Biochemical Pharmacology, Vol. 30, No. 23, pp. 3159–3163, 1981.
Printed in Great Britain.

0006-2952/81/233159-05 $02.00/0
Pergamon Press Ltd.

INVOLVEMENT OF TISSUE SULFHYDRYLS IN THE FORMATION OF A COMPLEX OF METHYLMERCURY WITH SELENIUM

HEITAROH IWATA,* TOHRU MASUKAWA, HIROKAZU KITO and MASAAKI HAYASHI
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, Yamada-oka, Suita-shi, Osaka 565, Japan

(*Received* 22 *February* 1980; *accepted* 8 *April* 1981)

Abstract—Albumin-bound methylmercury was converted to a benzene-extractable form by the soluble fraction of rat liver, kidney or brain in the presence of selenite, but not in its absence. The factors in the soluble fraction causing this conversion were investigated by column chromatography. Sephadex G-25 chromatography showed that effective factors were present in non-protein and protein fractions. It was concluded from ion exchange and Sephadex G-200 chromatography that these factors in the non-protein and protein fractions were reduced glutathione (GSH) and protein sulfhydryl groups respectively. Because GSH and the soluble protein could be replaced by sulfhydryl compounds, such as cysteine and 2-mercaptoethanol, as well as by a purified protein with sulfhydryl groups, reduced ribonuclease (RNase), respectively, it was concluded that sulfhydryl groups of GSH and/or proteins in the soluble fraction were needed for selenite-induced conversion of methylmercury to a benzene-soluble form. Among the various selenium compounds tested, only H_2Se (the reduced metabolite of selenite) was found to react directly with methylmercury to form a benzene extractable mercury compound in the absence of the soluble fraction. These findings suggest that the conversion of methylmercury to a benzene-soluble form occurs by reaction of methylmercury with selenium (possibly H_2Se) reduced by GSH and/or protein sulfhydryl groups in the soluble fraction. Thin-layer chromatography showed that benzene-extractable mercury consists mainly of bis(methylmercuric) selenide (BMS). A minor component, trismethylmercuric selenonium, was also detected by mass spectrography.

Selenium is known to counteract methylmercury toxicity in experimental animals [1–6], although it does not increase the elimination of methylmercury from the body in the urine and feces [7]. The mechanism of the protective effect of selenium on the toxicities of inorganic mercury and cadmium has been suggested to be due to transfers of these metals from critical proteins to less critical ones [8, 9], but selenium does not change the distribution of methylmercury among proteins [10].

Recently, Sumino *et al.* [11, 12] found that, in the presence of blood cells or homogenates of rat tissues, methylmercury that was bound to albumin was converted to free methylmercury extractable with benzene on addition of selenite, and they suggested that this phenomenon may be associated with the protective effect of selenium on methylmercury toxicity. It is unknown, however, what kind of tissue homogenate components participate in selenite-induced conversion of protein-bound methylmercury to a benzene-extractable form. In the present study we characterized the endogenous components found in tissues involved in this conversion by selenite of methylmercury to a different form, and examined the nature of the benzene-extractable methylmercury.

* Author to whom all correspondence should be addressed: Prof. Heitaroh Iwata, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, Yamada-oka, Suita-Shi, Osaka 565, Japan.

MATERIALS AND METHODS

Chemicals. Methylmercuric chloride (MMC) was obtained from Wako Chemicals, Osaka, Japan. Sodium selenite and sodium selenate were purchased from Nakarai Chemicals, Kyoto, Japan. Selenocystine and selenomethionine were from the Sigma Chemical Co., St. Louis, MO. Bovine serum albumin (Fraction V) was from the Armour Pharmaceutical Co., Kankakee, IN. Dowex 1X1 anion exchange resin (50–100 mesh) was from Muromachi Kagaku, Tokyo, Japan. Sephadex G-25 (fine) and G-200 (coarse) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Silica gel plates (Kieselgel 60) were from Merck, Darmstadt, West Germany. Bovine pancreatic ribonuclease (RNase) was from Boehringer, Mannheim, West Germany. All other chemicals used were commercial products of analytical grade.

Animals. Male Wistar rats weighing 150–200 g were decapitated and the liver, kidney and brain were rapidly removed, exsanguinated and homogenized in 2 vol. of 0.15 M KCl. The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant fraction was recentrifuged at 105,000 *g* for 60 min. The tissue-soluble fraction obtained was used in further experiments.

Column chromatography. For fractionation of the tissue-soluble fraction, gel filtration column chromatography was carried out on Sephadex G-25 or G-200 at 4° in 0.01 M Tris–HCl buffer (pH 7.4). A Dowex 1X1 anion exchange resin column was used for isolation of GSH and cysteine by the method of

Furano [13]. Activity for conversion of methylmercury to a benzene-soluble form in the presence of selenite was measured as described below. Sulfhydryl and reduced glutathione (GSH) contents were measured by the methods of Ellman [14] and Hissin and Hilf [15] respectively.

Preparation of reduced RNase, seleno-RNase, glutathione selenotrisulfide (GSSeSG) *and hydrogen selenide* (H_2Se). Reduced RNase and seleno-RNase were prepared by modifications of the method of Ganther and Corcoran [16]. In this method, native bovine pancreatic RNase was reduced with $NaBH_4$ [17] in place of 2-mercaptoethanol, which interferes with measurement of activity for conversion of methylmercury to a benzene-soluble form. GSSeSG was synthesized by the method of Ganther [18], and H_2Se was prepared by reaction of Al_2Se_3 with H_2O [19].

Selenite-induced methylmercury converting activity. NMC was bound to albumin by incubation of 10 μM MMC with 1.0% albumin in 0.05 M Tris–HCl buffer (pH 7.4) for 30 min at 37°. The reaction mixture for measurement of selenite-dependent methylmercury converting activity contained the following reagents in a total volume of 4 ml: 2.50 μM MMC bound to 0.25% albumin, 2.50 μM sodium selenite and the indicated amounts of test materials. The reaction was started by adding the test materials and was stopped after incubation for 30 min at 37° by extraction with 2.0 ml of benzene. Activity for conversion of methylmercury to a benzene-extractable form was expressed as the percentage of total methylmercury completely extracted with benzene on acidification. Mercury in the benzene layer was measured by cold-vapor atomic absorption spectrometry [20].

Characterization of benzene-soluble mercury. The reaction mixture consisted of 25 μM MMC bound to 10% albumin, 5 mM GSH and 25 μM selenite. The reaction was carried out for 5 min at room temperature. Then the mixture was shaken with 1/8 volume of benzene and centrifuged at 3000 rpm for 10 min. A portion of the benzene layer was used for mass spectrography (JMS-D300 mass spectrometer, JEOL, Japan) and applied to a silica gel plate. The plate was developed to a distance of 10 cm from the origin with petroleum benzin–diethyl ether (7:2, v/v), and mercury was located by spraying the plate with 0.1% dithizone–chloroform solution. The plate was cut into twenty sections of 0.5 cm width, and mercury and selenium in each section were measured by the above method and that of Watkinson [21] respectively.

RESULTS

On Sephadex G-25 column chromatography of the soluble fractions from the liver, kidney and brain, activity for conversion of albumin-bound methylmercury to a benzene-extractable form in the presence of selenite was separated into two fractions (Fig. 1). The first fraction was eluted in the void volume and the second, with low molecular weight materials, in the position of sulfhydryl compounds. Active material in the latter fraction from the liver and brain was identified as GSH, whereas that from the kidney was identified as cysteine by further characterization of the sulfhydryl compounds by Dowex 1X1 anion exchange resin column chromatography (data not shown). Various sulfhydryl compounds, such as GSH, cysteine, 2-mercaptoethanol and 2,3-dimercaptopropanol, were as effective as the non-protein fraction in the selenite-induced methylmercury transformation (data not shown).

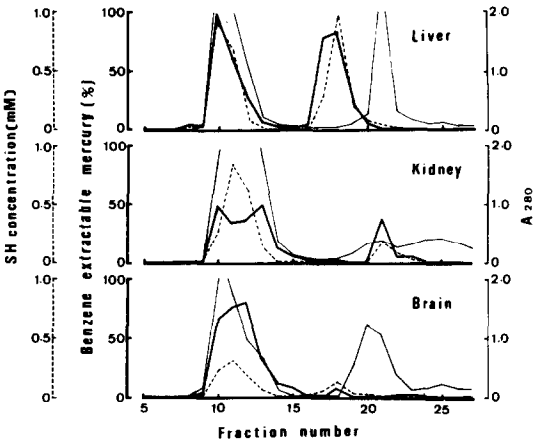

Fig. 1. Sephadex G-25 chromatographic pattern of selenite-dependent activities in the soluble fractions of rat liver, kidney and brain. The soluble fractions (2 ml) were applied to a Sephadex G-25 column (2 × 33 cm) that had been equilibrated with 0.01 M Tris–HCl buffer (pH 7.4), and material was eluted with the same buffer at a flow rate of 30 ml/hr. Fractions of 4 ml were collected, and 1.5 ml of each fraction was used for measuring activity.

The character of the soluble proteins with activity was examined by Sephadex G-200 column chromatography (Fig. 2). The activity of the soluble proteins was uniformly distributed from low to high molecular weight compounds. There was a correlation between

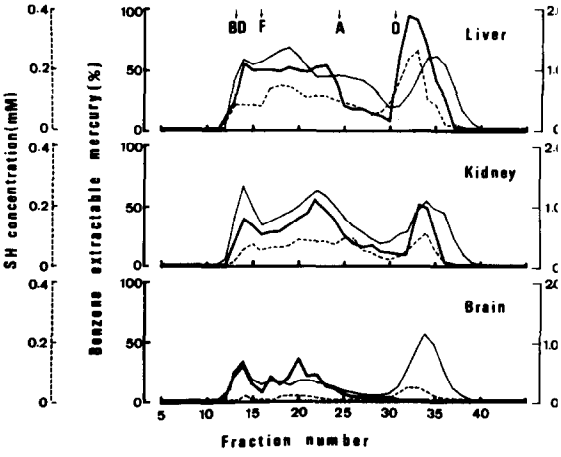

Fig. 2. Sephadex G-200 chromatographic pattern of selenite-dependent activities in the soluble fractions of rat liver, kidney and brain. The soluble fractions (4 ml) were applied to a Sephadex G-200 column (3 × 70 cm) that had been equilibrated with 0.01 M Tris–HCl buffer (pH 7.4), and material was eluted with the same buffer at a flow rate of 16 ml/hr. Fractions of 10 ml were collected, and 1.5 ml of each was used for measuring activity. The column was calibrated with the following standard proteins: BD (blue dextran); F (ferritin); A (aldolase) and O (ovalbumin).

Table 1. Activities of native, reduced, and seleno-RNase*

Treatment	Concn (μM)	Benzene-extractable mercury (%)
RNase		ND†
RNase + selenite	2.50	ND
RNase (reduced)		11.8
RNase (reduced) + selenite	0.10	24.3
	0.25	37.9
	0.50	60.9
	1.00	97.3
NEM-treated RNase + selenite	2.50	ND
Seleno-RNase (0.017 μmole Se/mg protein)		45.5

* A reaction mixture containing 2.5 μM MMC bound to 0.25% albumin, sodium selenite at the indicated concentration, and 100 μg/ml of purified protein was incubated for 30 min at 37°. Values are means of three determinations.

† ND: not detectable.

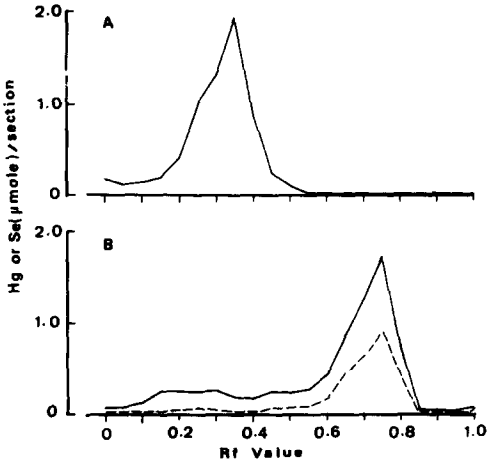

Fig. 3. Thin-layer chromatogram of benzene-extractable mercury showing the distribution of mercury and selenium. (A) MMC; (B) benzene-extractable mercury. Key: (——) mercury; and (– – –) selenium.

sulfhydryl concentration and activity of the eluate fractions

To examine whether sulfhydryl groups in proteins were responsible for this activity, we tested the effects of RNase and its derivatives as model proteins (Table 1). Native RNase, even in the presence of selenite, could not convert albumin-bound methylmercury to a benzene-extractable form, whereas reduced RNase had activity, depending on the concentration of selenite added. When the sulfhydryl groups in reduced RNase were alkylated with *N*-ethylmaleimide, this activity was completely lost. Seleno-RNase could also convert about 40 per cent of albumin-bound methylmercury to a benzene-soluble form, even in the absence of selenite. These results suggest that the non-specific reduction of selenite by sulfhydryl compounds or sulfhydryl groups of proteins was essential for the conversion.

Various selenium compounds, including reduced products of selenite with GSH, such as GSSeSG and H_2Se, were tested for activity. Selenite, selenate and

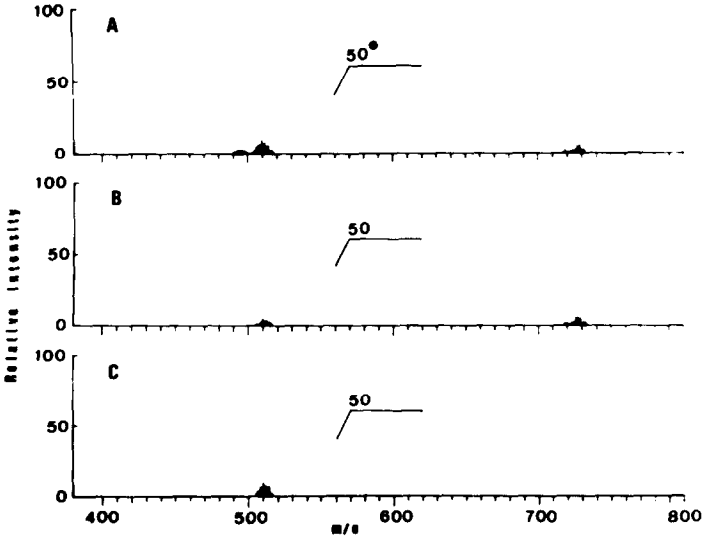

Fig. 4. Mass spectrum of benzene-extractable mercury. Mass spectrography was carried out under the following conditions: ionization voltage, 16 eV; ionization current, 350 μA; and chamber temperature, 180°. (A) Benzene-extractable mercury; (B) trismethylmercuric selenonium nitrate; and (C) bis(methylmercuric) selenide. An asterisk indicates magnification.

selenocystine had no effect in the absence of GSH, but they became effective on addition of GSH. The activity of selenite was much higher than that of selenate or selenocystine. Selenomethionine was not effective in the absence or presence of GSH. The activity of GSSeSG was higher than that of selenite in the presence of GSH. In the absence of GSH, GSSeSG was slightly effective, and bubbling with H_2Se was the most effective, suggesting that H_2Se was directly involved in the conversion. Other compounds such as sodium sulfide, sodium sulfate, cupric chloride, zine chloride, cadmium chloride and sodium tellurite were not effective at concentrations of up to 100 μM (data not shown). These findings indicate that the conversion activity was specific for selenium compounds.

The nature of the benzene-soluble form of mercury obtained with selenite and GSH was examined by thin-layer chromatography and mass spectrometry. Under the conditions of thin-layer chromatography used, the benzene-extractable mercury had an R_f value of 0.73, which was identical with that of bis(methylmercuric) selenide (BMS) synthesized by the method of Breitinger and Morell [22]. MMC, used as a reference, had an R_f value of 0.35. The atomic ratio of selenium to mercury in the dithizone-positive spot with an R_f value of 0.73 was about 1:2 (Fig. 3). Under the conditions used, trismethylmercuric selenonium nitrate, synthesized as a reference, gave two broad spots, one located at the origin and the other at the same position as BMS with tailing, suggesting that the former may have decomposed during development of the chromatogram. However, the mass spectrum of the benzene-extractable form of mercury showed slight peaks of trismethylmercuric selenonium (peaks near *m/e*, 727) (Fig. 4). From these results and the fact that BMS is much more soluble in benzene than trismethylmercuric selenonium nitrate, the benzene-extractable mercury was ascribed mainly to BMS.

DISCUSSION

The present study indicates that sulfhydryl groups of proteins as well as non-protein sulfhydryl groups are involved in the interaction between protein-bound methylmercury and selenite. The non-protein substance, with selenite-dependent activity that converted methylmercury to a benzene-soluble form, was identified as GSH in the liver and brain, and, in the kidney, as cysteine. Cysteine in the kidney was probably formed by decomposition of GSH during the separation process, because the non-protein sulfhydryl groups in the kidney have been reported to be mainly attributable to GSH [15, 23]. Thus, GSH may be the major substance, in the non-protein fraction with conversion activity.

Selenite is known to be reduced by GSH by the following reactions: $SeO_3^{2-} \rightarrow$ GSSeSG $\rightarrow$ GSSeH $\rightarrow H_2Se$ [18, 24]. Table 1 shows that, in the presence of GSH, the activity of GSSeSG was higher than that of selenite. Moreover, H_2Se, a reduced form of selenite, converted albumin-bound methylmercury completely to a benzene-extractable form, suggesting that H_2Se may be directly related to the development of this activity. With selenate, the above reactions may proceed after its reduction to selenite. The effect of selenocystine (CySeSeCy) may be due to an active selenide, such as selenocysteine (CySeH), formed by reduction with GSH. Therefore, the formation of H_2Se or —SeH may be essential for methylmercury-converting activity. Selenomethionine was probably ineffective because it does not react with GSH. The activity in the protein fraction seems to have been due to free sulfhydryl groups in the proteins. Selenite is thought to be reduced by protein sulfhydryl groups, such as those of reduced RNase, forming selenotrisulfide derivatives [16]. Although it is unknown whether H_2Se or —SeH is formed, the results in Fig. 2 and Table 2 suggest that both protein sulfhydryl groups and GSH can reduce selenite to an active selenide with a high affinity to methylmercury. Albumin, used as a methylmercury binding protein, apparently had no effect, indicating the low reactivity of its sulfhydryl groups.

The transformation of methylmercury from an albumin-bound form to a benzene-soluble one was

Table 2. Activities of various selenium compounds*

Treatment	Concn (μM)	Benzene-extractable mercury (%) GSH (−)	GSH (+)
None		ND†	ND
Selenite	10	ND	76.9
	20	ND	96.9
Selenate	20	ND	13.8
	50	ND	18.3
Selenocystine	20	ND	16.3
	50	ND	41.5
Selenomethionine	50	ND	ND
GSSeSG	5	9.1	64.2
	10	9.3	100.0
H_2Se‡		99.3	

* A reaction mixture containing 2.5 μM MMC bound to 0.25% albumin and a selenium compound at the indicated concentration with or without 250 μM GSH was incubated for 30 min at 37°. Values are means of three determinations.

† ND: not detectable.

‡ H_2Se, generated by the reaction of Al_2Se_3 and H_2O, was bubbled through 4 ml of solution containing 2.5 μM MMC bound to 0.25% albumin for 5 min.

associated with the formation of a benzene-soluble complex between methylmercury and selenium. Recently, it was shown that the benzene-soluble form of mercury formed by reaction of methylmercury with selenite in the presence of blood or GSH was BMS [25]. In the present study, however, although trismethylmercuric selenonium was detected in the benzene layer, the thin-layer chromatogram showed that the benzene-soluble mercury was mainly BMS. We are now studying the conditions suitable for formation of trismethylmercuric selenonium.

The binding of methylmercury to critical proteins in the tissues is presumably involved in its toxicity [26]. Although it is uncertain whether BMS, including trismethylmercuric selenonium, has affinity to proteins, the conversion of methylmercury to complexes probably induces a significant alteration in the interaction between methylmercury and proteins, resulting in modification of methylmercury toxicity. The protective action of selenium against the toxicities of heavy metals such as inorganic mercury and cadmium is apparently due to the formation of a biologically inactive complex with selenium [9, 27].

Recently, we found that BMS was formed transiently in the blood and tissues of rats injected with MMC and selenite, and that it was intimately related to the selenite-induced redistribution of methylmercury,* which has been reported to be one of the mechanisms of protection by selenium against methylmercury toxicity [10]. Further investigations are necessary, however, to evaluate the possible involvement of the present phenomena, and especially of methylmercury–selenium complexes, in the protective action of selenium against methylmercury toxicity.

Acknowledgement—This research was supported by a grant from the Takeda Science Foundation.

* T. Masukawa, H. Kito, M. Hayashi and H. Iwata, unpublished data.

REFERENCES

1. H. E. Ganther, C. Goudie, M. L. Sunde, M. J. Kopecky, R. Wagner, S. H. Oh and W. G. Hoekstra, *Science* **175**, 1122 (1972).
2. H. Iwata, H. Okamoto and Y. Ohsawa, *Res. Commun. Chem. Path. Pharmac.* **5**, 673 (1973).
3. S. L. Johnson and W. G. Pond, *Nutr. Rep. Int.* **9**, 135 (1974).
4. S. Potter and G. Matrone, *J. Nutr.* **104**, 638 (1974).
5. G. Ohi, S. Nishigaki, H. Seki, Y. Tamura, T. Maki, H. Maeda, S. Ochiai, H. Yamada, Y. Shimamura and H. Yagyu, *Toxic. appl. Pharmac.* **32**, 527 (1975).
6. G. S. Stoewsand, C. A. Bache and D. J. Lisk, *Bull. envir. Contam. Toxic.* **11**, 152 (1974).
7. B. R. Stillings, H. Lagally, P. Bauersfeld and J. Soares, *Toxic. appl. Pharmac.* **30**, 243 (1974).
8. R. W. Chen, P. D. Whanger and S. C. Fang, *Pharmac. Res. Commun.* **6**, 571 (1974).
9. T. A. Gasiewicz and J. C. Smith, *Biochim. biophys. Acta* **428**, 113 (1976)
10. R. W. Chen, V. L. Lacy and P. D. Whanger, *Res. Commun. Chem. Path. Pharmac.* **12**, 279 (1975).
11. K. Sumino, R. Yamamoto and S. Kitamura, *Nature, Lond.* **268**, 73 (1977).
12. K. Sumino, R. Yamamoto and S. Kitamura, *Biochem. biophys. Res. Commun.* **86**, 735 (1979).
13. A. V. Furano, *Methods of Enzymology* Vol. XV, p. 509. Academic Press, New York (1971).
14. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1959).
15. P. J. Hissin and R. Hilf, *Analyt. Biochem.* **74** 214 (1976).
16. H. E. Ganther and C. Corcoran, *Biochemistry* **8**, 2557 (1969).
17. W. D. Brown, *Biochim. biophys. Acta* **44**, 365 (1960).
18. H. E. Ganther, *Biochemistry* **10**, 4089 (1971).
19. F. Feher, in *Handbuch der Präparative Anorganishen Chemie* (Ed. G. Brauer), p. 378. F. Enke, Stuttgart (1960).
20. L. Magos and T. W. Clarkson, *J. Ass. off. analyt. Chem.* **55**, 966 (1970).
21. J. H. Watkinson, *Analyt. Chem.* **38**, 92 (1966).
22. D. Breitinger and W. Morell, *Inorg. nucl. Chem. Lett.* **10**, 409 (1974).
23. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
24. H. E. Ganther, *Biochemistry* **7**, 2898 (1968).
25. A. Naganuma and N. Imura, *Res. Commun. Chem. Path. Pharmac.* **27**, 163 (1980).
26. S. Skerfving, *Envir. Hlth Perspect.* **25**, 57 (1978).
27. D. H. Groth, L. Stetler and G. Mackay, in *Effects and Dose Response Relationships of Toxic Metals* (Ed. G. F. Nordberg), p. 527. Elsevier, Amsterdam (1976).