitro*, it is clear that thionein has a low affinity for methylmercury in comparison to Cd^{++} and Hg^{++}. The small amounts of mercury found in the protein fraction corresponding to metallo-

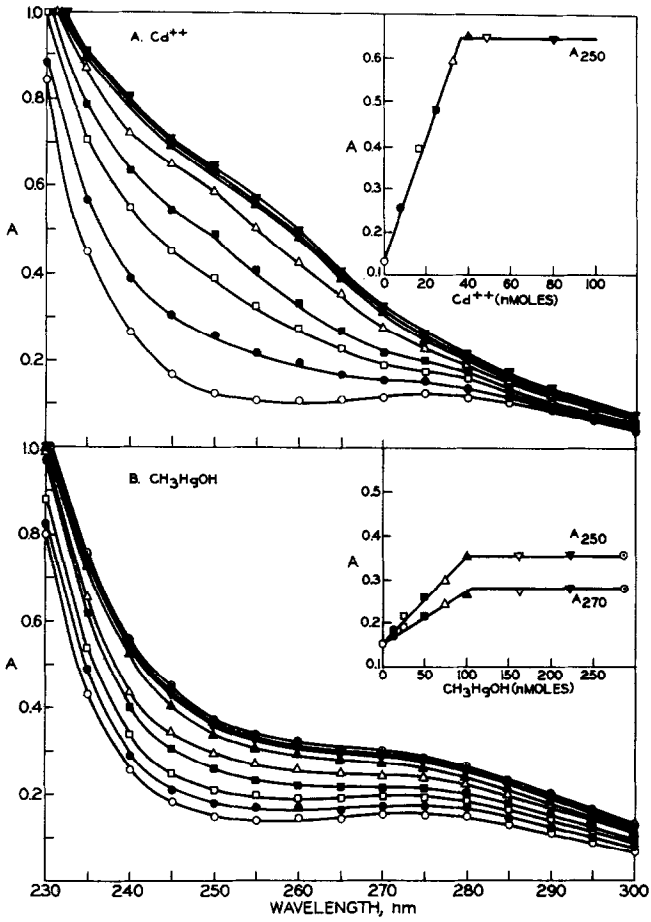

Figure 2. Spectrophotometric comparison of Cd^{++} and CH_3HgOH binding to purified thionein (horse kidney). To a cuvette containing 228 µgm of thionein in 1 ml of 5 mM sodium phosphate, pH 5.5, 25°, successive additions of $CdCl_2$ or CH_3HgOH were made and the spectrum recorded approximately two minutes after each addition. The spectra are corrected for dilution. Path length = 1 cm.

thionein after in vivo administration of CH_3HgOH probably represent Hg^{++} formed by metabolism. It is interesting that the amount of mercury in this fraction, on an absolute basis as well as a percentage basis, was greater in kidneys of rats fed the lower level of CH_3HgOH (2 ppm Hg) compared to the higher level (10 ppm Hg). Absolute amounts of Hg in the thionein peak from 10 ml of a 20% homogenate of rat kidney were 8.6 and 5.8 µg for the diet containing 2 ppm Hg plus or minus Se, respectively. Corresponding values were 4.6 and 2.3 µg for the diet containing 10 ppm Hg plus or minus Se.

Table II. Binding of Cd^{++}, Hg^{++}, and CH_3HgOH to purified thionein (horse kidney) after Sephadex G-25 chromatography.

Metal	nmoles added to 228 µg thionein	nmoles eluted with thionein	% Recovery
Cd^{++}	400	36.5	64.2
Hg^{++}	600	21.5	3.4
CH_3HgOH	1100	0.2	61.1

The indicated amounts of CH_3HgOH, $^{109}CdCl_2$, or $^{203}Hg(NO_3)_2$ were added to thionein (228 µgm) in 4 ml of 0.001M Tris-0.05M KCl buffer, pH 8.6, and stirred for 30 minutes at 4°. Each mixture was chromatographed on a Sephadex G-25 column (2.5 x 43 cm) which was preequilibrated and eluted with the same buffer at 4°. The fractions eluting at the void volume (thionein) were pooled and assayed for the metal. Subsequent fractions up to V_e/V_o = 6.7 were pooled, assayed, and summed with the thionein peak to measure recovery: $\left(\frac{\text{metal eluted}}{\text{metal applied}} \times 100\right)$.

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