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THE INTERRELATIONSHIP BETWEEN NON-PROTEIN BOUND THIOLS AND THE BILIARY EXCRETION OF METHYLMERCURY

LASZLO MAGOS, THOMAS W. CLARKSON and JUDITH ALLEN

MRC Toxicology Unit, Carshalton, Surrey, England and
Department of Radiation Biology and Biophysics, University of Rochester, School of Medicine and Dentistry, Rochester, NY U.S.A.

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Abstract—The biliary excretion of methylmercury was investigated in male rats injected with various thiol compounds, methionine and 3′-methyl-4-dimethyl-azobenzene (3′-MeDAB) after the i.v. administration of 1.0 mg/kg of Hg as $Me^{203}HgCl$. All the thiol compounds (2 m-moles of glutathione (GSH) and 4 m-moles/kg of cysteine, D-penicillamine or dimercaptosuccinic acid (DMSA) reduced the blood concentration of $Me^{203}Hg^+$, but only cysteine, GSH and D-penicillamine increased the liver content of mercury and its biliary excretion. No significant changes were seen in the volume of bile flow after treatment. There was no correlation between the increase in non-protein thiol concentration in blood and either the liver content or the biliary excretion of $Me^{203}Hg^+$. Although those thiol compounds that stimulated the biliary excretion of methylmercury also increased the liver concentration of non-protein thiol groups, there was no proportionality between either the extent or time course of these effects. An initial inhibition of excretion was caused by 4 m-moles/kg of cysteine though the concentration of non-protein thiols in the liver was at that time the highest. After the administration of cysteine and GSH, excretion increased from 30 to 120 min while the concentration of non-protein thiols in the liver declined. 3′-Methyl-4-dimethylazobenzene (3′-MeDAB) and methionine caused a larger increase in liver non-protein thiol concentration than GSH but their effect on the biliary excretion of $Me^{203}Hg^+$ was smaller.

On the assumption that D-penicillamine spares GSH from other metabolic processes, these results are consistent with the role of GSH in the biliary excretion of methylmercury. They do not support a direct relationship between free GSH and the biliary excretion of methylmercury.

The fecal excretion of methylmercury is determined by three factors: (1) biliary excretion, (2) shedding of the intestinal wall and (3) reabsorption [1]. Since reabsorption can be decreased by the oral administration of a non-absorbable polythiolated resin [2, 3], any method to increase in the biliary excretion of methylmercury is of therapeutic significance. However, very little is known about the mechanism of methylmercury excretion in bile. It has been shown that phenobarbitone, an inducer of glutathione S-transferase in the liver [4], increases the biliary excretion of methylmercury [5, 6]. Methylmercury in the bile has been shown to be complexed with cysteine [1, 7], glutathione [7], or a compound with a mol. wt between that of these two thiols [8] in agreement with the affinity of methylmercury for thiol groups [9].

Following the observation by Norseth [10] that the thiol compound, D-penicillamine increased the biliary excretion of methylmercury, a variety of thiol compounds were tested in the present work. These included two physiological compounds, L-cysteine and glutathione (GSH) and two compounds known to be effective in mobilizing methylmercury, D-penicillamine and dimercaptosuccinic acid (DMSA) [3, 11, 12].

To learn more about the mechanism of action of these compounds, changes in the biliary excretion of methylmercury were compared with changes in concentrations of non-protein thiols in blood and liver. The thiol compounds were given at doses that significantly affected non-protein thiols in tissues. Two other compounds were used to enhance tissue thiol concentrations: L-methionine and 3′-methyl-4-dimethylazobenzene (3′-MeDAB). The former is metabolized to homocysteine, cysteine and consequently to glutathione [13], the latter inhibits glutathione S-transferase thereby increasing the liver concentration of glutathione [14].

MATERIALS AND METHODS

Male rats of the Sprague–Dawley (Charles-River strain) and in experiments with methionine and 3′-MeDAB, Porton–Wistar strains of approximately 180 g body wt were used. Animals were anaesthetized with sodium pentobarbital (65 mg/kg) and kept at a constant body temperature of 37° on a mesh above a heated water bath throughout the experiments. The jugular vein and the bile duct were cannulated with PE-10 tubing. Methylmercury chloride (K & K Labs) labelled with ^{203}Hg (New England Nuclear, Boston, MA, U.S.A.) was injected into the jugular cannula in a dose of 1 mg Hg/kg. The volume of the injection solution was 1 ml/kg and the specific activity of Hg was approximately 2.5 $\mu Ci/\mu g$. Bile, collected every 30 min was weighed and ^{203}Hg determined in a gamma scintillation counter. Thiol compounds or L-methionine (Sigma Chemical Co., London, U.K.) were given by i.p. injections in

Table 1. Effects of different thiol compounds on the concentration of non-protein thiol groups in the plasma and liver*

Treatment	Dose in m-moles/kg	No.‡	Shift in thiol concentration (mean ± S.E.M.) compared with paired controls† μmoles/ml plasma 30 Min	60 Min	120 Min	μmoles/g liver 30 Min	60 Min	120 Min
L-Cysteine	4	5	0.54 ± 0.041§	0.29 ± 0.052§	0.05 ± 0.012§	3.43 ± 0.47§	1.77 ± 0.25§	1.24 ± 0.48§
Glutathione	2	6	0.13 ± 0.023§	0.03 ± 0.014	0.02 ± 0.018	1.63 ± 0.39§	2.16 ± 0.37§	1.40 ± 0.46§
D-Penicillamine	4	4	1.41 ± 0.147§	0.92 ± 0.181§	0.49 ± 0.094§	2.30 ± 0.37§	2.19 ± 0.61§	1.21 ± 0.70
DMSA	4	6	5.49 ± 0.63§	1.69 ± 0.307§	0.29 ± 0.111§	1.49 ± 0.21§	−0.14 ± 0.35	−0.19 ± 0.02

* Thiol compounds were i.p. administered in a volume of 5.0 ml/kg. Controls were given equal volume of saline.

† The control values were for plasma: 0.01 ± 0.001 μmoles/ml ($N = 21$) and for liver 6.00 ± 0.11 μmoles/g ($N = 34$). In a few cases the same control served as a pair for two experimental animals killed at the same time.

‡ Liver non-protein concentration was estimated only in four animals per group.

§ Significant increase with the directional Student's 't' test.

saline (volume 5 ml/kg, pH neutral) at the end of the fourth 30 min period. Bile flow and excretion of ^{203}Hg in the post-treatment period were expressed as a percentage of the values measured in the 1-hr period before treatment in order to minimize the individual differences unrelated to treatment. 3′-MeDAB (Eastman Kodak) was administered i.p. in arachis oil (6 ml/kg) 24 hr before the administration of methylmercury. The effects of thiol compounds and of methionine or 3′-MeDAB on tissue non-protein thiol levels were estimated in different animals which were not given methylmercury. Controls paired with the experimental ones received equal volumes of saline at the same time.

Animals were sacrificed by decapitation, blood was collected in a beaker, the liver and kidneys were removed, washed, weighed and their ^{203}Hg contents were estimated.

Non-protein thiol groups were estimated with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Two different procedures were used depending on whether only total non-protein thiols were estimated, or whether distinction was made between cysteine and GSH thiol groups. Procedure 1 was based on the method of Sedlak and Lindsay [15]. Whole blood was diluted in the ratio of 1 : 1 with distilled water. Plasma or diluted whole blood were precipitated with an equal volume of 10% trichloroacetic acid. Procedure 2 was based on the method of Ball [16] with slight modifications. A 2.5% (w/v) liver homogenate was made in 5% sulfosalicyclic acid. After centrifugation the supernatant was diluted in a ratio of 1 : 4 with 0.5 M phosphate buffer, pH 7.2, containing 20 mM EDTA. A sample of diluted supernatant (5 ml) was incubated at 60° with 1 ml 2% glyoxylic acid (Sigma) in EDTA phosphate buffer (each solution was pre-warmed before mixing). After a 5-min incubation, the sample was cooled to room temperature in melting ice and mixed with 1 ml 0.15% DTNB reagent. Reading against a reagent blank at 412 mμ enables estimation of the total non-protein thiol concentration minus the concentration of cysteine. For the estimation of total non-protein thiol concentration, 1 ml DTNB reagent was added to the 5 ml diluted supernatant followed by 1 ml 2% glyoxylic acid and the absorbance was measured at 412 mμ without incubation. Whole blood was first diluted in the ratio 1 : 1 with distilled water and precipitated by an equal volume of 10% sulfosalicylic acid.

RESULTS

Table 1 shows that 30 min after their administration, all the thiol compounds used in these experiments increased significantly the concentration of non-protein thiol groups in plasma and liver. After 30 min the plasma non-protein thiol concentration exponentially decreased in L-cysteine, D-penicillamine and DMSA-treated rats with half-times of 24, 51 and 21 min. The kinetics of decline of plasma thiol groups after glutathione can not be defined accurately because of the scatter of the points. It can be seen that at 30 min L-cysteine produced the highest increase in non-protein thiols in the liver followed closely by D-penicillamine. Glutathione and DMSA compared with L-cysteine and D-penicillamine cause significantly smaller increases, but from 30 to 60 min there was an increase in liver non-protein thiol concentration in glutathione treated rats. At 60 and 120 min after their administration there was no difference in non-protein thiol concentrations in the liver between L-cysteine, glutathione and D-penicillamine-treated rats. At this time, in DMSA-treated animals, the non-protein thiol concentration was below those of the controls.

Bile flow was minimally affected by these thiol compounds. A significant decrease occurred in DMSA-treated rats in the first hr while GSH, and penicillamine-treated rats showed an increase in the middle collection periods (Fig. 1). However, this change was never more than 25 per cent of the control value and could not account for changes in biliary excretion of methylmercury.

Figure 2 shows the change in the biliary excretion of ^{203}Hg after the administration of the same thiol compounds. It can be seen that the biliary excretion pattern does not follow either the change in plasma or in liver non-protein thiol groups. DMSA, which cause the highest increase in the plasma non-protein thiol groups, did not stimulate the biliary excretion of methylmercury and in the last two collection periods (at 150 and 180 min) depressed it. The other three thiol compounds produced significant increases in the biliary excretion of methylmercury although the time course differed from one compound to the

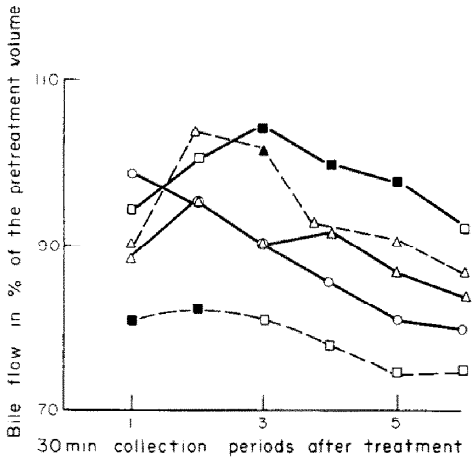

Fig. 1. Effect of thiol groups on bile flow in methylmercury-treated rats. Thiol compounds were administered i.p. 2 hr after the i.v. administration of 1 mg Hg/kg as $Me^{203}HgCl$ in the following doses: L-cysteine (△——△), D-penicillamine (△- - -△), and dimercaptosuccinic acid (□- - -□) 4 m-moles/kg; glutathione (□——□) 2 m-moles/kg. Controls (○——○) were given equivalent volume of saline. Solid symbols mark significant differences between the geometric means of the experimental and control animals (two directional Students 't' test, $P<0.01$). The number of animals were, control = 12; cysteine, glutathione and D-penicillamine = 6 each and dimercaptosuccinic acid $N = 9$.

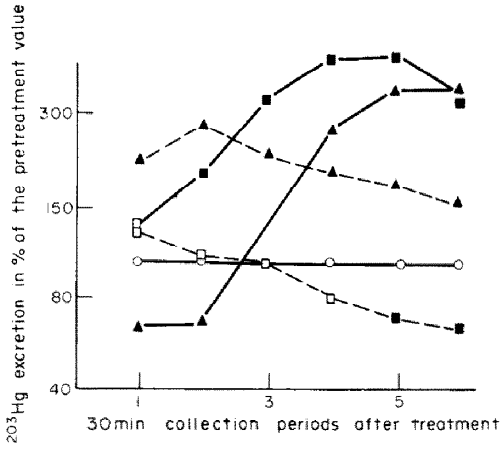

Fig. 2. The effect of thiol compounds on the biliary excretion of methylmercury. Thiol compounds were administered i.p. 2 hr after the i.v. administration of 1 mg Hg/Kg as $Me^{203}HgCl$ in the following doses: L-cysteine (△——△), D-penicillamine (△- - -△), and dimercaptosuccinic acid (□- - -□) 4 m-moles/kg; glutathione (□——□) 2 m-moles/kg. Controls (○——○) were given equivalent volume of saline. Solid symbols mark significant differences between the geometric means of the experimental and control animals (two directional Students 't' test, $P<0.01$). The number of animals were; control = 12, cysteine, glutathione and D-penicillamine = 6 each and dimercaptosuccinic acid $N = 9$.

other. D-Penicillamine produced a peak excretion in bile collected at 30 min followed by a progressive decrease; cysteine and glutathione produced the largest stimulation of biliary excretion, but this did not occur until 120–180 min. The time response to cysteine is remarkable: at 30 and 60 min collection times, the biliary excretion was actually significantly depressed by cysteine. As Table 2 shows, cysteine concentration in blood and liver declined at roughly the same rate with a half-time of about 30 min and in blood all the increase in the non-protein thiol concentration was caused by unchanged cysteine. In the liver the contribution of cysteine to thiol group increase was somewhat less. Again there was no similarity between the change in total thiol groups or cysteine on the one hand and in change of the biliary excretion of methylmercury.

All the thiol compounds decreased the concentration of methylmercury in blood (Table 3). In contrast, liver concentrations of methylmercury were elevated but only in the case of the two physiological compounds, cysteine and glutathione, was this increase in concentration significantly different from controls. The biliary excretion rate of methylmercury in the last collection period as reported in Fig. 2 shows a high correlation with the liver mercury levels as reported in Table 3, [$r^2 = 0.8$]. A multilinear regression comparing these same biliary excretion rates with both blood and liver mercury levels reported in Table 2 yields a slightly higher degree of correlation [$r^2 = 0.9$]. Although liver concentrations of mercury appeared to account for most of the variance, the possibility that blood levels may play a minor role in determining biliary excretion of methylmercury can not be excluded.

This close correlation between liver levels and

Table 2. The concentration of total non-protein thiol groups and cysteine in whole blood and liver after the administration of L-cysteine*

	Control	μmoles/g (mean ± S.E.M.) Time after treatment, min 30	60	120
	$N = 12$	$N = 4$	$N = 4$	$N = 4$
Total thiols in blood	0.98 ± 0.05	2.02 ± 0.32‡	1.47 ± 0.09†	1.19 ± 0.04
Cysteine in blood	0.10 ± 0.08	1.10 ± 0.18†	0.47 ± 0.07†	0.18 ± 0.14‡
Total thiols in liver	5.89 ± 0.15	9.45 ± 0.40†	7.27 ± 0.66‡	6.64 ± 0.43
Cysteine in liver	0.14 ± 0.06	2.18 ± 0.19†	0.67 ± 0.08‡	0.44 ± 0.20

* Animals were given cysteine, 4 m-moles/kg i.p. or an equal volume of isotonic saline. Controls were killed at the same time as the paired experimental animals.

† Significantly different with the directional Student's 't' test for paired control samples, $P<0.01$.

‡ Significantly different with the directional Student's 't' test for paired control samples, $P<0.05$.

Table 3. The effect of thiol compounds on the content of mercury in whole blood and liver five hours after $Me^{203}HgCl$

Treatment*	Dose m-moles/kg	No.	Micrograms Hg (mean ± S.E.M) In 1 ml blood	In total liver/100 g body wt
Saline	—	19	12.77 ± 0.89	7.99 ± 0.71
L-Cysteine	4	7	5.07 ± 0.21†	13.32 ± 1.95†
Glutathione	2	6	6.22 ± 0.56†	12.80 ± 1.53†
D-Penicillamine	4	6	5.57 ± 0.14†	11.45 ± 1.81
Dimercaptosuccinic acid	4	7	4.90 ± 0.71†	9.13 ± 1.80

* Thiol compounds were given i.p. 2 hr after 1 mg Hg/kg i.v. as $Me^{203}HgCl$.
† Significantly different from the controls with the non-parametric Mann–Whitney U test, $P < 0.01$.

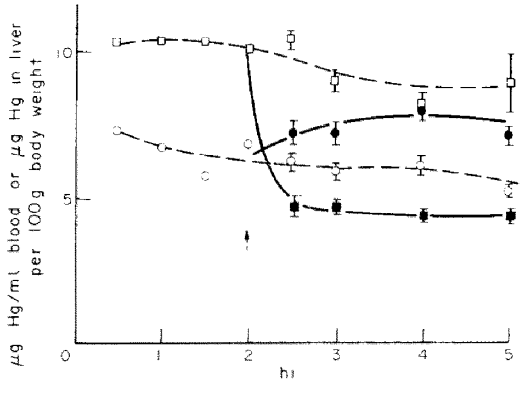

Fig. 3. Mercury in blood (squares) and liver (circles) in rats given 1 mg Hg/kg as methyl $^{203}HgCl$ i.v. and treated 2 hr later with saline (controls) or 4 m-moles/kg L-cysteine i.p. (arrow). The continuous line connects symbols of animals given cysteine. Solid symbols indicate significant differences from controls (two directional Student's 't' test, $P < 0.01$). Every point before treatment is the mean of two animals; at 2½ hr the mean of 3; at 3 hr, controls = 4, experimentals = 6; at 4 and 5 hr controls = 5, experimentals = 8. The standard errors are indicated by the horizontal bars.

biliary excretion of methylmercury is not valid for the whole time course as evidenced by the data in Fig. 3. The mercury content of blood and liver were investigated in rats treated with L-cysteine, the only thiol compound which showed a biphasic effect on biliary excretion. The data in Fig. 3 indicates that 4 m-moles/kg cysteine resulted in a 50 per cent decrease in the blood levels of methylmercury within 30 min and that these levels remained at 50 per cent of control values up to 5 hr. The liver content of mercury in the cysteine-treated animals increased by only 15 per cent in the first 60 min and thereafter remained approximately constant. It should be noted that in periods after 60 min, pronounced stimulation in biliary excretion of methylmercury had occurred (Fig. 2). Indeed, the switch from depressed methylmercury excretion rates to highly stimulated excretion rates, which occurred just after the 60 min collection, was accompanied by no significant changes in either blood or liver concentrations of methylmercury.

The biphasic effect of cysteine was found to be dose-dependent. When a lower dose (0.4 m-mole/kg cysteine) was used, the initial inhibition of methylmercury excretion disappeared. In the first 30 min collection period, biliary excretion of mercury increased to 152 per cent of the pretreatment value

Table 4. The effect of L-methionine on the biliary excretion of methylmercury chloride and liver non-protein thiol concentration*

Collection period post mercury, min	Biliary excretion of ^{203}Hg % of the pretreatment excretion		Liver non-protein thiol concentration μmoles/g	
	Control $N = 5$	Methionine $N = 7$	Control	Methionine
0–30	91.1 ± 4.83	91.2 ± 4.01		
30–60	98.0 ± 7.56	95.5 ± 5.99	4.75 ± 0.62 $N = 4$	7.92 ± 0.55† $N = 4$
60–90	92.1 ± 2.63	100.1 ± 7.56		
90–120	102.3 ± 3.02	101.2 ± 6.20	4.93 ± 0.26 $N = 4$	7.06 ± 0.16† $N = 4$
120–150	87.7 ± 5.33	110.0 ± 5.85†		
150–180	94.9 ± 4.50	115.7 ± 8.43†	5.31 ± 0.24 $N = 12$	7.74 ± 0.32† $N = 12$

* L-Methionine (2 m-moles/kg, i.p.) was given either to untreated rats (for thiol group estimation) or 2 hr after an i.v. injection of 1 mg Hg/kg as $Me^{203}HgCl$ (for bile collection). Controls received saline. Bile was collected immediately after administration of mercury. The biliary excretion of mercury for the 1 hr pretreatment period was 6.17 ± 0.22 μg/kg/hr for controls ($N = 5$); and 5.83 ± 0.31 μg/kg/hr for treated animals ($N = 7$). Animals were sacrificed at 1, 2 and 3 hr post-treatment for thiol estimations.
† Significantly different from the control with the directional Student's 't' test, $P < 0.05$.

Table 5. Effects of 3′-MeDAB on liver non-protein thiol concentration and the biliary excretion of methyl mercury*

	Liver thiol concentration μmoles/g (Mean ± S.E.M.)	Biliary excretion of Hg μg/kg/Hr (Mean ± S.E.M.)
Control	4.52 ± 0.14 $N = 8$	6.11 ± 0.33 $N = 8$
3′-MeDAB	7.69 ± 0.55† $N = 5$	7.73 ± 0.33† $N = 5$

* $Me^{203}HgCl$ was i.v. administered at a dose of 1.0 mg Hg/kg 24 hr after the i.p. administration of 165 mg/kg of 3′-MeDAB or saline. Bile was collected for 5 hr after treatment.

† Significantly different from the control with the two directional Student's 't' test for paired samples, $P < 0.01$.

and for the following 30 min collection periods it was: 322, 306, 290, 237, and 209 per cent. Thirty min after the administration of this dose of cysteine, no detectable change could be found in plasma non-protein thiol groups ($N = 4$) but an average of 0.92 μmole/g increase was found in the liver. This increase was lower than that seen at higher doses of cysteine and with other thiol compounds. Nevertheless the increase in biliary excretion within the first 30 min was higher than that produced by the other thiol compounds except for penicillamine. A ten times lower dose of cysteine [0.04 m-mole/kg] had no effect on either the biliary excretion of methylmercury or on the concentration of liver non-protein thiol groups.

A dose of 2 m-moles/kg methionine was able to increase biliary excretion of methylmercury only 2 hr after administration (Table 4). The increase was much less than that seen with either cysteine or glutathione after 120 min although methionine was able to increase liver concentrations of non-protein thiols by 3 μmoles/g above controls about 1 hr prior to the significant elevation in biliary excretion rates of methylmercury.

3′-Methyl-4-dimethylazobenzene (an inhibitor of glutathione transferase activity) was able to significantly elevate liver non-protein thiol concentrations by slightly more than 3 μmoles/g (Table 5). Compared with controls, this was accompanied by a small (23 per cent) increase in methylmercury excretion in bile over a 5-hr period.

DISCUSSION

Our results have indicated that several sulfur-containing compounds increased biliary excretion of methylmercury and these increases were accompanied or preceded by elevations in non-protein bound thiol concentrations in liver. Since no quantitative correlation could be found, the increase in biliary excretion of methylmercury can not be due directly to elevated non-protein thiol concentrations in liver or plasma. Thus the form of the non-protein thiols seems to be critical. The biliary excretion of methylmercury was increased by 0.4 m-mole/kg cysteine immediately although it was inhibited temporarily by 4 m-moles/kg. As a high proportion of the increase in plasma and liver non-protein thiols was due to the presence of unchanged cysteine, it seems reasonable to suppose that cysteine above a certain threshold actually inhibited the biliary excretion of methylmercury. As concentration of cysteine declined the biliary excretion of methylmercury increased.

It may be, as evidenced in one report in the literature [7] that methylmercury is excreted as the glutathione complex. This would be consistent with our findings that glutathione appears to be the most effective thiol compound in elevating biliary excretion of mercury in rats. The stimulatory effects of cysteine and methionine could be explained by the metabolism of these compounds to glutathione. D-penicillamine, by replacing glutathione in other metabolic processes, could make a substantial proportion of liver glutathione free for promoting methylmercury excretion. However, our data do not allow elucidation of the precise role that glutathione plays. Elevation of free GSH in liver is not, in itself, responsible for increased mercury excretion. For example, the stimulatory effect of cysteine was not accompanied by a significant increase in free thiol concentration except for cysteine itself. Furthermore, methionine or the inhibitor of glutathione transferase produced a larger increase in non-protein thiols in liver than glutathione and had only minimal effects on the biliary excretion of methylmercury.

Finally we must consider firstly that the GSH concentration in the liver was three order higher than the concentration of methylmercury; and secondly that the affinity constant of methylmercury for thiol groups is $Log_{10}K = 17$ [9]. Thus even a substantial change in the concentration of GSH could not affect the availability of GSH for $MeHg^+$. Consequently a mechanism more complicated than the direct effect of free GSH on the biliary excretion of methylmercury must be considered. Such mechanisms might include interaction of GSH and mercury [17, 18] with proteins, like ligandin and the facilitated diffusion of methylmercury in tissues.

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