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POSSIBLE ROLE OF HEPATIC GLUTATHIONE IN TRANSPORT OF METHYLMERCURY INTO MOUSE KIDNEY*

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Abstract—The mechanism of the renal uptake of methylmercury was studied in mice. Preadministration of 1,2-dichloro-4-nitrobenzene (DCNB), which is a reagent that depletes hepatic glutathione (GSH) without affecting the renal GSH level, 30 min before injection of methylmercury significantly decreased the renal accumulation of mercury. The renal accumulation of mercury in mice receiving methylmercury–GSH intravenously was significantly higher than that in mice receiving methylmercuric chloride. These results suggest the possibility that hepatic GSH, as a source of extracellular GSH, plays an important role in the renal accumulation of methylmercury. No significant difference in renal mercury accumulation between bile duct-cannulated mice and normal mice was observed, indicating that the enterohepatic circulation of methylmercury is not an important factor in the renal accumulation of methylmercury in mice. Pretreatment of mice with acivicin, a potent inhibitor of γ-glutamyl transpeptidase (γ-GTP), significantly depressed the renal uptake of methylmercury and increased the urinary excretion of GSH and methylmercury. In *in vitro* reactions, methylmercury-GSH was degraded into methylmercury-cysteinylglycine by γ-GTP, and this product was then converted to methylmercury-cysteine by dipeptidase. These results suggest that methylmercury is transported into the kidney as a complex with GSH, and then incorporated into the renal cells after degradation of the GSH moiety by γ-GTP and dipeptidase, although the methylmercury bound to extracellular GSH can be reversibly transferred to plasma proteins in the bloodstream.

The highest concentration of methylmercury after administration to rats [1] and mice [2, 3] via any route is generally found in the kidney. Several studies have been carried out to clarify the mechanism of the renal uptake of methylmercury, but very little is actually known. Richardson and Murphy [4] examined the effects of glutathione (GSH)-depleting agents on the tissue deposition of methylmercury in rats, and suggested that renal GSH might be a determinant for renal methylmercury accumulation. Actually a methylmercury–GSH complex has been found in liver [5], brain [6], bile [7–9] and erythrocytes [10, 11], suggesting that the complexation may play an important role in the transport, accumulation and excretion of methylmercury. The renal accumulation and biliary secretion of methylmercury increase with co-administration of GSH or cysteine (Cys) [12–16]. Studies on the biliary secretion of methylmercury have suggested that methylmercury might be secreted from the liver into the bile as a complex with GSH [16, 17], and the GSH transport system might be the primary determinant of the biliary secretion of methylmercury [16, 18]. The enhancing effect of GSH or Cys administration on the biliary secretion of methylmercury [14–16] may be due to its ability to increase both the hepatic GSH level and the biliary secretion of GSH [15, 16]. The biliary secretion of methylmercury–GSH and the enterohepatic circulation of methylmercury may be of importance in the renal uptake of methylmercury in rats, since ligation [19] or cannulation [14] of the bile duct lead to a decrease in the renal mercury level in rats. In mice, however, the rate of methylmercury secretion into the bile is remarkably lower than that in rats [9], despite the fact that mouse kidney has a high affinity for methylmercury like rat kidney. A study using mice, in which the enterohepatic circulation of methylmercury is almost negligible, would probably provide more important information on the mechanism of renal accumulation of methylmercury in animals.

The purpose of the present study was, therefore, to determine the roles of hepatic and renal GSH, and the biliary secretion of GSH in the uptake of methylmercury by the kidney in mice, as a possible key mechanism in renal mercury uptake.

MATERIALS AND METHODS

Chemicals. $^{203}HgCl_2$ was obtained from New England Nuclear (Boston, MA). Methylcobalamine and L-Cys were purchased from Sigma Chemical Co. (St Louis, MO). *o*-Phthalaldehyde (OPT) was purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). Diethylmaleate (DEM), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 1,2-dichloro-4-nitrobenzene (DCNB) and methylmercuric chloride were obtained from Wako Pure Chemical Industries Ltd. (Osaka,

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Japan). GSH was obtained from Boehringer Mannheim GmbH (F.R.G.). L-(*S*,5*S*)-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin) was a gift from Upjohn Co. (Kalamazoo, MI). L-Cysteinylglycine (CysGly) was prepared through the enzymatic degradation of GSH with γ-glutamyl transpeptidase (γ-GTP), and purified by Sephadex G-15 chromatography. All other chemicals were of analytical grade and from commercial sources.

Preparation of ^{203}Hg*-labelled methylmercuric chloride.* ^{203}Hg-Labelled methylmercuric chloride ($CH_3{}^{203}HgCl$) was prepared by the method of Naganuma *et al.* [20]. To an HCl solution (pH 1–2, 1 ml) containing 0.1 mg of $^{203}HgCl_2$ (4.99 mCi/mg) was added a 5 molar equivalent of methylcobalamin (dissolved in 0.01 N HCl), and then the volume of the mixture was adjusted to 2 ml with 0.01 N HCl. The mixture was kept for 1 hr at room temperature in the dark to allow the formation of $CH_3{}^{203}HgCl$. The reaction mixture was then applied on a CM-Sephadex C-25 column (5 × 60 mm) equilibrated with 20 mM Tris–HCl buffer (pH 7.6). The column was eluted with the same buffer. The fractions containing $CH_3{}^{203}HgCl$ eluted from the column were pooled and then applied on a different size of CM-Sephadex C-25 column (20 × 25 mm) equilibrated with 20 mM Tris–HCl buffer (pH 7.6). After the application, the column was washed with 20 ml of the same buffer and then eluted with 0.9% NaCl solution. The peak fraction containing $CH_3{}^{203}HgCl$ was used in the present experiments. The lack of contamination by methylcobalamine and aquocobalamine, which are the reaction products, in the finally obtained $CH_3{}^{203}HgCl$ solution was confirmed spectrophotometrically [20]. Inorganic mercury was completely retained by the first column [20, 21]. The content of [^{203}Hg]-inorganic mercury in the $CH_3{}^{203}HgCl$ solution was only 0.007%, as determined by the method of Omata *et al.* [22].

Tissue distribution. Male ICR mice (weighing about 22–25 g) were purchased from Charles River Japan, Inc.

$CH_3{}^{203}HgCl$ (12 μCi/5 μmol/kg) was injected intravenously (i.v.) into the mice. The specific activity of $CH_3{}^{203}HgCl$ (2.4 μCi/μmol) was adjusted with non-radioactive methylmercuric chloride. DEM (3.1 mmol/kg) or DCNB (2.5 mmol/kg) with olive oil was injected intraperitoneally (i.p.) 30 min before the $CH_3{}^{203}HgCl$ administration. The ^{203}Hg contents of the liver, kidney, plasma and erythrocytes were determined by counting with an Aloka Auto well gamma system (Aloka, Japan) at 5 or 30 min after administration of $CH_3{}^{203}HgCl$. The tissue distribution of ^{203}Hg was also determined in mice injected i.v. with $CH_3{}^{203}HgCl$ (12 μCi/5 μmol/kg) premixed with GSH (5 μmol/kg).

Cannulation of bile duct. Mice were anaesthetized with sodium pentbarbital (70 mg/kg), and a constant body temperature was maintained with warming lamps. The bile ducts were cannulated with polyethylene tubing (i.d., 0.2 mm; o.d., 0.5 mm), and then $CH_3{}^{203}HgCl$ (12 μCi/5 μmol/kg) was injected i.v. Bile samples were collected for 30 min or 3 hr after the $CH_3{}^{203}HgCl$ injection. At the end of the bile collection period, blood and tissues were removed for ^{203}Hg determination.

Acivicin pretreatment. Acivicin (2.5 mmol/kg) was administered i.p. to mice 30 min prior to $CH_3{}^{203}HgCl$ (12 μCi/5 μmol/kg, i.v.) injection. Each mouse was kept in a metabolic cage (T-488 type; Tokiwa Co. Ltd., Japan), which enabled separate collection of urine and feces. Urine samples were collected during the 2 hr after the $CH_3{}^{203}HgCl$ injection, and the kidneys and other tissues were removed at the end of the urine collection period.

Preparation of enzymes. Partially purified γ-GTP, dipeptidase and aminopeptidase M were obtained from brush-border membranes of mouse kidney by a slight modification of the method of Kozak and Tate [23]. The brush-border membranes were isolated from a 3.3% kidney homogenate (in 2 mM Tris–HCl buffer (pH 7.6) containing 50 mM D-mannitol) by the calcium precipitation method described by Malathi *et al.* [24]. The membranes, suspended in 5 mM Tris–HCl buffer (pH 7.6) containing 50 mM NaCl and 10 mM 2-mercaptoethanol, were incubated with papain (1 mg/10 mg of membrane protein) at 37° for 3 hr. The solubilized proteins were separated from the membranes by centrifugation at 40,000 *g* for 20 min, and then precipitated by ammonium sulfate fractionation (45–90%). The ammonium sulfate fraction was dissolved in 25 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, and then chromatographed on a Sephadex G-150 column (2.6 × 90 cm). The activities of aminopeptidase M and γ-GTP in each fraction eluted from the column were measured. The fractions showing the highest aminopeptidase M and γ-GTP activities were used as the respective preparation. As the dipeptidase preparation, the fraction showing no aminopeptidase M activity that was eluted just before the foot of the γ-GTP peak from the Sephadex G-150 column was used [23].

Assaying of enzyme activities. γ-GTP activity was determined by the method of Tate and Meister [25] using L-γ-glutamyl-*p*-nitroaniline and glycylglycine (GlyGly) as a substrate and γ-glutamyl acceptor, respectively. Aminopeptidase M activity was measured by the method of Rankin *et al.* [26] using L-leucine-*p*-nitroanilide as a substrate.

Enzymatic reaction. $CH_3{}^{203}Hg$-GSH, $CH_3{}^{203}Hg$-CysGly and $CH_3{}^{203}Hg$-Cys were obtained by mixing an equimolar amount of $CH_3{}^{203}HgCl$ with GSH, CysGly or Cys in 100 mM Tris–HCl buffer (pH 7.6). The reaction of $CH_3{}^{203}Hg$-GSH (2.0 mM) and γ-GTP was performed in a reaction mixture consisting of 0.05 M Tris–HCl buffer (pH 8.0), 75 mM NaCl and 20 mM GlyGly at 37° for 30 min. The reaction of $CH_3{}^{203}Hg$-CysGly (0.5 mM) and aminopeptidase M or dipeptidase was carried out in 100 mM Tris–HCl buffer (pH 7.6) at 37° for 30 min. After the reaction, each reaction mixture was applied on a Sephadex G-15 column (19 × 450 mm). The column was eluted with 100 mM Tris–HCl (pH 7.6), and the eluate was collected in fractions of 2 ml each. Then, the elution position of ^{203}Hg was compared with those of $CH_3{}^{203}Hg$-Cys, $CH_3{}^{203}Hg$-CysGly and $CH_3{}^{203}Hg$-GSH.

Determination of GSH and non-protein thiols. The concentrations of GSH and non-protein thiols (NPSH) were determined by the modifications of the methods of Cohn and Lyle [27] and Beutler *et*

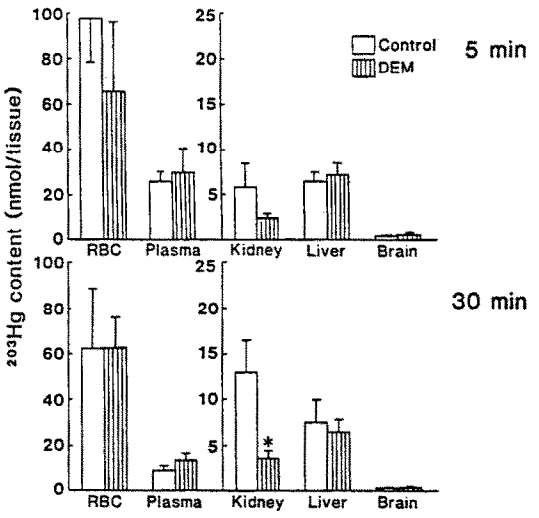

Fig. 1. Effect of pretreatment with DEM (3.1 mmol/kg) 30 min prior to i.v. injection of $CH_3{}^{203}HgCl$ (5 μmol/kg) on the tissue distribution of ^{203}Hg at 5 min or 30 min after $CH_3{}^{203}HgCl$ injection. * Significantly different ($P < 0.005$) from the control. RBC, red blood cells.

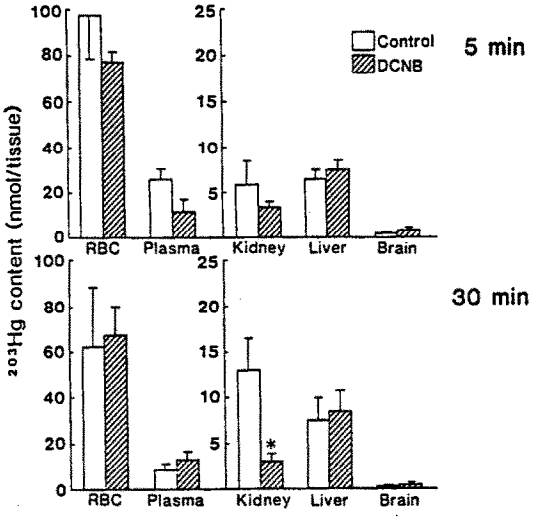

Fig. 2. Effect of pretreatment with DCNB (2.5 mmol/kg) 30 min prior to i.v. injection of $CH_3{}^{203}HgCl$ (5 μmol/kg) on the tissue distribution of ^{203}Hg at 5 min or 30 min after $CH_3{}^{203}HgCl$ injection. * Significantly different ($P < 0.005$) from the control.

al. [28], respectively. The tissues (50 mg of the liver and 200 mg of kidney) and urine (200 μl) were homogenized in 3 ml of 10 mM EDTA containing 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 800 *g* for 10 min to remove proteins, and then the supernatant was divided into two portions (20 μl and 1.0 ml) for determination of GSH and NPSH, respectively. Five milliliters of 0.4 M Tris–HCl buffer (pH 8.9) containing 10 mM EDTA and 250 μl of OPT in methanol (1 mg/ml) were added to a 20 μl sample of the supernatant. After about 5 min reaction the GSH content was determined fluorometrically (excitation, 342 nm; emission, 428 nm). For NPSH determination, 1.0 ml of the supernatant obtained as above was mixed with 4.0 ml of 0.4 M Tris–HCl buffer (pH 8.9) containing 10 mM EDTA and 0.1 ml of 10 mM DTNB. Following 10 min reaction the NPSH content was determined spectrophotometrically (412 nm) using GSH as a standard.

Statistics. Data were statistically analyzed by Student's *t*-test.

RESULTS

Role of GSH in mercury transport into kidney

Pretreatment of mice with DEM 30 min prior to injection of $CH_3{}^{203}HgCl$ significantly decreased the renal accumulation of ^{203}Hg (Fig. 1), as reported earlier in the case of rats [4, 14]. Administration of DEM 2 hr after $CH_3{}^{203}HgCl$ had no effect on the tissue (kidney, liver, plasma and erythrocytes) distribution of mercury (data not shown). DEM has the ability to decrease the NPSH concentration in both the liver and kidney, as reported by Richardson and Murphy [4] (see Table 1). After several series of experiments in a search for a reagent that specifically depresses hepatic GSH, DCNB was selected among the various GSH-binding substances tested. As shown in Table 1, DCNB administration significantly decreased the GSH concentration in the liver without affecting the renal GSH and NPSH levels at least during the 1 hr after the treatment. Figure 2 shows the effect of pretreatment with DCNB on the distribution of methylmercury. The renal accumulation of

Table 1. Concentrations of GSH and NPSH in liver and kidney of mice administered DEM or DCNB

		NPSH (μmol/g tissue)			GSH (μmol/g tissue)		
		Time after injection (hr)			Time after injection (hr)		
		0	0.5	1	0	0.5	1
Liver	DEM	6.42 ± 1.46	1.27 ± 0.41	1.25 ± 0.42	6.17 ± 1.98	0.54 ± 0.29	1.45 ± 0.30
	DCNB		2.47 ± 0.64	2.54 ± 1.00		1.42 ± 0.52	1.53 ± 0.84
Kidney	DEM	1.79 ± 0.28	0.51 ± 0.13	0.52 ± 0.20	0.29 ± 0.03	0.33 ± 0.14	0.25 ± 0.50
	DCNB		1.56 ± 0.11	1.74 ± 0.19		0.24 ± 0.04	0.31 ± 0.07

DEM (3.1 mmol/kg) or DCNB (2.5 mmol/kg) was administered i.p. Values are means ± SD (4 mice).

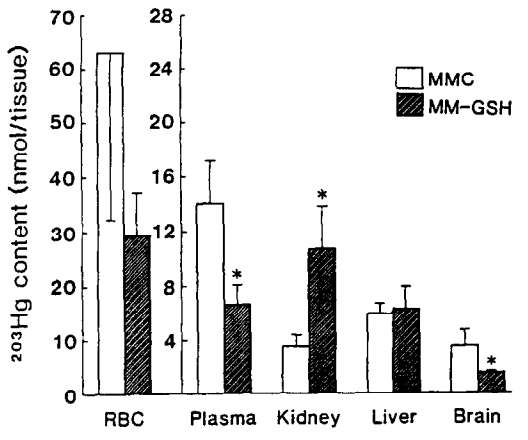

Fig. 3. ^{203}Hg incorporation into tissues 5 min after i.v. administration of $CH_3{}^{203}HgCl$ (MMC, 5 μmol/kg) alone or premixed with GSH (MM-GSH, 5 μmol/kg). * Significantly different ($P < 0.005$) from the case of MMC.

methylmercury decreased with the DCNB-induced depression of hepatic GSH, similarly to the case of DEM treatment. This suggests that hepatic GSH rather than renal GSH plays an important role in the methylmercury accumulation in the kidney.

Fate of methylmercury-GSH in plasma and role of biliary secretion in methylmercury metabolism

Figure 3 shows the distribution of mercury after i.v. injection of methylmercury–GSH. In comparison with the case of mice administered methylmercuric chloride, significantly higher renal accumulation of mercury was observed in mice receiving methylmercury–GSH. On the other hand, no effect of bile duct cannulation on mercury accumulation in the kidney or other tissues subjected to mercury determination (liver, lung, heart, spleen, testis, brain, plasma and erythrocytes) was observed at either 30 min or 3 hr after administration of methylmercuric chloride (data not shown). In these cannulated mice, the amount of mercury secreted into the bile during the period of 30 min–3 hr after methylmercury administration was only 0.62 nmol (mean for 5 mice), but the mean value for the renal mercury content increased by 4.1 nmol during this period.

Role of renal γ-GTP in mercury uptake by kidney

Table 2 shows the effects of pretreatment of mice with acivicin, a potent and irreversible inhibitor of γ-GTP [29, 30], on the renal accumulation and urinary excretion of methylmercury. In this study, 99.5% of the renal γ-GTP activity was found to be inhibited at 30 min after administration of the inhibitor (2.5 mmol/kg). Significant depression of mercury uptake by the kidney was observed 2 hr after methylmercury administration in acivicin-pretreated mice. Urinary excretion of mercury and GSH in these mice during the 2 hr after methylmercury administration was about 300-fold and 17-fold higher than those in the untreated mice, respectively.

In the *in vitro* reaction, it was confirmed that methylmercury–GSH was degraded into methylmercury–CysGly by partially purified γ-GTP obtained from mouse kidneys, and this product was then converted to methylmercury-Cys by either aminopeptidase M or dipeptidase (Fig. 4).

DISCUSSION

Methylmercury can easily bind to GSH and other thiol compounds without the involvement of GSH-*S*-transferase [6, 10, 17]. The binding of methylmercury with GSH is reversible and proteins can deprive the methylmercury–GSH complex of methylmercury [9, 11]. Although methylmercury–GSH has been found in several GSH-rich tissues [5–11], most of the plasma methylmercury was bound to proteins and methylmercury–GSH has hardly been detected in plasma, which contains only a trace amount of free GSH [31, 32]. However, the present study using DCNB, a specific depletor of GSH in the liver, demonstrated that hepatic GSH plays an important role in the renal accumulation of methylmercury in mice (Fig. 2). It is well known that hepatic GSH, a major source of plasma GSH, and its *S*-conjugates with electrophilic xenobiotics are released from the liver into both the plasma and bile [33], and these compounds having been introduced into the plasma are preferentially extracted by the kidney [33–35]. Using an *in vivo* blood-dialysis technique, Takahashi *et al.* [36] recently revealed that a trace amount of methylmercury–GSH actually exists in rabbit plasma. Thus, there is a possibility that methylmercury is translocated to the kidney as a complex with GSH.

In rats, a considerable amount of methylmercury

Table 2. Effects of pretreatment with acivicin on the renal accumulation and urinary excretion of methylmercury in mice

Acivicin (mmol/kg)	^{203}Hg content: Kidneys (nmol/tissue)	^{203}Hg content: Urine (nmol/2 hr)	GSH excreted in urine (nmol/2 hr)
0	22.5 ± 4.1	0.03 ± 0.01	120.5 ± 9.5
2.5	7.1 ± 1.0	8.74 ± 2.58	2056.0 ± 522.8

Mice were pretreated with acivicin (2.5 mmol/kg; i.p.) or saline 30 min prior to $CH_3{}^{203}HgCl$ injection (5 μmol/kg; i.v.). The mercury content in the kidneys, and the mercury and GSH contents in pooled urine were determined 2 hr after methylmercury injection. Values are means ± SD (4 mice).

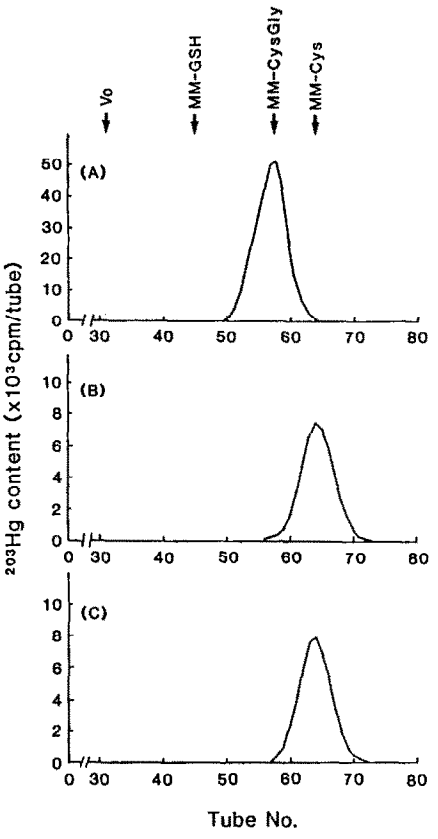

Fig. 4. Sephadex G-15 chromatography of reaction products obtained on treatment of $CH_3{}^{203}Hg$–GSH with γ-GTP (A), $CH_3{}^{203}Hg$–CysGly with aminopeptidase M (B) and $CH_3{}^{203}Hg$–CysGly with dipeptidase (C), respectively. MM–GSH, methylmercury–GSH; MM–CysGly, methylmercury-CysGly; MM–Cys, methylmercury–Cys.

is excreted into the bile, and more than 90% of the methylmercury is reabsorbed from the gut [37, 38]. A methylmercury–GSH complex has been identified in liver [5], and constitutes the main form of methylmercury in bile [7, 9]. Alexander and Aaseth [14] reported that the biliary secretion and reabsorption of methylmercury in rats may be of importance in the renal uptake of methylmercury, since the accumulation of methylmercury in the kidney decreases with cannulation and ligation of the bile duct. As mentioned under Introduction, however, the amount and rate of biliary secretion of methylmercury in ICR mice employed in the present study were much lower than those in rats [9]. In fact, although significantly higher accumulation of methylmercury in the kidney was observed in mice receiving methylmercury–GSH intravenously in comparison with methylmercuric chloride-injected mice (Fig. 3), as in the case of rats [12, 14], bile-duct cannulation hardly affected the renal uptake of methylmercury in mice. These results suggest that the enterohepatic circulation of methylmercury is not so important in renal accumulation of methylmercury, at least in mice.

In the kidney, extracellular GSH and its *S*-conjugates are hydrolyzed through the actions of γ-GTP and dipeptidase(s) into their constituent amino acids, which are rapidly taken up by kidney cells [33]. Pretreatment of mice with acivicin, a potent inhibitor of γ-GTP, significantly depressed the renal accumulation of methylmercury and increased the urinary excretion of methylmercury and GSH, as shown in Table 2. This indicates that the complexation of methylmercury with GSH and the hydrolysis of the GSH by γ-GTP are important steps in the renal accumulation of methylmercury in mice, supporting the above assumption that methylmercury is transported to the kidney as a complex with GSH. Moreover, the 300-fold increase in urinary excretion of methylmercury due to the inhibition of renal γ-GTP (Table 2) also suggests that the form of methylmercury filtered through the glomerulus is almost completely methylmercury–GSH, and more than 99.5% of the methylmercury is reabsorbed by the proximal tubules.

Hirata and Takahashi [8] reported that a crude preparation of γ-GTP containing dipeptidase(s) was able to decompose methylmercury–GSH to methylmercury–CysGly and -Cys. Aminopeptidase M has been found to catalyze the hydrolysis of both CysGly and *S*-blocked CysGly, such as cystinyl-bis-Gly and *S*-methyl-CysGly [23]. In contrast, dipeptidase can hydrolyze *S*-blocked CysGly but not Cys-Gly, and its activity is inhibited by CysGly [23]. The present study showed that methylmercury–GSH could be hydrolyzed to methylmercury–CysGly by γ-GTP, and that both aminopeptidase M and dipeptidase are capable of hydrolyzing methylmercury–CysGly to methylmercury–Cys (Fig. 4). This suggests that dipeptidase can recognize methylmercury–CysGly as an *S*-blocked CysGly and efficiently hydrolyze it to methylmercury–Cys, although the methylmercury of the methylmercury–GSH complex may be redistributed to other thiol-containing peptides, such as CysGly and Cys, which are possibly derived from GSH through enzymatic cleavage. Since the renal activity of dipeptidase is several hundred-fold greater than that of aminopeptidase M as to the hydrolysis of *S*-blocked CysGly [23], methylmercury–CysGly formed through the action of γ-GTP from methylmercury–GSH may be hydrolyzed to methylmercury–Cys mainly by dipeptidase in the kidney. The final product, methylmercury–Cys, may be taken up by kidney cells as in the case of cysteine *S*-conjugates with electrophilic xenobiotics [39].

Although some preliminary reports suggested the role of γ-GTP in the renal uptake and urinary excretion of methylmercury [40, 41], very little is actually known about the mechanism of methylmercury accumulation in the kidney. The present study demonstrated that methylmercury is translocated into the kidney as a complex with GSH, and then incorporated into renal cells as methylmercury–Cys after degradation of the GSH moiety by γ-GTP and dipeptidase. Both the hepatic GSH (as a source of extracellular GSH) and renal γ-GTP appear to be important determinants for the renal accumulation of methylmercury. The present findings provide a new aspect of the role of GSH in the transport and metabolism of metals which can reversibly bind to GSH.

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