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THE DEPENDENCE OF BILIARY METHYLMERCURY SECRETION ON LIVER GSH AND LIGANDIN

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Abstract—The biliary secretion of methylmercury was investigated in male rats which were given i.p. 400 μmoles/kg azathioprine or 96 μmoles/kg benziodarone 2 hr after the i.v. injection of 5 μmoles/kg MeHgCl. A group of rats were given 400 mg/kg *trans*-stilbene oxide (TSO) for 4 days before treatment with 10 μmoles/kg MeHgCl. A common link between these three compounds is their interference with ligandin. Azathioprine is a competitive inhibitor of glutathione *S*-transferase, benziodarone is covalently bound to ligandin and TSO is an inducer of liver ligandin. Although only azathioprine depletes liver GSH stores, both azathioprine and benziodarone inhibited the biliary secretion of methylmercury. As there is published proof that the reaction of $MeHg^+$ with GSH does not require enzymatic help, the inhibitory effect of azathioprine and benziodarone confirms the role of ligandin in the transport of methylmercury or its GSH complex. However, the biliary secretion of methylmercury was increased only slightly by TSO pretreatment, but when 2 hr after the injection of MeHgCl animals received 2 mmoles/kg GSH, secretion increased twice as much in TSO pretreated than in control rats. This indicates the dual dependance of biliary methylmercury secretion on liver GSH and ligandin.

Glutathione (GSH) occupies a key role in the biliary secretion of methylmercury ($MeHg^+$). Firstly the biliary secretion of this organomercurial is increased by the administration of cysteine or GSH [1]. Secondly GSH depletion in liver caused by the enzymatic conjugation of methyliodide [2] or acrylamide [3, 4] is associated with a decrease in the biliary secretion of $MeHg^+$ [5, 6]. Thirdly MeHg in bile is associated with GSH [7] or GSH degradation products [8–10] and therefore it is reasonable to suppose that it is secreted as a methylmercury–glutathione (MeHg–SG) complex.

Nevertheless the biliary secretion of $MeHg^+$ does not depend solely on the availability of GSH. After the injection of GSH or 3′-methyl-4-dimethylazobenzene (3′-MeDAB), both given in doses which produced approximately 2.0 μmoles/g increase in the hepatic concentration of non-protein thiol groups, GSH increased the biliary secretion of methylmercury more than 200% while 3′-MeDAB increased it only by 23% [1]. Bromosulphophthalein (BSP) and indocyanine green inhibited the biliary secretion of $MeHg^+$ [11], although only BSP is conjugated by glutathione *S*-transferase [12], and indocyanine green is a non-substrate ligand for ligandin [13]. Bilirubin, another ligand, did not exert an inhibitory effect [11], probably because it is released to the endoplasmic reticulum for glucuronidation [14].

The aim of the work presented here was to investigate further the biliary secretion of $MeHg^+$ in relation to GSH and ligandin or glutathione *S*-transferase activity. The compounds selected were azathioprine, benziodarone and *trans*-stilbene oxide (TSO). Azathioprine [15] in the liver undergoes decomposition catalysed by glutathione *S*-transferase resulting in glutathione depletion [16]. Benziodarone is a non-competitive inhibitor of glutathione *S*-transferase [17] and does not affect the liver concentration of GSH, but decreases the biliary secretion of BSP [18]. TSO is an inducer of glutathione transferases including ligandin [19], therefore it is expected that if ligandin is involved in the biliary secretion of $MeHg^+$, TSO will stimulate it.

MATERIALS AND METHODS

Male Porton–Wistar rats of 200–250 g body weight were anaesthetized with sodium pentobarbital (65 mg/kg, i.p.) and kept at a constant body temperature of 37° in an incubator throughout the bile collection. In experiments with azathioprine and benziodarone the bile duct and jugular vein were cannulated with PP-10 tubing and in the TSO experiments only the bile duct was cannulated. The experimental schedules were as follows.

(A) After cannulation methylmercury chloride (K & K, Plainview, N.Y.) labelled with $Me^{203}HgCl$ (Amersham International) was injected into the jugular cannula in a dose of 5 μmoles/ml saline/kg with a specific activity of 0.5 μCi/μmole. After a 2 hr bile collection, a freeze-dried pharmaceutical preparation of azathioprine (Wellcome), dissolved in distilled water, pH adjusted to 9.0 with HCl, was injected i.p. in a dose of 400 μmoles/5 ml/kg. Controls were given distilled water.

(B) The treatment schedule and MeHgCl dose were the same as in group A, but instead of azathioprine, animals were given i.p. benziodarone (S. A. Labaz) in a dose of 96 μmoles/8 ml arachis oil/kg; the corresponding controls received arachis oil only.

(C) Animals were given *trans*-stilbene oxide

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(Aldrich Chem. Co., Milwaukee, WI) i.p. for 4 days in daily doses of 400 mg/5 ml corn oil/kg or corn oil only. Twenty-four hours after the last dose, animals were cannulated and MeHgCl in a dose of 10 μmoles/1 ml saline/kg with a specific activity of 0.3 μCi/μmole was injected into the dorsal vein of the penis. After 2 hr one group of animals were given reduced glutathione (Sigma Chem. Co.) i.p. in a dose of 2 mmoles/5 ml saline/kg.

Bile was collected every 30 min for 5 hr and at the end of the tenth collection period the animals were killed by decapitation, blood was collected and liver removed. All the samples were weighed and assayed for ^{203}Hg content by counting in a well-shaped NaI crystal (well diameter, 8 cm; depth, 8 cm) linked to an automatic scintillation spectrometer. The counting efficiency was 43%. As 5 hr after MeHgCl administration about 99% of total ^{203}Hg in liver [20] and more than 95% in bile [21] remains in the organic form, concentrations of methylmercury in bile and liver samples were calculated from radioactivity without correcting for inorganic mercury. Changes in bile flow and mercury secretion caused by GSH, azathioprine or benziodarone are expressed in relation to mean values measured in the same rat in the last two collection periods before treatment. Non-protein thiol groups in liver samples were estimated by the method of Sedlak and Lindsay [22].

Statistical significances were calculated with non-directional Student *t*-test when one treatment group was compared with the control group and with the multicomparison procedure of Dunnett [23] when two treatment groups were compared with one control group. As variances were not always homogeneous at the P = 0.05 level, significance was tested at the P = 0.01 level. Another alternative was to reduce the degree of freedom according to the Satterthwaite's equation for two samples with non-homogeneous variances [24]. However, as the possibility of false positives increases with increasing numbers of comparisons the more conservative approach offered a more reliable test. The possibility of false positive was confirmed when data presented in Fig. 2 gave significant differences for biliary methylmercury secretion at the P = 0.05 level only in one (the seventh) collection period.

RESULTS

The biliary secretion of $MeHg^+$ was inhibited both by azathioprine and benziodarone (see Fig. 1), although as Table 1 shows, only azathioprine decreased the liver concentration of GSH. Neither of them had any effect on the liver concentration of $MeHg^+$, although azathioprine accelerated the clearance from blood. Azathioprine also increased bile flow. The effects of azathioprine on bile flow and methylmercury secretion started during the first collection period, while benziodarone decreased biliary $MeHg^+$ secretion only after a considerable delay.

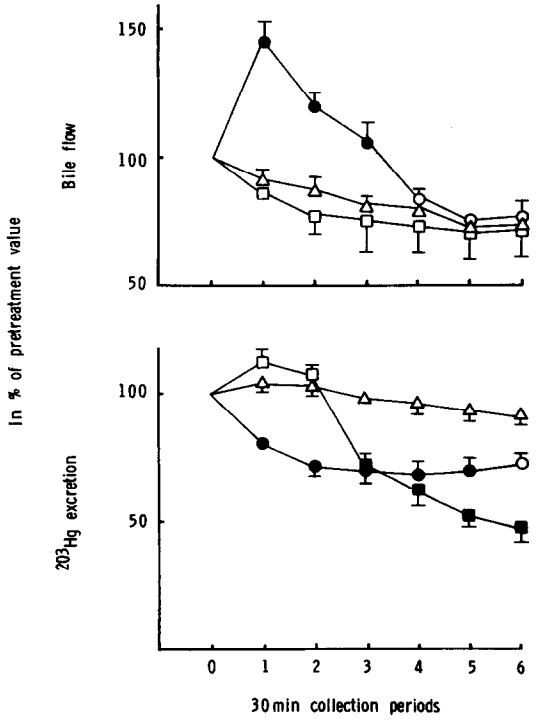

Fig. 1. The effects of azathioprine (400 μmoles/kg) or benziodarone (96 μmoles/kg) on bile flow and biliary methylmercury secretion. Azathioprine (circles) and benziodarone (squares) were given i.p. 2 hr after the i.v. administration of 5 μmoles/kg $Me^{203}HgCl$ (zero time on the graph). Control animals (triangles) were given saline. The number of animals were: control, 12; azathioprine, 8; benziodarone, 4. Solid symbols indicate significant difference from the control at the P < 0.01 level. Vertical bars indicate S.E.M. when larger than symbol size.

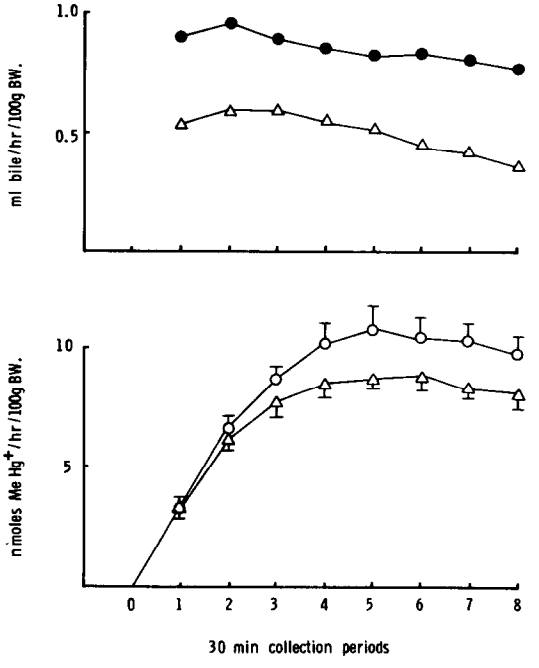

Fig. 2. Bile flow and biliary secretion of methylmercury in control (triangles) and *trans*-stilbene oxide (TSO) (circles) pretreated rats. $Me^{203}HgCl$ was given i.v. in a dose of 10 μmoles/kg 24 hr after the last of 4 daily doses of 400 mg/kg TSO. The number of animals were: control 4; TSO, 5. Solid symbols indicate significant difference from the control at the P < 0.01 level. Vertical bars indicate S.E.M. when larger than symbol size.

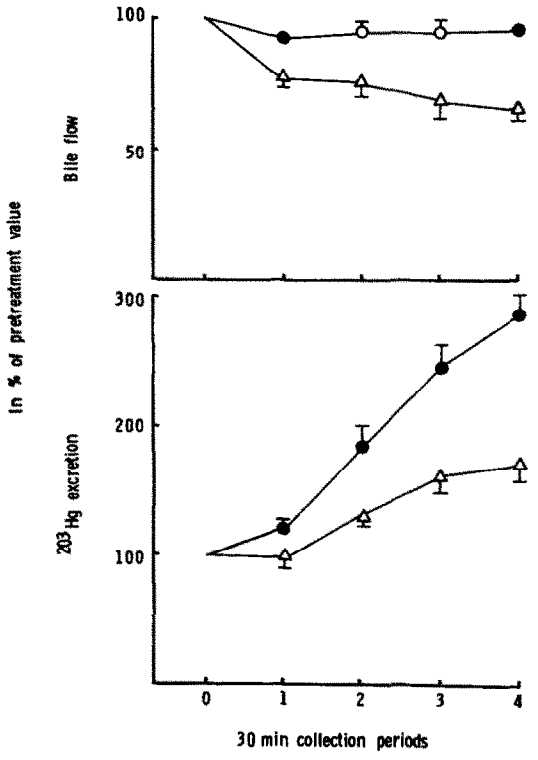

Fig. 3. The effect of GSH on the biliary excretion of methylmercury in control (triangles) and TSO pretreated rats (circles). Me^{203}HgCl (10 μmoles/kg) was given i.v. 24 hr after the last of 4 daily doses of 400 mg/kg TSO. The effect of GSH (2 μmoles/kg) given i.p. 2 hr after methylmercury is expressed in % of the mean biliary methylmercury excretion of individual rats measured in the last two 30 min collection periods before GSH. The number of animals were: control, 6; TSO, 7. Solid symbols indicate significant difference from controls at the $P < 0.01$ level. Vertical bars indicate S.E.M. when larger than symbol size.

The biliary secretion of $MeHg^+$ was in every collection period about 15% higher in TSO pretreated than in control rats, but the difference was not significant (see Fig. 2). The total mercury secretion during the 4 hr period was 29.5 ± 1.28 nmoles/100 g body weight in controls, equivalent to 5.9% of the dose) and 35.8 ± 2.58 nmoles/100g body weight in TSO pretreated rats equivalent to 7.0% of dose). The difference was not significant even at the $P < 0.05$ level. When control and TSO pretreated rats were given 2 mmoles/kg GSH 2 hr after methylmercury, biliary $MeHg^+$ excretion increased significantly more in TSO pretreated than in control rats (see Fig. 3). Between 3 and 4 hr after the administration of methylmercury the biliary secretion of methylmercury expressed as a percentage of the dose was raised by GSH from 1.6% to 2.3% in control (corn oil pretreated rats) and from 2.0% to 4.4% in TSO pretreated rats. Bile flow which was significantly increased by TSO, was not affected by GSH. Table 2 shows that GSH but not TSO accelerated $MeHg^+$ clearance from blood. In agreement with an earlier report [1] GSH increased the liver concentration of $MeHg^+$, but in the enlarged liver of TSO treated rats (5.2 ± 0.82 g/100 g body weight, N = 12, vs 3.8 ± 0.49 g/100 g body weight, N = 10, in controls) the total liver mercury was diluted to a lower concentration. Liver weight in relation to body weight was not affected by GSH either in control (corn oil) or TSO treated rats as the ratio of $MeHg^+$ in whole liver per 100 g body weight to liver concentration clearly indicates.

DISCUSSION

The inhibitors of methylmercury secretion can be divided into the following two groups. The first group includes those inhibitors which are ligands for ligandin but do not deplete liver GSH stores such as indocyanine green [11] and benziodarone. The second group includes compounds which, like methyliodide [5], acrylamide [6] and azathioprine, are both ligands for ligandin and depletors of liver GSH stores. It has been suggested by Refsik [5] that the key step in the biliary secretion of methylmercury is the conjugation of $MeHg^+$ with GSH by ligandin. However, as $MeHg^+$ reacts nonenzymatically with GSH with a rate constant of 10^{-6} sec^{-1} [25] and with an association constant of 10^{-6} M [26], this reaction does not require enzymatic intervention, and therefore the role of ligandin must be restricted to the transport process. The finding that BSP and indocyanine green inhibit both the biliary secretion of methylmercury and GSH [27] actually suggests that the secretion of the methylmercury–glutathione complex and GSH depends on the same transport process.

Table 1. The effects of azathioprine or benziodarone on the concentrations of nonprotein thiol groups in liver and methylmercury in blood and liver

	Mean ± S.E.M.				
		μmoles non-protein thiol groups per g liver		nmoles $MeHg^+$ per g	
Treatment	No.		No.	blood	liver
—	8	6.3 ± 0.19	12	72.3 ± 3.7	10.0 ± 0.5
Azathioprine	4	3.8 ± 0.56*	8	59.8 ± 2.2*	10.8 ± 0.5
Benziodarone	4	6.2 ± 0.43	4	79.0 ± 5.2	11.6 ± 0.6

MeHgCl was given i.v. in a dose of 5 μmoles/kg 2 hr before i.p. treatment with 400 μmoles/kg azathioprine or 96 μmoles/kg benziodarone. Animals were killed for GSH estimation 2 hr and for mercury estimation 3 hr after azathioprine or benziodarone.

* Significantly different from controls at the $P < 0.01$ level

Table 2. The effects of TSO pretreatment and GSH on the retention of methylmercury in blood and liver

TSO	GSH	No.	nmoles $MeHg^+$ (Mean ± S.E.M.) in 1 ml blood	in whole liver per 100 g body weight	in 1 g liver
−	−	4	139.0 ± 3.4	68.0 ± 2.2	17.8 ± 0.6
+	−	5	154.5 ± 4.4	81.0 ± 4.8	15.7 ± 0.90
−	+	6	112.0 ± 3.5*	81.5 ± 3.7	21.4 ± 0.60*
+	+	7	100.0 ± 1.4*	80.0 ± 3.3	15.6 ± 0.65

$Me^{203}HgCl$ was given i.v. in a dose of 10 μmoles/kg 24 hr after the last of 4 daily i.p. doses of 400 mg/kg TSO. GSH (2 mmoles/kg) was given i.p. 2 hr after the methylmercury. All animals were killed 4 hr after methylmercury.

* Significantly different from controls (without TSO and GSH) at the $P < 0.01$ level.

The dual dependence of biliary methylmercury secretion on liver GSH and ligandin is supported by the interaction of TSO pretreatment with GSH. Although the TSO pretreatment used in our experiments was reported to produce a 120% increase in the liver content of ligandin [28], it increased only slightly the biliary secretion of methylmercury. However, after the injection of GSH methylmercury secretion progressively increased in the next 2 hr by about 50% in control (corn oil pretreated) but by 200% in TSO pretreated rats (see Fig. 1). The possibility that GSH exerted this effect through increased transport from extrahepatic tissues into the liver can be rejected on the following argument. Firstly the effect of GSH both after intraperitoneal (ref. 1 and present experiment) and after intravenous administration [27] became evident only after a 30 min latent period when some of the injected GSH is already metabolised [29]. Secondly the extrahepatic hydrolysis of GSH and the hepatic uptake of constituents essential for GSH synthesis is so fast [29] that the concentration of GSH in bile is significantly increased 30 min after the i.v. injection of GSH [27].

In the kinetics of methylmercury biliary secretion is an important process. Phenobarbitone which increases the biliary secretion of methylmercury increased the daily faecal excretion of methylmercury in mice by 1.2% of the body burden and decreased biological half time by 15% [30]. In rats the faecal to urinary mercury excretion ratio is higher [31] than in the mouse [30], and therefore the biliary secretion is probably a more important step in the elimination process. A decrease in the biological half time of methylmercury from 20 days to 15 days — assuming that only 10% of the biliary methylmercury is excreted and 90% is reabsorbed [31] — should require the biliary secretion to be increased by 0.5% of the body burden per hour. Actually the combination of TSO and GSH increased the biliary secretion from 1.6% of the body burden (at the time dose approximates body burden) per hour to 4.4%, giving a difference of 2.6%. Such a secretion resulting in the faecal excretion of 0.44% of the body burden per hour — without any additional excretion — could decrease the biological half time of methylmercury from 20 days to 6.5 days and when the reabsorption of secreted methylmercury is prevented by the administration of a non-absorbable polythiol resin [32] then the half time could be reduced to 14 hr.

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