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ORGAN DISTRIBUTION AND CELLULAR UPTAKE OF METHYL MERCURY IN THE RAT AS INFLUENCED BY THE INTRA- AND EXTRACELLULAR GLUTATHIONE CONCENTRATION

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Abstract—Intravenous administration of CH_3HgCl (4 μmole/kg) premixed with glutathione or cysteine (8 μmole/kg) to female rats caused a rapid uptake of mercury in the kidney and a depressed content in the liver and blood as compared to CH_3HgCl given alone. GSH depletion in the tissues, produced by injection of diethylmaleate, DEM (3.9 mmole/kg) did not influence the kidney uptake of mercury from administered CH_3Hg^+-GSH, whereas the uptake of injected CH_3HgCl was depressed. Both GSH and cysteine (8 μmole/kg) promoted the biliary excretion of methyl mercury. In suspensions of rat erythrocytes and isolated hepatocytes, additions of GSH reduced the cellular uptake of CH_3Hg^+ from the medium, whereas this was increased in the hepatocytes by adding cysteine or methionine. Cysteine addition slightly reduced the uptake of CH_3Hg^+ in the erythrocytes. GSH-depletion as obtained by DEM pretreatment of the cells, reduced the CH_3Hg^+ uptake into hepatocytes by 40%, in contrast to only a negligible effect on the erythrocytes. Our results support previous reports that a physiological CH_3Hg^+-GSH-complexation takes place intracellularly, at least in liver cells. Our results are furthermore consistent with the assumption that biliary excreted CH_3Hg^+-GSH, which can be reabsorbed, only to a limited extent is taken up by the liver, whereas this GSH-complexation and reabsorption is of importance for the CH_3Hg^+-uptake in the kidneys.

Methyl mercury is rapidly excreted via the bile [1] bound to glutathione (GSH) in the rat [2, 3], but more than 70% of the metal compound is reabsorbed from the gut [1]. Previous studies indicate that biliary excretion of methyl mercury is of importance for the kidney uptake of methyl mercury, since both ligation [4] and cannulation [5] of the bile duct lead to a decreased kidney content of mercury. Reabsorbed methyl mercury from the gut is only to a small extent taken up by the liver and reexcreted into the bile [6]. The intracellular level of GSH in the liver plays a role for the biliary excretion of methyl mercury [3]. Depletion of GSH from rat tissues by administration of GSH-binding substances influences the organ uptake of methyl mercury [7] and inhibits the biliary excretion of methyl mercury [3].

By using different biological model systems, viz. rats, isolated rat hepatocytes, rat erythrocytes and *in vitro* binding studies in rat plasma, we have further investigated the role of extra- and intracellular presence of GSH for the organ distribution, and the biliary excretion of methyl mercury.

MATERIALS AND METHODS

Chemicals

Methyl mercuric chloride labelled with ^{203}Hg was supplied by New England Nuclear, Boston, MA. L-Cysteine and glutathione were supplied by Sigma Chemical Co., St. Louis, MO. All the other chemicals used were commercially obtained and of high purity.

Animal studies

Female Wistar rats, weighing about 180–250 g, of our own breed (Institute of Occupational Health) and fed a commercial pellet diet, were used in the study.

Organ distribution. Either $CH_3{}^{203}HgCl$ (4 μmole/kg) alone, or $CH_3{}^{203}HgCl$ premixed with L-cysteine or glutathione (8 μmole/kg) was injected intravenously (i.v.) into the rats. In some experiments diethylmaleate (DEM) (3.9 mmole/kg) was injected intraperitoneally (i.p.) 30 min prior to mercury. The rats were killed after appropriate time intervals by blood collection from the abdominal aorta, and the organs were removed for mercury and GSH determination.

Biliary excretion. The bile duct was cannulated during barbiturate anaesthesia (pentobarbitone sodium, mebumalum NFN). A tracheal tube was inserted to ensure free airways during the collection period, and the body temperature was kept constant, using a warming lamp. A single i.v. injection of either 4 μmole $CH_3{}^{203}HgCl$/kg alone, or premixed with L-cysteine or glutathione (8 μmole/kg) was administered shortly after finishing the surgical procedures, when a proper bile flow was observed (0.5–1.0 ml/hr). The bile was collected in preweighed vials and the content of mercury was determined.

Studies using erythrocyte suspensions

Freshly drawn blood from female Wistar rats,

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mixed with citrate and glucose, was centrifuged, and plasma, white blood cells and platelets were discarded. The red blood cells were washed 3 times in saline buffered to pH 7.4 with 5 mM Tris, containing 1 mM glucose and bovine albumin 50 mg/mg. The erythrocytes were then suspended in 9 volumes of this same buffer, and mixed with methyl mercuric chloride (4 μmole/l) as previously described [8]. The thiols to be studied were added in a small volume, simultaneously with the addition of mercury. Diethylmaleate (10 μl) was added 15 min prior to methyl mercury. During the whole incubation period (240 min), the vials were slowly rotated and kept at room temperature. Aliquots of the red blood mixture were withdrawn after various intervals, and centrifuged. Radiomercury in the blood cell suspension before centrifugation (here set to 100%) and radiomercury in the supernatant were determined in a Packard autogamma spectrometer.

Binding studies in rat plasma

Rat plasma was prepared from freshly drawn heparinized blood. One millilitre of plasma was mixed with 1.5 nmole $CH_3{}^{203}HgCl$ with or without 0.1 mmole GSH 10 min prior to fractionation of a Sephadex G-25 coarse column (1.5 × 30 cm) eluted with 0.1 N Tris–HCl (pH = 7.85) in fractions of 0.5 ml.

Studies using hepatocyte suspensions

Isolated hepatocytes were prepared from female Wistar rats, weighing 200–250 g, which had free access to food and water. The isolation procedure was basically the same as described previously by Seglen [9, 10] and others [11]. The buffers [10] used in this procedure were pre-oxygenated and oxygenated continuously when in contact with liver cells. The animal livers were initially perfused *in situ* at a flow rate of 35 ml/min for 7 min with calcium-free Hank's solution [10]. The liver was excised and the perfusion continued for 15 min with a modified Hank's solution containing 0.05% collagenase (Sigma Chemical Co., C-0130) and 4.8 mM Ca^{2+}. After transfer to a petri dish, the liver capsule was gently disrupted with scissors and forceps and the cells dispersed by gentle agitation by hand in 50 ml of cold balanced salt solution with Hepes [10] and 5 mM glucose and 5 mg/ml bovine albumin. Connective tissue and cell clumps were removed by filtration through gauze. The cells were washed 3 times in the buffer and each time centrifuged for 30 sec at 50 *g*. Subsequently, the cells were resuspended in the above mentioned buffer containing Ca^{2+} and glucose, filtered through a 100 μm mesh gauze, and diluted to a suspension of 0.8–1.2 × 10^6 cells/ml for use in studies of CH_3Hg^+-binding. The viability of the cells was routinely assessed by the trypan blue exclusion test (>95% viability accepted). Glutathione measurements were performed before and after the cell studies with mercury. Mixtures of radiolabelled (^{203}Hg) methyl mercuric chloride (4 μM final concentration) and the thiol to be tested were added to the isolated cells. The cell suspensions also contained bovine albumin (5 mg/ml). The suspensions were slowly rotated for the whole incubation period (240 min). Aliquots of the suspensions were withdrawn at various intervals. Radioactivity was determined in the whole suspension aliquots, and in the supernatant after centrifugation at 2500 *g*. The measurements were done in a Packard autogamma scintillation spectrometer. Mercury bound to the hepatocytes could then be calculated in per cent of total amount of mercury added to the suspensions.

Determination of GSH

Rat hepatocytes. Cellular GSH was measured by the following procedure. 2.5 ml of cell suspension (1 × 10^6 cells/ml) was centrifuged for 5 min at 50 *g*. The pellet was resuspended in 0.9% NaCl to 0.2 ml and 50 μl Triton X-100 120 was added for cell lysis, whereafter 100μl 0.02 M EDTA was added. Fifty μl ice cold 4.8 M $HClO_4$ was added to the cooled sample for protein precipitation followed by centrifugation. In 300 μl of the supernatant, the surplus of $HClO_4$ was precipitated with 16μl 2.5 M KOH and removed by centrifuging the sample. The latter supernatant (240 μl) was mixed 340 μl 0.1 M phosphate buffer and 50 μl (1 unit) glutathione (GSSG)-reductase and 100 μl 2 mM NADPH. The sample was mixed and allowed to stand for 15 min at room temperature before 2.1 ml ethanol was added for removal of the reductase. The pH was adjusted to 8.0 in the supernatant by 120 μl Tris 0.5 M/EDTA 0.02 M before 100 μl 2 mM DTNB (Ellman's reagent) [12] was added. The sample could after 10 min of incubation be read at 412 nm. Standard curves were made by adding a known amount of GSH and/or GSSG in 0.02 M EDTA after treatment of the sample with Triton. All solutions used in the GSH assay were pregassed with N_2. GSH determination was not influenced by the presence of 50 μM methyl mercury.

Red blood cells and rat organs. GSH was determined essentially as described by Tietze [13]. That is, a sample of 200 μl red blood cells was haemolyzed in 2 ml water by freezing and thawing. The liver and kidney sample (approx 0.2–0.25 g) was homogenized in 2 ml water. The proteins in the erythrocyte lysate and organ samples were precipitated by adding 0.8 ml 2 M TCA followed by centrifugation. The supernatant was diluted 1:10 with 0.1 M phosphate buffer pH 6.0 and 200 μl of the samples was mixed with 2 ml of 0.1 M phosphate buffered 0.6 mM DTNB, pH 7.5 with 0.01 M EDTA, and 100 μl GSSG-reductase (25 U/ml). The reaction was started by adding 50 μl 10 mM NADPH and the reaction rate was read at 412 nm. Standard curves were made by adding known amounts of GSH to the homogenates.

Mercury determination

Mercury determination in organs and bile was done in a γ-scintillation well counter (Packard) by comparing it with a standard sample of the injection solution with known specific activity.

Statistical treatment

Differences between groups of treated and control samples were evaluated by the Wilcoxon two-sample, two-tailed test and considered significant when $P < 0.05$.

Incubation experiments, using hepatocytes and red blood cells, were performed at least in triplicates and typical results are shown in the figures.

RESULTS

Organ distribution

Intravenous administration of cysteine or GSH (8 μmole/kg) together with methyl mercury (4 μmole/kg) caused a distinct change in the organ distribution of mercury. The content in blood and liver was depressed after 1 hr, whereas a significant increase in the kidney content of mercury was seen in the animals given the methyl mercury complexes (Fig. 1). The difference in the renal content of mercury was even more prominent 30 min after injection, the treated animals reaching the same kidney level as that at 1 and 4 hr (Fig. 2). Pretreatment with DEM (3.9 mmole/kg) reduced the GSH content in the liver, kidneys and blood cells from 6.6, 1.6 and 1.54 μmole/g, respectively, to below 10% of the pretreatment value. The kidney content of mercury after methyl mercury injection was reduced in DEM pretreated rats. However, the renal level of mercury after combined treatment with GSH was not affected by intracellular GSH depletion (Fig. 2). After 4 hr the difference in the kidney content between controls and treated was only slight, but in the same direction as earlier (Fig. 1). In rats with cannulated bile duct, the treated animals had still a content of mercury in the kidney significantly higher than that of the controls (not shown). The liver content of mercury was depressed in the rats given the methyl mercuric mercaptides at 1 hr, whereas there was no difference at 4 hr (Fig. 1). The mercury content in blood was depressed in the GSH treated animals (Fig. 1).

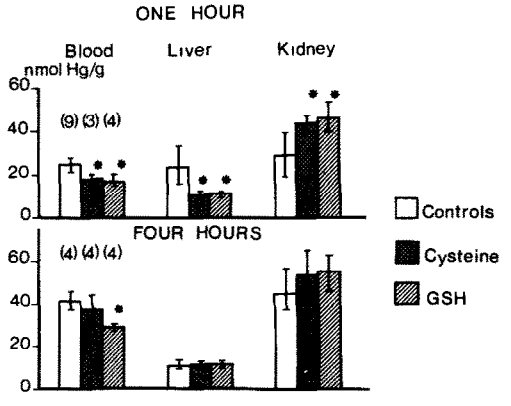

Fig. 1. The effect of glutathione (8 μmole/kg) or L-cysteine (8 μmole/kg) given i.v. premixed with methyl mercuric chloride (4 μmole/kg) on the organ distribution of mercury. Upper panel shows values for 1 hr after injection and lower panel shows values for 4 hr after injection. Each value and vertical bar represents the mean mercury concentration and experimental range. The number of rats is given in parentheses. * Represents values significantly different ($P < 0.05$) from the control values.

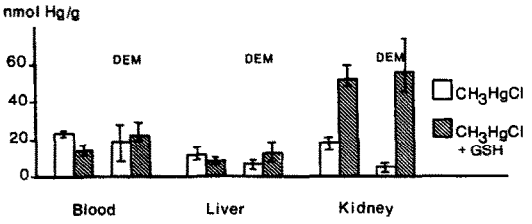

Fig. 2. The effect of pretreatment with diethylmaleate (DEM) (3.9 μmole/kg) 30 min prior to i.v. injection of CH_3HgCl (4 μmole/kg) alone or premixed with glutathione (GSH) (8 μmole/kg) on the organ distribution of mercury 30 min after methyl mercury injection. Each value and vertical bar represents the mean mercury concentration and experimental range of groups of 4 rats.

The organ to plasma ratios of the GSH treated animals at 30 min were depressed in blood and liver, whereas it was increased in the kidney (Table 1).

Rats with cannulated bile duct given the methyl mercury and GSH (4 and 8 μmole/kg, respectively) in duodenum, showed a slight increase in the kidney content of mercury and depressed content in the liver: 14.6 (range: 13.7–15.3) nmoles Hg/kg vs 17.0 (range: 15.8–19.1) nmoles Hg/g in the controls 4 hr after treatment. No such difference was seen for cysteine treated rats.

Biliary excretion

When cysteine (8 μmole/kg) or GSH (8 μmole/kg) was given premixed with methyl mercury (4 μmole/kg), the biliary excretion of methyl mercury was significantly promoted (Fig. 3). The increase was most prominent during the first hour and then there was a decline to the curves of the rats given only methyl mercury. The bile (bile sample 15–30 min) to plasma (30 min after injection) ratio was 6.9, 9.2 and 11.9 for controls, cysteine treated and GSH treated, respectively. The bile to liver ratio in the same interval was 0.17, 0.28 and 0.29 for controls, cysteine treated and GSH treated, respectively.

Red blood cells and blood plasma

In the presence of GSH, the uptake of methyl

Table 1. Organ to plasma ratio of methyl mercury

	Blood	Liver	Kidney
Control*	13.2 (11.2–15.9)	5.9 (5.2–6.7)	9.6 (9.3–9.8)
GSH†	5.5‡ (5.9–6.9)	3.4‡ (3.2–3.7)	21.6‡ (20.4–22.7)

* Rats were given 4 μmole CH_3HgCl/kg i.v. and killed after 30 min. Mean and experimental range of 4 rats in each group are given.

† GSH/kg (8 μmole) was mixed with CH_3HgCl prior to i.v. injection.

‡ Significantly different from controls, $P < 0.05$.

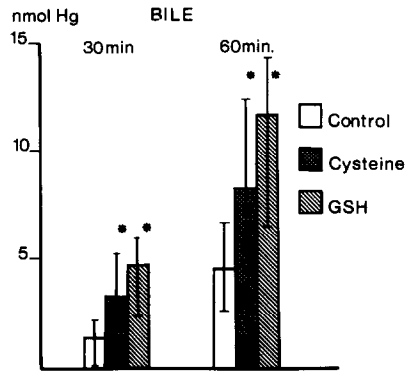

Fig. 3. The effect of glutathione (8 μmole/kg) or cysteine (8 μmole/kg) given i.v. premixed with methyl mercuric chloride (4 μmole/kg) on the biliary excretion of mercury. Each value and vertical bar represents the mean and range of total mercury excreted into the bile during the first 30 and 60 min, respectively. There were seven control rats, five glutathione treated rats and four cysteine treated rats. * Represents values significantly different ($P < 0.05$) from the control values.

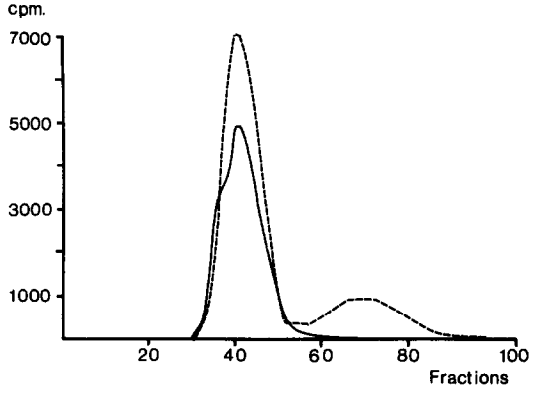

Fig. 5. Chromatographic separation of methyl mercury in rat plasma. One millilitre of rat plasma was mixed with 1.5 nmole $CH_3{}^{203}HgCl$ with or without 0.1 μmole glutathione prior to fractionation on a Sephadex G-25 coarse column (1.5 × 30 cm) eluted with 0.1 Tris–HCl, pH = 7.85 in fractions of 0.5 ml. $CH_3{}^{203}HgCl$ (———), $CH_3{}^{203}HgCl$ + GSH (- - - -).

mercury in the erythrocytes was depressed (Fig. 4). L-Cysteine caused a slight depression of mercury uptake in the red blood cells to 87% mercury binding, in keeping with their low capacity of cysteine uptake [14]. Pretreatment of the erythrocytes with DEM (10 μl) reduced the GSH content from 1.6 mM in untreated cells to <0.2 mM. GSH-depleted red blood cells accumulated slightly less CH_3Hg^+ than the untreated cells (Fig. 4); accordingly, it has been reported that methyl mercury is almost exclusively bound to proteins, and not GSH, within rat erythrocytes [15]. Methyl mercury, added *in vitro*, is protein bound in plasma. When GSH is present CH_3Hg^+ is also bound to a low molecular weight fraction (Fig. 5).

Hepatocytes in suspension

During the incubation time, the GSH content was stable, varying from 40 to 60 nmole GSH/10^6 cells, which accords well with the findings of others [11]. When the cells were treated with diethylmaleate (DEM) (3 μmole/ml), a rapid decrease of the GSH level below 5 nmole GSH/10^6 cells was seen and the GSH content did not increase significantly during the incubation time. Cells pretreated with DEM accumulated slower and less methyl mercury as compared to the control cells (Fig. 6). Small amounts of cysteine (8 μm) and glutathione (8 μM) mixed with methyl mercury (4 μM) prior to the addition to the hepatocyte suspension, had no significant effect on the uptake of methyl mercury into the hepatocytes (not shown). Larger amounts of GSH (0.6 mM)

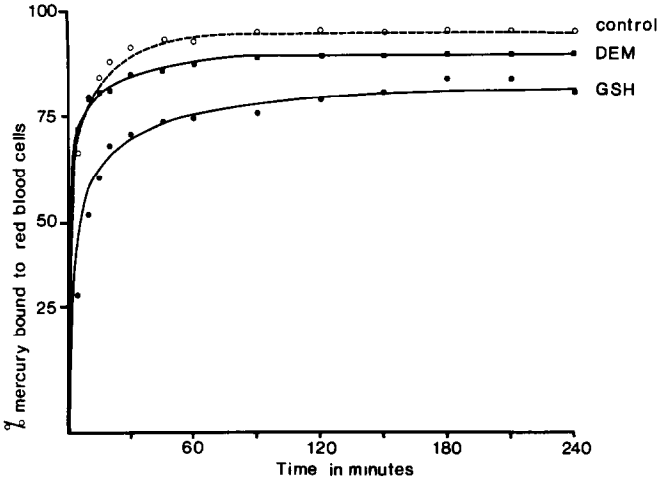

Fig. 4. The effect of glutathione (GSH) (1 mM) and GSH-depletion on the uptake of methyl mercury (5 μM) into rat erythrocytes incubated as described in Materials and Methods. Five μmole $CH_3{}^{203}HgCl$/l was added to the erythrocyte suspension premixed with thiol to be tested. Diethylmaleate (10 μl) was added 15 min prior to methyl mercury.

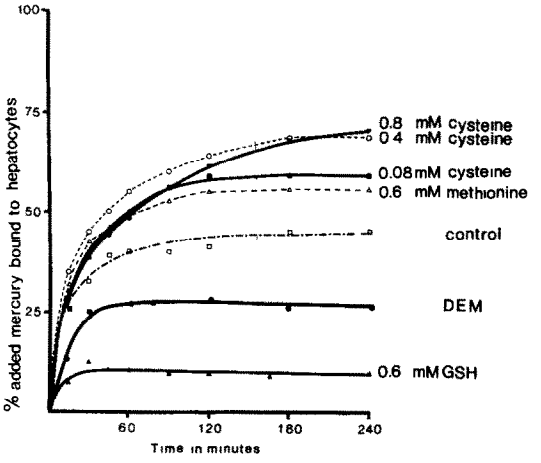

Fig. 6. The influence of glutathione (GSH), methionine, cysteine and GSH-depletion on the uptake of methyl mercury into rat hepatocytes. Four μmole $CH_3{}^{203}HgCl$/l was added to the hepatocyte suspension premixed with the various substances to be tested. Diethylmaleate (3 μmole/ml) was added 30 min prior to medium shift and addition of methyl mercury.

reduced the uptake, whereas cysteine and methionine increased the uptake (Fig. 6). The hepatocytes accumulated methyl mercury slower when larger doses of cysteine (0.8 mM) were used. Glutathione precursors, such as glycine and glutamate, had no effect alone and did not increase the effect of cysteine (not shown). Histidine had no effect.

DISCUSSION

When CH_3Hg^+-GSH is introduced into blood plasma, it is rapidly extracted by the kidneys (Fig. 1), as is GSH alone [16]. A slight increased kidney content was also obtained when giving CH_3Hg^+-GSH into duodenum, but not by such CH_3Hg^+-cysteine administration. Tripeptides in the gut, e.g. GSH, can be absorbed without catabolization [17], and our results accord with the suggestion that a part of the CH_3Hg^+-GSH complex from bile [3] is reabsorbed unchanged to the circulation, although a partial catabolism to CH_3Hg^+-cysteine may take place [18]. This is of physiological importance, because biliary excreted methyl mercury complexes, i.e. CH_3Hg^+-GSH formed in the liver, are previously suggested to reappear in the circulation as small amounts of low molecular weight methyl mercury [19]. CH_3Hg^+ introduced directly into rat plasma *in vitro* is bound solely to proteins (Fig. 3). In contrast to the high molecular weight CH_3Hg^+-protein complexes, the low molecular weight CH_3Hg^+-GSH complex (Fig. 3) is extracted rapidly into the kidneys across the glomerular membrane into primary urine, where the GSH moiety can be rapidly catabolized by the highly active γ-glutamyltranspeptidase on the luminal outside of the tubular cell membrane [16, 20, 21, 22]. It seems (Figs. 1 and 2) as if CH_3Hg^+ introduced from the gut, is thereby translocated to, and retained in, the kidney. The described translocation of CH_3Hg^+(-GSH) to the kidney is decreased by cannulation or ligation of the bile duct which interrupts the excretion/reabsorption of GSH-complexes [4, 5]. DEM also depressed the kidney content of mercury after CH_3HgCl administration (Fig. 2), and this may be due to interrupted formation/excretion of the CH_3Hg^+-GSH complex from the liver [3], or reduced GSH-levels in the kidneys. The renal uptake of mercury following direct intravenous CH_3Hg^+-GSH infusion was, however, not influenced by such reduced GSH-levels (Fig. 2). In contrast to the kidneys [16, 20], erythrocytes [23] and hepatocytes [20] do not take up GSH, which may explain the reduced uptake of CH_3Hg^+-GSH (as compared to CH_3HgCl) into these cells (Figs. 1, 4 and 6).

Following methyl mercury exposure, the physiological formation of the CH_3Hg^+-GSH complex is supposed to take place mainly intracellularly [24, 25] because the plasma concentration of GSH is only about 0.1% of the cellular GSH-levels [13, 16]. The liver cells (GSH-concentration approx. 7 μmole/g) [13, 26], which do not take up GSH or CH_3Hg^+ complex, have high GSH-synthesizing ability [26] and are apparently able to conjugate the CH_3Hg^+-GSH complex [24]. Liver cells seem to differ from other cells as they can also export large amounts of the CH_3Hg^+-GSH complex rapidly, into the bile [2, 3]. This reduces the liver content of CH_3Hg^+ rapidly, as compared to other organs (Fig. 1). In hepatocytes, DEM-induced GSH-depletion caused a 40% reduction of the CH_3Hg^+ uptake. A GSH-binding of about 25% of CH_3Hg^+ in the liver cytosol has previously been reported [24]. Cysteine addition increased the amount of methyl mercury binding by the hepatocytes, probably by increasing the intracellular level of both GSH and cysteine [27]. A similar effect was also seen by the GSH precursor methionine [27, 28]. Both cysteine and GSH increased the biliary excretion of methyl mercury as previously reported [29]. This effect of cysteine was probably caused by its ability to increase the synthesis of GSH [27] which carries CH_3Hg^+ into the bile [2, 3]. In order to produce the same effect, injected GSH has to be metabolized to cysteine, presumably in the kidneys [20], since the hepatocytes are unable to take up GSH [20].

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