Effect of Mercury on Erythrocyte Glutathione Reductase Activity. *In vivo* and *in vitro* Studies

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The toxicity of mercury has been widely studied. The most probable cause of mercury toxicity is the affinity of mercury for SH-groups. Mercury readily binds to the SH-groups of proteins to make them biologically inactive. A high proportion of the total blood Hg in methylmercury poisoning is found in the erythrocytes (GAGE, 1965). Glutathione reductase is an erythrocyte enzyme which contains active site SH-groups (ICEN, 1967). It catalyses the following reaction: $TPNH + H^+ + GSSG = TPN^+ + 2GSH$. GSH is the most abundant SH-compound in the cells, and appears to function in maintaining the normal integrity of the erythrocytes via glutathione peroxidase (COHEN and HOCHSTEIN, 1963). Glutathione reductase is known to be easily inhibited by various sulfhydryl reagents in vitro (ICEN, 1967), and is among those enzymes most sensitive to Hg^{++} (WEBB, 1963). It would thus be expected that the feeding of methylmercury might depress erythrocyte glutathione reductase. Previous studies have shown that experimental methylmercury poisoning decreases glutathione reductase activity in rat brain, but not, however, in rat liver (PEKKANEN and SANDHOLM, 1972).

The purpose of these experiments was to study the effect of chronic methylmercury administration on erythrocyte glutathione reductase activity, to see if the possible decrease in erythrocyte glutathione reductase activity might serve as an early indicator for mercury poisoning, as in the case of red blood cell δ-amino levulinic acid dehydratase in Pb poisoning. In addition, some experiments were done to determine the effect on glutathione reductase activity when methylmercury and mercuric chloride were added to erythrocyte hemolysates in vitro. Since dietary selenium has been shown to decrease the toxicity of methylmercury (GANTHER et al., 1972), the effect of selenium on glutathione reductase activity in erythrocytes exposed to mercurials was also studied.

MATERIALS AND METHODS

Blood samples from rats and Japanese quail (Coturnix coturnix japonica) were used. There were three different studies. Table 1 shows the levels of mercury and selenium added to the diets.

Table 1

The levels of mercury and selenium added to the diet.

Group	Exp. 1[a] ppm		Exp. 2[b] Hg (ppm)		Exp. 3[c] Hg (ppm)	
	Hg	Se	Basal	Basal+Se[d]	Basal	Basal+Se[e]
1	0	0	0	0	0	0
2	0	6	1	1	5	5
3	20	0.3	2	2	15	15
4	20	6	4	4	30	30
5			6	6		
6			10	10		

[a]Japanese quail (1 day old) were fed the diets 28 days, then blood was taken from one bird in each group

[b]Rats (4 weeks old) were fed the diets 29 weeks, then blood was taken from 5 animals in each group (4 animals in group 5)

[c]Japanese quail (1 day old) were fed the diets 32 days, then blood was taken from 6 birds in each group

[d]0.5 ppm Se as sodium selenite

[e]12 ppm Se as sodium selenite

Except for Experiment 1, mercury was fed at each of several levels, either with or without supplemental selenium. Mercury was added to the diet as methylmercury hydroxide and selenium as sodium selenite. The diet for quail was a corn-soya diet containing approximately 0.15 ppm Se, while a purified casein diet containing approximately 0.02 ppm Se was fed in the case of rats (GANTHER et al., 1972). The diets were fed ad libitum. Blood samples were taken by heart puncture into heparinized tubes which were

kept in ice. Pooled blood samples were hemolyzed by a method modified from BEUTLER (1969). One milliliter of blood was pipeted into a heavy wall centrifuge tube. Red blood cells were separated by centrifuging the samples at 1500 x g for 10 min. Supernatant was discarded. The cells were washed three times in 4-5 volumes of isotonic sodium chloride (0.9% NaCl). To lyse the cells, 8.0 ml of distilled water was added to the cells and the solutions were frozen and thawed. To separate the stroma, samples were centrifuged at 10,000 x g for 15 min. The samples were stored at 4 °C for further studies.

Enzyme assay. The activity of glutathione reductase was determined by using a method modified from MASSEY and WILLIAMS (1965). Into a one milliliter cuvette the following solutions were pipetted: 0.1 ml of 0.3 M sodium phosphate, pH 7.6; hemolysate (Experiment 1 0.05 ml, Experiment 2 0.20 ml and Experiment 3 0.10 ml); 0.10 ml 1 mM TPNH; water to 0.99 ml. After incubation for 5 min at 25 °C, 0.01 ml of 0.3 M GSSG was added to start the reaction. In the blank, water replaced the GSSG-solution. The activity of glutathione reductase was determined as the change in absorbance at 340 nm in a spectrophotometer connected to a recorder. Activities are reported as the change in absorbance at 340 nm per minute per ml whole blood (ΔA/min/ml whole blood), corrected for the blank.

RESULTS AND DISCUSSION

In vivo studies. The effect of dietary methylmercury on glutathione reductase activity of rat or quail erythrocytes is shown in Table 2.

Table 2

Effect of dietary methylmercury on erythrocyte glutathione reductase activity.

	Glutathione reductase activity (ΔA/min/ml whole blood)				
	Exp. 1 (quail)	Exp. 2 (rats)		Exp. 3 (quail)	
Group		Basal	Basal+Se	Basal	Basal+Se
1	1.28	0.35	0.37	2.56	2.32
2	2.40	0.43	0.38	2.32	2.24
3	2.88	-	0.33	2.64	2.40
4	2.56	0.40	0.42	2.80	2.40
5		0.40	0.45		
6		0.35	0.38		
mean	2.28	0.38	0.39	2.58	2.37

Each value is the average of 4 determinations in Experiment 1, and 3 in Experiment 2 and Experiment 3.

Suprisingly, there was no inhibitory effect of dietary methylmercury on enzyme activity of hemolysates in any of these experiments. The low value of the control (group 1) in Experiment 1 is hard to explain other than by individual variation (one animal per group) or by experimental error. The mean values for quail erythrocyte glutathione reductase activity in Experiments 1 and 3 are similar (2.28 and 2.48). The slightly lower value for the Se-supplemented groups in Experiment 3 might be due to the very high content of selenium in the diet (12 ppm). The activity of erythrocyte glutathione reductase in quail was much higher than in rats (quail:rat ratio 6.0); the data of MANSO and WROBLEWSKI (1958) indicate a value of 2.5 for the ratio chicken:rat.

Since methylmercury is known to be taken up by erythrocytes, but does not inhibit glutathione reductase, these findings suggest that methylmercury is preferentially bound to something other than glutathione reductase in erythrocytes. Part of the mercury might be bound to the membrane and unable to get inside the red blood cell, or part could be bound to proteins inside the cell, such as hemoglobin. In the next section the effect of mercurials added directly to hemolysates is described.

In vitro studies. Table 3 shows the effect of various amounts of $Hg(NO_3)_2$ added in vitro on hemolysate glutathione reductase activity in Experiment 1. Inhibition seemed to increase almost linearly with increasing mercury concentrations in the range of 0.64 to 3.20 ppm. Dietary treatments seemed to have little effect on the inhibition.

Table 3

Inhibition of quail erythrocyte glutathione reductase by mercuric nitrate added in vitro (Experiment 1).

Group	Inhibition (%)				
	Hg added: moles/l (or ppm)				
	3.2×10^{-6} (0.64 ppm)	8×10^{-6} (1.6 ppm)	16×10^{-6} (3.2 ppm)	32×10^{-6} (6.4 ppm)	320×10^{-6} (64 ppm)
1	12	38	75	-	100
2	20	37	80	-	100
3	-	25	67	89	-
4	-	35	75	88	-

In rats, the addition of 1.6 ppm Hg (8×10^{-6} M) as $Hg(NO_3)_2$ generally inhibited the rat glutathione reductase activity about 30% (Table 4), similar to the effect on quail hemolysates. Inhibition by Hg added *in vitro* was highest in group 6, which received the highest dietary mercury level (10 ppm). In all but one case, the inhibition by mercury *in vitro* was less when selenium was fed in the diet.

Table 4

Effect of mercuric nitrate added to rat blood hemolysates *in vitro* on glutathione reductase activity.

Group	Percentage inhibition[a]	
	Basal	Basal+Se
1	30.0	8.5
2	27.5	17.5
3	-	28.8
4	29.3	30.0
5	-	7.8
6	85.0	52.3

[a] 8×10^{-6} M (1.6 ppm) Hg added as $Hg(NO_3)_2$

Methylmercury was far less inhibitory to glutathione reductase than $Hg(NO_3)_2$ (Table 5). The great difference between inorganic and organic mercury is clearly seen in experiments with rat blood samples; for a 30-40% decrease in activity, 1.6 ppm (8×10^{-6} M) Hg as $Hg(NO_3)_2$ had to be used, whereas about 200 ppm Hg (1×10^{-3} M) as methylmercury was necessary for the same inhibition. In Se-supplemented groups no inhibition was seen, even with methylmercury levels as high as 200 ppm, again suggesting some degree of protection against mercury added *in vitro* when selenium was fed in the diet. Selenium in the diet may become incorporated nonspecifically into erythrocytes, forming high affinity binding sites that complex Hg and decrease its binding to glutathione reductase. The difference between

Table 5

Effect of methyl mercury added to rat blood hemolysates *in vitro* on glutathione reductase activity.

Diet	Group	Mercury (ppm) Diet	Blood[a]	Added *in vitro*[b]	Inhibition (%)
Basal	1	0	0.008	40	0
	2	1	12	160	10.5
	5	6	55	200	36.5
	6	10	76	120	11.7
Basal + Se	1	0	0.016	80	0
	2	1	5.4	180	0
	5	6	52	200	0
	6	10	70	140	0

[a]Total Hg in whole blood was analyzed in this laboratory by M. J. Kopecky, using the flameless atomic absorption procedure of MUNNS and HOLLAND (1971)

[b]The levels added to Basal + Se hemolysates were inadvertently higher than for Basal in all but one case.

organic and inorganic mercury in the inhibition of glutathione reductase activity might be due to structural differences, causing a preference of glutathione reductase for binding mercuric mercury, or to a preference of hemoglobin in the hemolysate for binding methylmercury. Methylmercury was found to have a lower affinity for metallothionein compared to mercuric ion (CHEN *et al.*, 1973). Alkyl mercurials are largely associated with hemoglobin (TAKEDA *et al.*, 1968). Possibly both effects are operating. We have not used purified glutathione reductase to compare the effects of the two mercurials in the absence of other proteins.

SUMMARY

Glutathione reductase activity was determined in erythrocyte hemolysates of rats and quail fed various levels of methylmercury. Dietary mercury as high as 30 ppm had no effect on the activity of this enzyme. Micromolar concentrations of inorganic mercuric nitrate added to stroma-free hemolysates *in vitro* resulted in a clear inhibition. To get the same decrease in activity with methylmercury *in vitro*, however, it was necessary to add a hundred-fold higher level of Hg. Dietary selenium seemed to have some protective effect against the inhibitory effect of mercury added *in vitro*.

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