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MECHANISM OF METHYLMERCURY EFFLUX FROM CULTURED ASTROCYTES

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Abstract—To study the mechanism of methylmercury (MeHg) efflux from the central nervous system cells, cultured astroglia obtained from neonatal rats were incubated with 10 μM MeHg-cysteine (CySH) for 30 min. After being washed four times, cells were incubated in Hg-free medium, and the release of MeHg from the cells was monitored. The amount of MeHg released in the medium approached a plateau level (*ca.* 31% of the loaded amount) at 4 hr. Treatment of the cells with a CySH precursor, 2-oxothiazolidine-4-carboxylic acid (OTC), resulted in a significant increase of cellular levels of CySH and glutathione (GSH). OTC also increased 1.5-fold the MeHg efflux from the loaded cells. Another GSH enhancer, GSH isopropyl ester, also stimulated MeHg export from the cells. Ion-exchange column chromatography using DEAE-Sephadex revealed that the MeHg metabolite thus released was exclusively MeHg-GSH conjugate, both with and without OTC. Since the MeHg efflux was suppressed significantly by the presence of probenecid, the efflux occurred via the probenecid-sensitive organic acid transport system. Even though the cellular GSH levels were depleted drastically by treatment with L-buthionine-(*S,R*)-sulfoximine (BSO), a considerable level (90% of the control) of Hg efflux was detected. Since neither GSH- nor CySH-MeHg was detected in the culture medium of the BSO-treated cells, GSH depletion may trigger some other secretion system(s) in the cells. These results suggest that conjugation with GSH is the major pathway for MeHg efflux in rat astroglia, and that elevation in the cellular GSH level would possibly be a logical therapy for MeHg poisoning, promoting the accelerated elimination of MeHg from the critical tissues.

Key words: methylmercury; astrocytes; cell culture; glutathione; efflux; organic anion transporter

Methylmercury (MeHg‖) is well known to be a cause of the severe neurological syndrome called Minamata disease, which includes cerebellar signs, concentric constriction of the visual field, and sensory disturbance [1]. Exposure to MeHg occurs mostly by consumption of MeHg-contaminated fish. MeHg is readily absorbed from the gastrointestinal tract and transferred into the blood, through which it is distributed rapidly among various tissues including those in the brain [2]. The presence of cysteine (CySH) was reported to accelerate the intestinal absorption of MeHg [3]. CySH also increases MeHg uptake by the whole brain [4–7], brain microvessels [8], and astroglia [9]. Since MeHg easily reacts with CySH to form a conjugate with a structure similar to methionine, the conjugate is suggested to be taken up by the cells through the L neutral amino acid carrier transport system [3–9].

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‖ Abbreviations: MeHg, methylmercury; GSH, glutathione; CySH, cysteine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBS, Hanks' balanced salt solution containing 15 mM HEPES (pH 7.4); GFAP, glial fibrillary acidic protein; LDH, lactate dehydrogenase; OTC, 2-oxothiazolidine-4-carboxylic acid; GSH-OPr^i, glutathione isopropyl ester; BSO, L-buthionine-(*S,R*)-sulfoximine; and SBD-F, 4-fluoro-7-sulfobenzofurazan ammonium salt.

The MeHg thus accumulated is gradually eliminated from various tissues and finally from the body, mainly into the bile and renal tubules. *In vivo* experiments suggested that, in both processes, MeHg is secreted as its glutathione (GSH) conjugate via the organic acid transport system [10–12]. In urine, however, MeHg was found as a CySH conjugate because of tripeptide moiety degradation by the tubular enzymes [12]. Contrary to the excretory processes, very little information is available on the process by which MeHg is eliminated from neural tissues. Aschner *et al.* [13] speculated that the MeHg efflux from cultured astrocytes would be the reverse of its uptake pathway as a CySH conjugate. Since the organic acid secretion system through which GSH-MeHg is translocated is a common one among various cell types, this system may also exist in neural tissues.

In the present study, to confirm the functional elimination pathways of MeHg, primary cultured rat astrocytes were loaded with MeHg. Efflux rates were monitored in the presence or absence of reagents that affected the cellular GSH levels. Then, the conjugation form(s) of secreted MeHg was identified, using ion-exchange chromatography.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's Medium (DMEM) and Ham's F12 Medium were purchased

from the Nissui Pharmaceutical Co., Ltd. Fetal bovine serum (FBS) was purchased from GIBCO/BRL Life Technologies, Inc. GSH and CySH were obtained from Wako Pure Chemical Industry Ltd. (Osaka) and the Nakarai Chemical Co. (Tokyo), respectively. 4-Fluoro-7-sulfobenzofurazan, ammonium salt (SBD-F) and L-buthionine-(*S,R*)-sulfoximine (BSO) were purchased from Dojindo Laboratories (Kumamoto) and the Sigma Chemical Co. (St. Louis, MO), respectively. GSH isopropyl ester (GSH-OPr^i) was provided by the Yamanouchi Pharmaceutical Co. (Tokyo). All other reagents used were of analytical grade. MeHg conjugates of CySH and GSH were synthesized by reacting equivalent amounts of MeHg chloride (Tokyo Chemical Industry Co., Ltd., Tokyo) and each thiol compound in PBS, and then using them immediately in the experiment. Each MeHg compound thus prepared showed a single peak on DEAE-Sephadex A-25 column chromatography with a recovery of above 90%.

Cell culture. Primary cultured rat astroglia were prepared from the cerebral hemispheres of neonatal rats according to the method of McCarthy and de Vellis [14]. Briefly, meninges were removed and tissue was disrupted by passing through a sterile nylon sieve (250 μm). Cells were plated in a 75-cm^2 flask at 4×10^5/cm^2 in the medium [DMEM/Ham's F12 containing 4 mM glutamine, 0.5% glucose, penicillin (100 U/mL), streptomycin (100 μg/mL), mycostatin (100 U/mL)] with 15% FBS. The cultures were incubated at 37° in a humidified 95% air/5% CO_2 atmosphere. The culture medium was changed on days 3, 5, 7, and 9. On days 10 and 11, the flasks were shaken for 20 hr at 200 rpm to remove microglia and oligodendrocytes. The astrocytes thus obtained were transferred to a new flask every 2 weeks, and were incubated in medium with 10% FBS. The third-passage astrocytes were used for the experiment. At this stage, more than 95% of the cells were positive for a marker of astrocytes, glial fibrillary acidic protein (GFAP) [15].

Cytotoxicity test. To evaluate the cytotoxicity of MeHg on the astroglia, the cells were plated onto a 96-well plate and exposed to MeHg-CySH (0–100 μM) in DMEM/Ham's F12 with 10% FBS for 24 hr at 37°. The viable cell numbers were estimated by the neutral red uptake assay [16]. Cell viability during uptake and efflux experiments was monitored by a lactate dehydrogenase (LDH) release test [17]. No abnormal LDH release was observed throughout either experiment.

Uptake of MeHg. Astroglia were grown and reached almost confluent density in a 35-mm dish. The cells were washed three times with Hanks' balanced salt solution (HBS) containing 15 mM HEPES (pH 7.4), and then were incubated in the presence of 10 μM MeHg-CySH (1.5 mL) at 37°. At indicated times, the cells were washed three times with HBS and solubilized in 2 N NaOH (1 mL). Total Hg and protein levels in the alkaline solutions were determined according to the oxygen combustion–gold amalgamation method [18] and the method of Lowry *et al.* [19], respectively. Hg analysis was carried out using a Rigaku mercury analyzer SP-3 and expressed as total Hg per cellular protein. Since the cellular Hg reached a plateau level at 30 min, the efflux experiment was carried out using the cells of this stage as follows.

Efflux of MeHg. Astrocytes loaded with MeHg for 30 min as above in a 35-mm dish were washed four times with HBS, and then cultured in one of the following buffers: HBS only, HBS containing 5 mM OTC or 5 mM GSH-OPr^i for 4 hr at 37°. At adequate intervals, the extracellular medium was collected, and the cells were immediately washed three times with HBS, and then were solubilized in 2 N NaOH. The combined fraction of medium and wash and the alkaline solution were subjected to Hg determination. Each Hg value was expressed as total Hg (ng) per cellular protein (mg). To evaluate a possible demethylation of MeHg, inorganic Hg in the whole culture was quantified [20] at the final stage of the experiment.

Analysis of thiol compounds. Another series of cells preloaded with MeHg were incubated in the presence or absence of OTC (5 mM) or GSH-OPr^i (5 mM) to determine thiol compounds inside and outside the cells. To determine the extracellular levels, the medium was diluted with an equal volume of ice-cooled 5% perchloric acid containing 1 mM EDTA. After washing three times with HBS, the cells were homogenized in 5% perchloric acid (1 mM EDTA) to determine the cellular thiol levels. The acidic mixtures thus obtained were centrifuged at 12,000 *g* for 3 min; the supernatant was washed four times with benzene to remove MeHg as previously reported [21], and then reacted with SBD-F according to Toyo'oka and Imai [22]. To evaluate oxidized GSH and cystine, the reaction was carried out in the presence of tri-*n*-butylphosphine (0.25%). The labeled thiol compounds were analyzed using a Waters radial pack C_{18} column and detected by a Hitachi 650 fluorometer.

Analysis of MeHg conjugates. To analyze the MeHg metabolite(s) released in the medium, DEAE-Sephadex A-25 (1.5 × 34 cm) column chromatography was carried out. The samples containing Hg metabolite(s) (about 100 ng of Hg) were eluted by 50 mM phosphate buffer (pH 7.0), and 4.7-mL fractions were collected. Hg content in each fraction was determined as described above and expressed as total Hg per fraction.

Effect of BSO on cellular GSH and MeHg efflux. The astrocytes were treated with 1 mM BSO for 24 hr at 37° in HBS. The cells were washed and then incubated with MeHg-CySH for 30 min. After washing with HBS, the cells were incubated with Hg-free HBS at 37°, and the MeHg released was monitored. Evaluation of intra- and extracellular GSH levels and ion-exchange chromatography of MeHg metabolite(s) released were carried out as described above.

Effects of probenecid on MeHg efflux. The MeHg-loaded astrocytes were cultured in one of the following buffers: HBS only, HBS containing 5 mM OTC, 1 mM probenecid or 5 mM OTC plus 1 mM probenecid for 2 hr at 37°. Hg contents in the extracellular fractions were determined as above.

Statistical analysis. Time-course data were analyzed by two-way ANOVA, followed by a Tukey

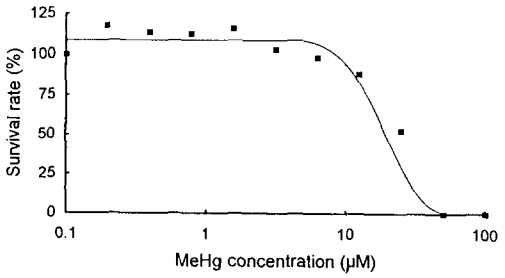

Fig. 1. Cytotoxicity of MeHg evaluated by a neutral red uptake assay. Cells on a 96-well plate were exposed to MeHg in DMEM/Ham's F12 with 10% FBS for 24 hr, cultured in the same medium including 50 μg/mL neutral red for 2 hr, then fixed and washed with 1% calcium chloride/1% formaldehyde, and solubilized in 1% acetate/50% ethanol. Absorption at 540 nm was measured by a microplate reader. Each point is the mean value of 8 wells, and standard deviations of 8 wells were less than 10% of the mean values. The curve fitting the data was calculated by Log-Logit (using Softmax R: software for maxline microplate readers from Wako Pure Chemical Industry Ltd.).

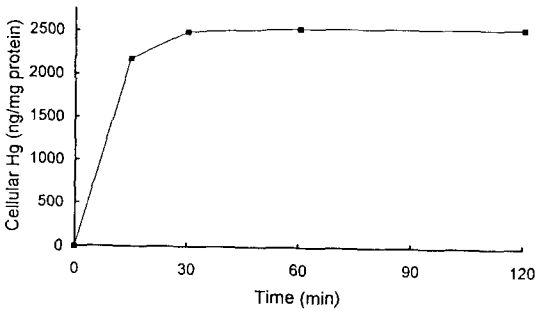

Fig. 2. Uptake of MeHg by primary cultured astroglia. Cells were cultured in HBS containing 10 μM MeHg-CySH. At the indicated time, cells were washed three times with HBS and solubilized with 2 N NaOH. The mercury content of the alkaline solution was determined and expressed as total Hg (ng) per cellular protein (mg). Each point is the average of three samples, and the standard deviation was less than 10% of the average.

test for individual comparisons where differences were indicated. Other data were analyzed by Student's *t*-test.

RESULTS

MeHg is reported to be taken up into the tissue as a MeHg-CySH conjugate via the L neutral amino acid carrier transport system [3–8]. In a preliminary experiment using cultured astrocytes and several kinds of MeHg-S conjugates possibly present in blood plasma (e.g. conjugates of GSH, CySH, cysteinylglycine and mercaptalbumin), the most effective Hg uptake by the cells was detected when MeHg-CySH was added to the medium (data not shown). Accordingly, in the present study we employed this conjugate for loading MeHg onto the astrocytes. The cytotoxicity of MeHg-CySH to the astrocytes was evaluated by the neutral red uptake method. After a 24-hr exposure at 37° to various levels of MeHg-CySH, the LC_{10} and LC_{50} values were found to be 15 and 25 μM, respectively (Fig. 1). We used an MeHg concentration of 10 μM for the loading, which was low enough to cause no cellular death throughout the experiments. By incubating the astrocytes in the presence of 10 μM CySH-MeHg, the cellular Hg accumulation reached a plateau level of about 2500 ng/mg protein at 30 min, which accounted for 33% of the added amount, and the level remained unchanged at least for the following 90 min (Fig. 2). During the period from 30 to 90 min, the rates of influx and efflux could possibly be at an equilibrium. Accordingly, the Hg loading was carried out by cell incubation for 30 min at 37° with 10 μM MeHg-CySH.

MeHg loaded onto the astroglia, as described above, was spontaneously released to the Hg-free HBS at 37° as shown in Fig. 3. The curves of the release approached a pseudo steady state, which

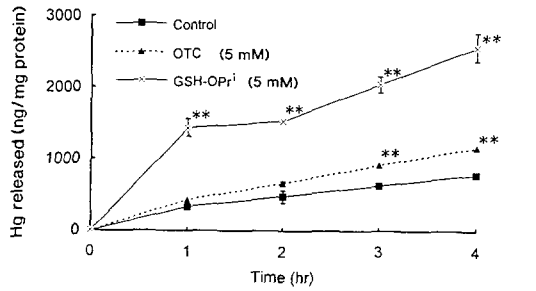

Fig. 3. Efflux of MeHg from astroglia. Cells loaded by MeHg using 10 μM MeHg-CySH for 30 min were washed four times with HBS, and then incubated in HBS. Hg efflux was monitored for HBS alone, in the presence of 5 mM OTC or 5 mM GSH-OPr^i. Hg levels in the extracellular medium were determined and expressed as total Hg (ng) per cellular protein (mg). Each value is the mean ± SD obtained from three experiments. Key: (**) significantly different ($P < 0.01$) from control (HBS alone).

presumably represented a balance of efflux and influx. The proportion of Hg thus released was about 31% of the loaded Hg after 4 hr. OTC, a precursor of CySH [23], was reported to increase both CySH and GSH levels [24] or only the CySH level [25] in rat brain. In the present study, when the astrocytes were incubated with OTC (5 mM), the levels of both thiol compounds increased significantly (Fig. 4). In the presence of OTC at this level, the efflux rate of MeHg increased by 1.5-fold, accounting for 45% of the total cellular Hg at 4 hr. Similarly, GSH-OPr^i, which was reported to function as a GSH precursor and increase the cellular GSH level, also enhanced the Hg release from the cells. As much as 99% of the cellular MeHg was released after 4 hr in the presence of 5 mM GSH-OPr^i (Fig. 3). A greater increase in the cellular GSH levels was caused by incubation with GSH-OPr^i than with OTC (Fig. 4).

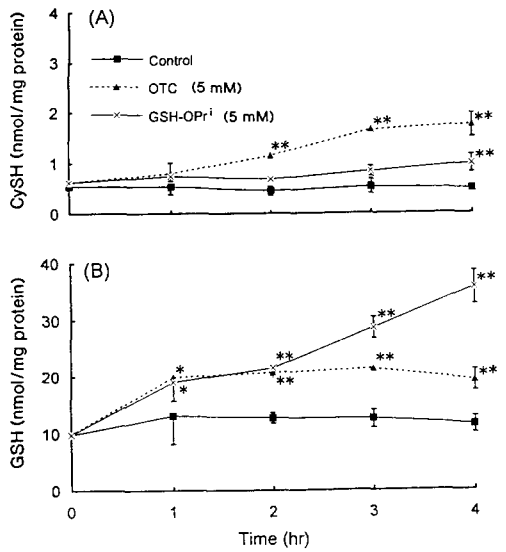

Fig. 4. Effects of OTC and GSH-OPr^i on cellular thiol levels in the astrocytes. MeHg-loaded cells were incubated in HBS or HBS containing 5 mM OTC or GSH-OPr^i for the indicated time. Cells were washed three times with HBS, and extracted with 5% perchloric acid containing 1 mM EDTA. CySH (A) and GSH (B) levels in these samples were measured according to Toyo'oka and Imai [22]. Each value is the mean ± SD obtained from three experiments. Significant differences from control were shown by * ($P < 0.05$) and ** ($P < 0.01$).

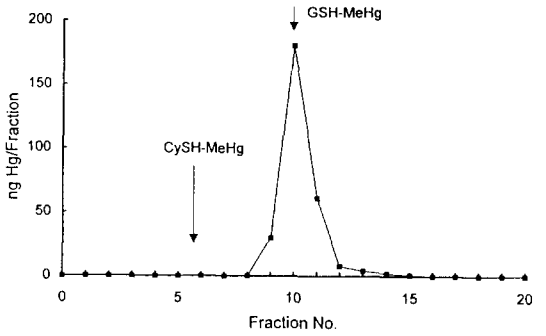

Fig. 5. Ion-exchange chromatogram of MeHg metabolites secreted from astrocytes. After a 2 hr incubation of MeHg-preloaded cells in HBS, an aliquot of the extracellular medium was chromatographed in a column of DEAE-Sephadex A-25 (1.5 × 34 cm). MeHg metabolites were eluted by 50 mM phosphate buffer (pH 7.0), and 4.7-mL fractions were collected. The Hg content in each fraction was determined and expressed as total mercury per fraction. Arrows represent peak positions obtained from authentic CySH- and GSH-MeHg.

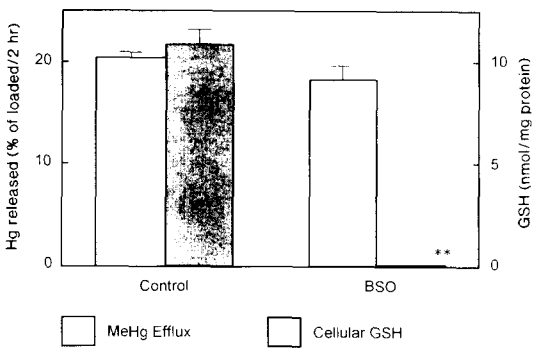

Fig. 6. Effects of BSO on Hg efflux and cellular GSH levels. Cells were incubated with 1 mM BSO for 24 hr, followed by MeHg loading with 10 μM MeHg-CySH for 30 min. After washing, the cellular GSH levels and Hg released in the extracellular medium for 2 hr were determined. Each value is the mean ± SD obtained from three experiments. Key: (**) significantly different ($P < 0.01$) from control by Student's t-test.

Since no appreciable level of inorganic Hg could be detected at the final stage of the experiment (data not shown), the Hg–C bond would be sufficiently stable under the present circumstances. Furthermore, virtually all of the Hg added was found in the combined medium and cellular fractions throughout the experiment, suggesting that the loss of MeHg (evaporation and/or adsorption on the dish) would be very little.

To know what secretion system(s) functioned in the MeHg efflux, it was necessary to determine the conjugation form(s) of the organic metal released. The media that contained MeHg released from the astrocytes were chromatographed using a DEAE-Sephadex A-25 column. In both spontaneous and OTC-stimulated effluxes, GSH-MeHg was the exclusive form in the HBS (Fig. 5). When GSH-OPr^i was present in the medium, MeHg conjugate of the sulfhydryl ester was the exclusive form detected (data not shown). To confirm the possible presence of other thiol or disulfide species in the extracellular media, into which MeHg was released, the perchloric acid-treated media were reacted with SBD-F to label the thiols. HPLC analysis of the fluorescent compounds [22] revealed that the reduced form of GSH was the exclusive component in the media of spontaneous and OTC-enhanced secretion; no GSH disulfide (GSSG), CySH or cystine was detected (data not shown). These results implied that GSH-MeHg would be the exclusive MeHg species that was secreted from the astrocytes. When there was a great excess of GSH-Opr^i, MeHg secreted as its GSH conjugate would rapidly be bound to the sulfhydryl ester. Thus, a chelating effect of the extracellular GSH-OPr^i might contribute, in part, to the increase of Hg release.

It should be noted that even if the astrocytes were treated with BSO, a specific inhibitor of GSH biosynthesis, the cells still released a considerable amount (90% of the spontaneous release) of MeHg, though the cellular GSH was depleted to an undetectable level (Fig. 6). DEAE-Sephadex chromatography revealed the presence of a single peak of MeHg metabolite that eluted much faster than CySH- or GSH-MeHg. Some additional

Table 1. Effect of probenecid (PBC) on MeHg secretion from astrocytes

Medium	MeHg released from astrocytes for 2 hr (% of loaded amount)
HBS	20.8 ± 5.8
HBS + PBC	5.6 ± 0.3*
HBS + OTC	27.2 ± 8.4
HBS + OTC + PBC	8.5 ± 0.2*

Cells preloaded with MeHg were cultured in the presence or absence of PBC (1 mM) or OTC (5 mM) for 2 hr. Hg content in each extracellular medium was determined and expressed as a percentage of the loaded amount (3100 ± 280 ng Hg/mg protein). Values are means ± SD obtained from three experiments.

* Significantly different ($P < 0.01$) from the values without PBC by Student's *t*-test.

transport system(s) may function in the GSH-depleted cells. Despite the extreme decline in the cellular GSH, no appreciable increase of LDH was observed in these cells even after the MeHg loading, suggesting that the plasma membrane had remained intact during the experiment.

Since GSH and its S-conjugates of xenobiotics were documented to be secreted via the probenecid-sensitive organic acid transport system [26], the effects of probenecid on MeHg efflux were examined. As shown in Table 1, the spontaneous efflux was suppressed by 70% in the presence of probenecid. This was also observed in OTC-stimulated efflux, suggesting that in the MeHg secretion by astrocytes GSH served as a carrier via the organic acid secretion system.

DISCUSSION

Although many reports have dealt with MeHg metabolism *in vivo* [2], it is not understood in detail at the cellular level. A few investigators suggested that MeHg was taken up by cells as MeHg-CySH conjugate through the L neutral amino acid transport system [3–9]. However, there are very few investigations concerning the efflux mechanism of MeHg. Aschner *et al.* [13] investigated the kinetics of radiolabeled MeHg with rat primary cultured astroglia, and speculated that MeHg would be exported as CySH conjugate via the same transport system used for its uptake.

The present study was carried out to examine the possibility of another transport system for the MeHg efflux besides the neutral amino acid carrier system suggested by Aschner *et al.* [13]. We demonstrated here that conjugation with GSH was the major pathway for efflux of MeHg in cultured rat astroglia. The secretion is suspected to be mediated by the probenecid-sensitive organic acid transport system. Furthermore, the increase of the intracellular GSH concentration would promote the efflux.

Since MeHg has a high affinity for sulfhydryl groups and forms complexes with various thiol compounds, such as GSH, CySH and cysteinyl residues of proteins [27], its chemical form in some tissues would be determined by the population of co-existing thiol compounds. If a portion of the MeHg secretion occurred as CySH conjugate, as suggested by Aschner *et al.* [13], its metal moiety might quickly be transferred to GSH, because our results showed that GSH-MeHg was the exclusive form detected in the extracellular medium. Therefore, the CySH thus formed in the extracellular space would easily be oxidized to cystine. Accordingly, some cystine should be detected in the media to which MeHg was released. The fact that thiol and disulfide analysis detected only GSH indicated that GSH-MeHg must be the exclusive form for the secretion of this heavy metal in the astrocytes. Alternatively, if the astrocytes have a secretion system for CySH-MeHg as suggested by Aschner *et al.* [13], this metabolite might quickly be reabsorbed by the cells. However, the extracellular CySH-MeHg level might be appreciable in GSH-depleted cells, in which GSH-MeHg secretion would be lowered markedly. It should be noted that the BSO-treated astrocytes, whose GSH levels decreased to an undetectable level, still released a considerable amount of MeHg. However, neither CySH- nor GSH-MeHg was detected in the extracellular space by DEAE-Sephadex chromatography. Some other transport system(s) for MeHg secretion might function in the BSO-treated cells. Further study must be conducted to clarify this transport system.

GSH is the major low molecular weight hydrophilic thiol compound in various tissues including the brain [28]. Since the level of GSH in the astroglia was about fifteen times higher than that of CySH (Fig. 4), MeHg taken up by the cells as MeHg-CySH could readily be transferred to GSH conjugate inside the cells. In the whole brain homogenate, the GSH conjugate was also reported to be the major component of MeHg metabolites [29, 30]. Thus, MeHg-GSH, which was formed in the cytosol, would be secreted into extracellular spaces via the probenecid-sensitive transport system. Furthermore, the chemical form of MeHg exported from the rat astrocytoma C6 and the human glioblastoma T98G, which were classified as the same group of astrocytes, was also found to be GSH conjugate (data not shown). The probenecid-sensitive organic anion transport system observed here might be a common

secretion system for cellular MeHg in this kind of cell.

In most animal species, the major MeHg elimination occurs via bile and renal tubule secretion. The conjugation forms in both secretions were suggested also to be GSH-MeHg [10–12]. Naganuma *et al.* [11] showed that depletion of hepatic GSH in MeHg-treated mice caused lowered renal Hg accumulation due to the reduced Hg supply from the liver to the circulation. Previously, we demonstrated that hepatic and renal secretion rates of GSH were closely related to the rates of MeHg elimination from these tissues [31]. Thus, the secretion system for GSH might also function as an MeHg elimination system in various tissues. Recently, Miura and Clarkson [32] reported that PC12 mutant cells with a high cellular GSH and high efflux rate showed much greater resistance to MeHg toxicity than other mutants with the normal level and efflux rate of the tripeptide. In this light, the present results also suggest that drugs which elevate cellular GSH levels could be used to treat MeHg poisoning by enhancing the elimination of intracellular MeHg.

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