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ENZYMATIC OXIDATION OF MERCURY VAPOR BY ERYTHROCYTES

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Summary

The formation of glutathione radicals, the evolution of nascent oxygen or the peroxidatic reaction with catalase complex I are considered as possible mechanisms for the oxidation of mercury vapor by red blood cells. To select among these, the uptake of atomic mercury by erythrocytes from different species was studied and related to their various activities of catalase (hydrogen-peroxide : hydrogen-peroxide oxidoreductase, EC 1.11.1.6) and glutathione peroxidase (glutathione : hydrogen-peroxide oxidoreductase, EC 1.11.1.9). A slow and continuous infusion of diluted H_2O_2 was used to maintain steady concentrations of complex I. 1% red cell suspensions were found most suitable showing high rates of Hg uptake and yielding still enough cells for subsequent determinations. The results indicate that the oxidation of mercury depends upon the H_2O_2-generation rate and upon the specific acticity of red-cell catalase. The oxidation occurred in a range of the catalase-H_2O_2 reaction where the evolution of oxygen could be excluded. Compounds reacting with complex I were shown to be effective inhibitors of the mercury uptake. GSH-peroxidase did not participate in the oxidation but rather, was found to inhibit it by competing with catalase for hydrogen peroxide. These findings support the view that elemental mercury is oxidized in erythrocytes by a peroxidatic reaction with complex I only.

Introduction

It is well known that erythrocytes incorporate mercury vapor at high rates, and the present conception is that the atomic mercury becomes oxidized in the cell interior, thus not being able to permeate outwards through the membrane

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due to its charge [1,2]. In studies with suspensions of red cells [3,4] or glutathione solutions [5] Nielsen-Kudsk concluded that catalase (hydrogen-peroxide : hydrogen-peroxide oxidoreductase, EC 1.11.1.6) or the glutathione-peroxidase (glutathione : hydrogen-peroxide oxidoreductase, EC 1.11.1.9) system could participate in the oxidation of mercury vapor. Until now, all exposures of intact erythrocytes to mercury vapor had been carried out at approximately normal hematocrit values from 36—50% [2,3,4,6], whereas recently it was shown that, in incubations with low homogenate concentrations, the improved availability of the elemental metal to the tissue increases the rate of Hg uptake [7]. Furthermore, in most of the previous investigations, the specific activity of erythrocyte catalase was not measured and, in none of them, that of glutathione peroxidase.

We have reexamined the question of how red blood cells perform the oxidation of elemental mercury, whether GSH peroxidase plays a role, and to which extent catalase contributes to this either by the peroxidase reaction or by release of nascent oxygen from the catalase reaction. In general, all evidence obtained so far suggests that the oxidation of mercury is linked closely to the intracellular reactions of H_2O_2. To select among these reactions, three approaches seemed feasible: (1) variation of the H_2O_2 concentration; (2) specific inhibitors of the enzymes; (3) selection of genetically different erythrocytes according to their enzyme activities.

Materials and Methods

Erythrocytes and chemicals

Erythrocytes were obtained from human blood (supplied by the hospital blood bank) or from common farm ducks which were bled from the wing vein. The third and fourth group of red cells were obtained by heart puncture under ether anesthesia from normal (Cs^a) or acatalasemic (Cs^b) mice from the colonies of Dr. R.N. Feinstein, Argonne National Laboratory, Argonne, Illinois [8]. The following reagents were supplied by Sigma Chemical Company, Saint Louis, Missouri: 3-amino-1,2,4-triazole, GSH, GSSG reductase, NADPH, EDTA, NaN_3 and Drabkin's reagent. Iodoacetic acid was obtained from Eastman Kodak, Rochester, New York. All other chemicals were of the highest purity commercially available.

Assay for mercury and hemoglobin content

Red cells were washed 3 times in isotonic saline and suspended in a modified Krebs-Ringer solution which contained 1 g/l glucose. 2 ml 1% cell suspension were placed in the main compartment and 0.1 ml metallic mercury in the side arm of Warburg vessels (without center well). All vessels were closed with rubber stoppers and incubated for 45 min at 37°C in a Dubnoff metabolic incubator at a shaking rate of 80 strokes/min. At the end of the incubation period, the suspensions were centrifuged and, in some experiments, the Hg concentration was determined in the supernatant according to Magos and Clarkson [9]. A hemolysate was prepared from the packed cells by addition of 1.0 ml. An aliquot of this lysate was always taken to estimate hemoglobin [10] and another aliquot was used in some series to measure the enzyme activities. The

remaining lysate was then diluted with an appropriate volume of 0.9% NaCl to carry out the analysis of intracellular total mercury [9].

As shown by Oshino et al. [11], it is of prime importance to generate H_2O_2 at low and continuous rates in order to form only catalase complex I, since at higher generation rates, the evolution of O_2 may also occur. In the work reported here, 0.01—0.5 mM solutions of H_2O_2 were infused at a rate of 0.15 ml/h by means of a micro pump (type "Unita", B. Braun, Melsungen, G.F.R.) and a thin polyethylene tubing passing through the rubber stoppers into the incubation medium. By these precautions, local and transient high concentrations could be avoided.

Enzyme assays

Catalase activity was determined with an oxygen electrode in a closed and pressurized reaction vessel and calculated from the first-order rate constant (k) of the catalatic breakdown of H_2O_2 [12]. The specific activity (k_{Hb}) was expressed as the rate constant per hemoglobin concentration in the reaction vessel [$s^{-1} \cdot g^{-1} \cdot ml$]. GSH peroxidase activity was estimated as the consumption of NADPH in the coupled reaction described by Paglia and Valentine [13], the units being μmol NADPH $\cdot$ $min^{-1} \cdot g^{-1}$ Hb. Before any blood samples were processed for the exposure to Hg vapor, the activities of the two enzymes in the red cells were measured to exclude any deviating samples.

Results

Hydrogen peroxide generation and mercury uptake

Initially, the normal specific activities of the enzymes were determined (Table I). The results indicate that human, duck and normal and acatalasemic mouse red cells offer a wide range of activities of catalase and GSH peroxidase. Noteworthy is the very high activity of the latter in mouse erythrocytes (similar in both genetic types).

The dependence of the rate of mercury uptake from the H_2O_2 generation rate was investigated for the various types of red cells. Human erythrocytes showed a rapid rate of increase in Hg content up to an addition of 5.63 nmol H_2O_2 and beyond that, a slower rate of uptake. Also, the cells from normal

TABLE I

NORMAL SPECIFIC ACTIVITIES OF CATALASE AND GLUTATHIONE PEROXIDASE IN RED CELLS FROM VARIOUS SPECIES

Means ± S.E. from (n) determinations.

	Erythrocytes			
	Human	Duck	Cs[a] mice	Cs[b] mice
Catalase activity ($s^{-1} \cdot g^{-1} \cdot ml$)	354 ± 13 (18)	1 ± 0.1 (6)	174 ± 6.4 (9)	13 ± 0.3 (6)
GSH peroxidase activity ($\frac{\mu moles\ NADPH}{min \cdot g\ Hb}$)	22 ± 2.5 (6)	88 ± 8.5 (3)	297 ± 11.5 (3)	305 ± 8.7 (3)

mice oxidized more mercury with increasing addition of H_2O_2 (Fig. 1). In the erythrocytes from ducks and acatalasemic mice, the uptake could not be stimulated by H_2O_2. It may be seen that the rate of uptake depends upon the generation of peroxide and upon the activity of the red cell catalase, but does not reflect the GSH peroxidase activity (Table I).

Catalase may be involved indirectly in the oxidation of mercury vapor via the release of nascent oxygen. The catalatic reaction is thought to begin when the ratio of H_2O_2 generation rate to catalase-heme concentration exceeds a value of 60/min [11]. Under our experimental conditions, this value would correspond to the infusion of 111 nmol H_2O_2/mg hemoglobin per 45 min in a 1% suspension of human red cells, with approximately 2.5 mg hemoglobin/ml, if one assumes for the pure human enzyme a specific activity of $3.4 \cdot 10^7$ ($s^{-1} \cdot M^{-1}$), [14] and 4 hemes per molecule [15]. On account of their lower catalase concentrations, the critical H_2O_2 infusion rate decreased to 55 for normal mice, to 4.1 for acatalasemic mice and to 0.3 for duck erythrocytes. However, for the last 3 types of cells, the actual generation rate of peroxide needed for oxygen evolution may exceed the figures given above, since the highly active GSH peroxidase may reduce the amount of intracellular H_2O_2 available to catalase.

To test whether oxygen evolution was absent in our experiments, the O_2 tension in 1% suspensions of human red cells not exposed to mercury vapor was continuously recorded with the oxygen electrode while hydrogen peroxide was generated at 11.25 nmol/(mg Hb · 45 min). No change in pO_2 was observed before, during and after the 45 min period of H_2O_2 infusion. This indicates that nascent oxygen was not involved in the oxidation of mercury vapor. Nonenzymatic oxidation of mercury within the cells does not account for the Hg uptake as can be seen from the duck and Cs^b erythrocytes (Fig. 1). A further series of incubations was carried out to assess the direct oxidation of elemental Hg by hydrogen peroxide in the absence of enzymes. Indeed, the mercury concentra-

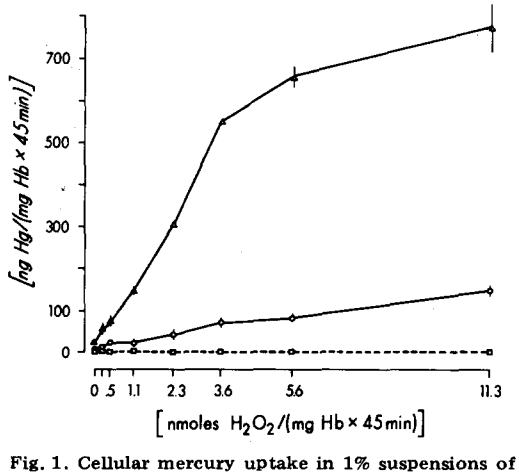

Fig. 1. Cellular mercury uptake in 1% suspensions of erythrocytes from various species in dependence of the H_2O_2 generation rate. Means from 4 experiments for human red cells (△) and from 3 experiments for normal (○) and acatalasemic mice (□) and ducks (- - - - -). S.E. shown only where greater than symbols.

tion in the buffer only increased 2.4 times upon infusion of the highest amount of H_2O_2 (Table II). In the supernatant from red cell suspensions, the mercury concentration actually decreased at high H_2O_2 generation rates.

Effect of mercury on enzyme activities

At the highest rates of peroxide generation, the concentration of mercury in the red blood cells at the end of the 45 min incubation period approached values as high as 1 mM (Table II). Similar concentrations of mercuric chloride have been known to cause enzyme inhibition in vitro [16]. The inhibition of catalase or other enzymes possibly involved in the oxidation of mercury might explain the fact that the rate of uptake slows down beyond 600 ng Hg/mg hemoglobin per 45 min (Fig. 1). It was decided to test catalase and GSH peroxidase activities in 1% suspensions of human red cells which were divided in two identical groups except for the presence or absence of mercury vapor. The hemolysates prepared after the incubations served for an immediate determination of enzyme activities as well as for the estimation of mercury in samples exposed to the vapor. As shown in Table III, there is a slight decrease in catalase activity with increasing H_2O_2 generation rate. However, at the same generation rate, the enzyme is not inhibited by the presence of Hg vapor as compared to the sample without the vapor. The activity of glutathione peroxidase remained also unchanged except at the maximal rate of mercury uptake where a significant inhibition occurred (Table IV). It may be inferred from these findings that enzyme inhibition by mercury is negligible at H_2O_2 generation rates below 5.63 nmol/mg hemoglobin per 45 min.

Effects of catalase and glutathione peroxidase inhibition on mercury uptake

The inhibition of the cellular mercury uptake was studied by the use of two other substrates for the peroxidase reaction with complex I (ethanol and for-

TABLE II

ENZYMATIC VERSUS NONENZYMATIC MERCURY OXIDATION

Columns (a) and (b) represent red cells and supernatants from the same incubations of 1% suspensions of human erythrocytes. Column (c) shows incubations of buffer only. For comparison all uptakes are given per ml; the cellular Hg concentration was calculated from the uptake per mg hemoglobin by assuming $29 \cdot 10^{-9}$ mg hemoglobin/red cell and a single-cell volume of $88 \cdot 10^{-12}$ ml [20]. Means ± S.E. from (n) experiments.

H_2O_2 added in 45 min (nmol/mg Hb or nmol/0.4 ml)	Mercury uptake in 45 min			t-test (b)—(c) $2P$
	(a)	(b) with red cells	(c) no red cells	
	μg Hg / ml red cell (n = 3)	ng Hg / ml medium (n = 3)	ng Hg / ml medium (n = 4)	
—	5.9 ± 2.6	31 ± 1 *	34 ± 8 **	>0.8
2.25	77.6 ± 4.1	27 ± 2	41 ± 6	>0.1
5.63	181.3 ± 12.7	20 ± 3	64 ± 11	<0.025
11.25	208.0 ± 13.9	19 ± 3 *	82 ± 13 **	<0.02

* $2P < 0.02$.
** $2P < 0.025$.

TABLE III

EFFECT OF MERCURY VAPOR ON CATALASE ACTIVITY IN 1% SUSPENSIONS OF HUMAN RED CELLS

Simultaneous data from one experiment; means ± S.E. for the specific activity form 3 determinations.

H_2O_2 generation rate ($\frac{\text{nmol } H_2O_2}{\text{mg Hb} \cdot 45 \text{ min}}$)	Hg uptake ($\frac{\text{ng Hg}}{\text{mg Hb} \cdot 45 \text{ min}}$)	Catalase activity ($s^{-1} \cdot g^{-1} \cdot ml$)	
		With Hg	Without Hg
—	41.7	370.4 ± 1.5	367.5 ± 10.3
1.13	157.8	355.1 ± 5.6	362.7 ± 6.7
2.25	313.5	354.3 ± 15.3	344.3 ± 10.1
5.63	540.5	348.9 ± 3.3	355.3 ± 3.8
11.25	737.1	328.5 ± 17.7	334.0 ± 3.5

mate [17]) and of aminotriazole as an irreversible inhibitor. The rates of mercury uptake were reduced considerably in the presence of 2 mM formate and even more so in the presence of 2 mM ethanol (Fig. 2). The displacement of the uptake curves to lower values suggests that the capacity of the peroxidase reaction at a given H_2O_2 generation rate is divided between the oxidation of these substrates and of mercury.

Aminotriazole did not inhibit the mercury uptake at low rates of peroxide generation. Only at higher rates did the reduction in uptake become more prominent. This could be due to the fact that the inhibition of the enzyme depends on the amount of complex I formed [18]. A further identical experiment, except for the omission of mercury showed that the inhibition of red cell catalase by 60 mM aminotriazole increased with increasing H_2O_2 generation rates. Hence, it could be demonstrated that the difference in mercury oxidation between the control and aminotriazole-treated erythrocytes correlates sufficiently with the inhibited catalase activity ($r^2 = 0.94$) (Fig. 3). The close correlation between catalase activity and Hg oxidation and the fact that the linear regression line passed nearly through the origin, lends further support to the conclusion that pathways of oxidation other than the H_2O_2-catalase system are quantitatively insignificant.

TABLE IV

EFFECT OF MERCURY VAPOR ON GLUTATHIONE PEROXIDASE ACTIVITY IN 1% SUSPENSIONS OF HUMAN ERYTHROCYTES

Simultaneous data from one experiment; means ± S.E. for the specific activity form 3 determinations.

H_2O_2 generation rate ($\frac{\text{nmol } H_2O_2}{\text{mg Hb} \cdot 45 \text{ min}}$)	Hg uptake ($\frac{\text{ng Hg}}{\text{mg Hb} \cdot 45 \text{ min}}$)	GSH peroxidase activity (μmol NADPH/min · g Hb)	
		With Hg	Without Hg
—	34.0	15.9 ± 1.0	17.5 ± 1.2
1.13	114.4	17.8 ± 1.1	17.0 ± 1.7
2.25	243.3	17.9 ± 1.5	18.6 ± 1.3
5.63	502.5	16.9 ± 1.9	16.4 ± 2.0
11.25	631.8	13.7 ± 0.4 *	17.9 ± 0.6 *

* t-test: $2P < 0.005$.

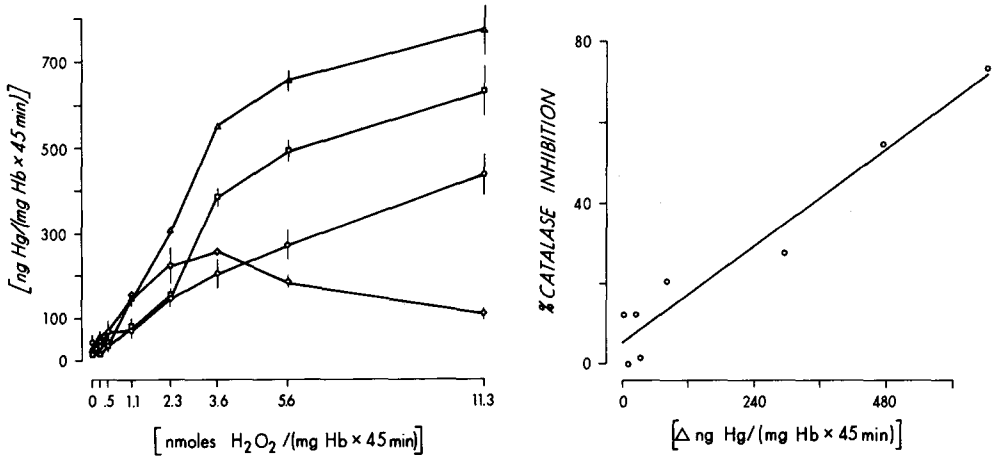

Fig. 2. Cellular mercury uptake in 1% suspensions of human erythrocytes in the presence of various inhibitors. Means from 4 experiments for the control (△) and from 3 experiments for 2 mM formate (□), 2 mM ethanol (○) and 60 mM aminotriazole (◇). S.E. shown only where greater than symbols.

Fig. 3. Inhibition of cellular mercury uptake in 1% suspensions of human red cells and of catalase activity by 60 mM aminotriazole. Each point represents the mercury uptake and the enzyme activity at one H_2O_2 generation rate. The inhibition of the uptake is expressed as the difference from means of 4 experiments without and 3 with aminotriazole; means of catalase inhibition from 3 determinations. Line represents the regression; correlation coefficient $r^2 = 0.94$.

In contrast to the close correlation seen in Fig. 3, the difference in the rate of Hg uptake in human versus Cs^a mouse cells did not correspond to that expected from the difference in catalase activities (Fig. 1, Table I). The rate of uptake by Cs^a was relatively too low as compared to human cells. One explanation is that the high GSH peroxidase activity in the mouse cells diverted hydrogen peroxide from the catalase pathway, which could be tested by a GSH peroxidase inhibitor. It was found that the GSH peroxidase activity dropped in the presence of 1 mM iodoacetic acid to 4.2% of the control after 10 min, to 2.6% after 20 min and to 1.6% after 45 min (means from three determinations). But because the same iodoacetic acid concentration raised the basic rate of mercury oxidation to 250 ± 12 ng Hg/(mg Hb · 45 min), (means ± S.E., $n = 3$) in the absence of exogenous peroxide (this could be due to an increase in endogenous H_2O_2) only the difference between the basic uptake and that under addition of peroxide was considered in these experiments. The results indicated that iodoacetate increased the H_2O_2-stimulated mercury oxidation by Cs^a cells to rates between those of untreated Cs^a cells and of human erythrocytes (Fig. 4).

It was also attempted to evaluate the stoichiometry of the mercury oxidation in the peroxidatic reaction from the data in Fig. 4. Theoretically, a 1 : 1 consumption can occur between peroxide and substrates for complex I, and this is approximated by the steepest part of the uptake curve by human red cells. The regression calculated from the eight values at the peroxide generation rates of 2.25 and 3.6 resulted in 0.9 unit mercury oxidized for one unit hydrogen peroxide added.

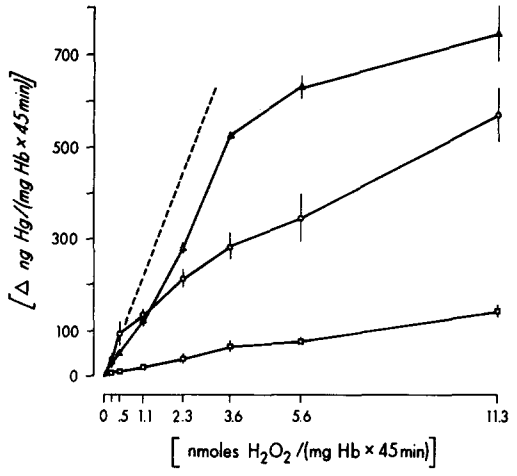

Fig. 4. Mercury uptake by red cells from normal mice in 1% suspensions with (○) and without (□) 1 mM iodoacetic acid. The rates of uptake in the presence of H_2O_2 generation were diminished by the basic uptake when no peroxide was added. Means from 3 experiments; S.E. indicated only where greater than symbols. For comparison, human red cells are also shown (△). The broken line represents the theoretical 1 : 1 stoichiometry between H_2O_2 and substrates for the peroxidase reaction.

Discussion

As observed with liver homogenates [7], the availability of the elemental Hg to the oxidizing system could be greatly enhanced by lowering the tissue concentration. Considering erythrocytes, this offers a wider range for cellular mercury uptake. Within this range, the variation of the hydrogen peroxide addition allowed a control of the Hg oxidation rate. In earlier investigations on the biological oxidation of mercury, hydrogen peroxide was generated either by addition of glucose and methyleneblue to red cell incubations [3,4,6], by diffusion of H_2O_2 vapor into a solution containing glutathione and catalase [5], or by stepwise injection of diluted H_2O_2 in incubations of liver homogenates [7]. All these procedures either did not allow a correct quantitation of the peroxide, or failed to take into account a possible interference of gaseous H_2O_2 with mercury vapor, or did not prevent transiently high concentrations of H_2O_2. The method used here provided for a continuous, controlled and slow addition of the peroxide, a prerequisite for constant concentrations of catalase-complex I [11].

Thus, in connection with the determination of catalase activity, it could be demonstrated that the mercury uptake depends upon the catalase concentration at a given H_2O_2 generation rate or varies with the peroxide generated in the same type of cells (Fig. 1). Evidence was also furnished locating these interactions clearly in the peroxidase range of the catalase-H_2O_2 reaction. Since at high peroxide generation rates the uptake by human red cells already tended to level off, it seems unlikely that an excess of H_2O_2 and the subsequent evolution of nascent oxygen could achieve another sizeable increase in mercury oxidation.

As for a possible participation of the glutathione-GSH peroxidase system, the in vitro experiments performed to this purpose by Nielsen-Kudsk [5] had an

inherent difficulty. Because no GSH peroxidase was present in these incubations, only the nonenzymatic oxidation of GSH by hydrogen peroxide was estimated, which proceeds much slower than the enzymatic oxidation [13]. In addition, nonenzymatic and enzymatic oxidations might form glutathione radicals in different ways. But, despite maintaining the cellular coexistence of GSH and enzyme in the experiments reported here, GSH-Px and its stimulation by H_2O_2 did not have any direct effect on the mercury oxidation as shown with the duck erythrocytes. On the contrary, it can act indirectly as an inhibitor of mercury uptake by consuming H_2O_2. This was made clear by treatment of red cells from normal mice with iodoacetate. However, the comparison of Cs^a and Cs^b erythrocytes reveals that even the highest GSH peroxidase activity cannot prevent the mercury vapor from becoming oxidized, provided there is sufficient catalase available for the formation of complex I.

The fact that those compounds which are known to react with the catalase-H_2O_2 intermediate all inhibited the uptake of mercury, also supports the view of the peroxidase reaction being the essential process for the oxidation of the metal. According to Stock [19], the concentration of elemental mercury does not exceed 0.1 μM in an aqueous medium, but the concentration of the alternative substrates had to be in the millimolar range in order to depress the Hg uptake (Fig. 2). Only future studies will determine why mercury should be such a highly preferred substrate by complex I and whether any mercury intermediates can be identified in the peroxidase reaction.

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