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ROLE OF CATALASE IN THE OXIDATION OF MERCURY VAPOR

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Abstract—In human red blood cells the slow rate of production of hydrogen peroxide limits the rate of oxidation of mercury vapor. When H_2O_2 was added to blood samples, the rate of mercury uptake increased six times, but the inhibitory effect of KCN was less pronounced than in samples without H_2O_2, possibly because H_2O_2 tends to destroy cyanide. Only in the absence of exogenous H_2O_2 did the inhibition of oxidation of mercury vapor by different concentrations of KCN parallel the inhibition of catalase. Addition of peroxide to liver homogenates was without effect. When 20% (w/v) liver homogenates were exposed to mercury vapor at 37°, neither acatalasemia in mice nor pretreatment of rats with 2 g/kg of aminotriazole produced an appreciable decrease in the uptake of mercury during the 90-min incubation period. However, it was clear that catalase was responsible for the oxidation of mercury from the correlation ($r^2 = 0.85$) between catalase activity and mercury uptake when 0.4 mg liver in 2 ml of incubation medium was exposed to mercury vapor. With higher concentrations of homogenates the availability of mercury limited the oxidation process. Experiments with horseradish peroxidase, beef liver catalase and inorganic catalysts of H_2O_2 decomposition indicate that the elemental mercury atom serves as an electron donor for complex I of catalase which is formed from the first reaction of catalase with H_2O_2. Other possible pathways of oxidation such as oxidation of elemental mercury by nascent oxygen released from H_2O_2 do not appear to be important.

Mercury vapor, due to its high diffusibility and lipid solubility, easily crosses biological membranes [1]. Suspensions of red blood cells in the presence of oxygen rapidly oxidize vapor (Hg^0) to divalent ionic mercury, (Hg^{2+}) [2]. Despite this rapid oxidation, sufficient amounts of inhaled vapor remained in the blood stream to reach the blood–brain and placental barriers [3, 4]. Once the vapor crosses into tissues, it is "trapped" by oxidation to ionic mercury. This model for the metabolism of inhaled mercury vapor [5] explains the finding that deposition of mercury in brain and in the fetus is much greater after inhalation of vapor than after an equivalent dose of ionic mercury.

Nielsen-Kudsk [6, 7] presented qualitative evidence that catalase played a role in the oxidation of mercury vapor by suspensions of red blood cells. His studies *in vitro* pointed to the importance of the catalase hydrogen peroxide complex (complex I) as playing a major role in this oxidation.

Nevertheless, the pattern of deposition *in vivo* of mercury vapor does not correspond, even on a qualitative basis, to published values of tissue catalase activities. For example, the initial deposition of inhaled vapor in the lung is approximately six times greater than in the liver [3] whereas the catalase activity in the liver is approximately twenty times greater than in the lung [8]. Furthermore, despite the fact that treatment of animals with aminotriazole greatly reduced catalase activity in the liver, this inhibitor actually increases the fraction of the inhaled mercury deposited in this organ [9].

The present study *in vitro* was aimed at investigating the role of catalase in the oxidation of mercury vapor and to attempt to resolve the apparent contradiction between tissue deposition *in vivo* of mercury and tissue catalase activities.

MATERIALS AND METHODS

All reagents were analytical grade if not otherwise stated. Human blood was supplied by the hospital blood bank and red blood cells were washed three times with Ringer solution (without glucose) immediately before use. Male Sprague–Dawley rats of approximately 200 g body weight and wild type mice of approximately 40 g body weight were used. A colony of mice having normal catalase activity (wild type) and a homozygote mutant having low catalase activity (acatalasemic) was kindly supplied by Dr. Feinstein of the Argonne National Laboratory, IL [10]. The animals were decapitated, exsanguinated and liver homogenates were prepared in Ringer solution (20%, w/v). Some of the rats were given 0.1 to 2.0 g/kg of 3-amino-1,2,4-triazole (Sigma Chem. Co.) 90 min before decapitation.

Samples were incubated in normal Warburg flasks without a central well. Metallic mercury (0.1 ml) was placed in the side arm and the sample in the main compartment. The flasks were glass stoppered and incubated in a metabolic shaking incubator at 37° for

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Table 1. Effect of H_2O_2 on mercury uptake by samples of human red blood cells (10 g/100 ml Hb) and rat liver homogenate (20%, w/v) exposed to mercury vapor for 3 hr at 37°*

H_2O_2	Hg uptake (mean ± S.E.M.) (ng/mg Hb)	(ng/mg liver)
Not added	3.2 ± 0.2	22.7 ± 1.0
	N = 5	N = 5
Added	23.7 ± 1.3†	24.1 ± 1.1
	N = 5	N = 5

* For details of incubation procedure, see Materials and Methods.

† Significantly different from the control by the Student's *t*-test ($P < 0.01$).

various periods of time. When H_2O_2 (1% in 0.1 M phosphate buffer, pH 7.2) or phosphate buffer was to be added to the sample during incubation, a rubber stopper was substituted for the central glass stopper. A volume of 0.1 ml H_2O_2 was injected every 10 min from 10 min on by means of a 1-ml syringe the needle of which passed through the rubber stopper.

Incubation of human red blood cells. The final hemoglobin concentration was 10/100 ml and the final glucose concentration 13.7 mM. The incubation medium consisted of 2 ml of red blood cell suspension in Ringer solution and 0.5 ml phosphate buffer with or without KCN. The stock solution of KCN was made with phosphate buffer (pH 7.2). After exposure to mercury as described above, red blood cells were washed three times with saline (0.85% NaCl, w/v). After the third washing, red blood cells were hemolyzed with distilled water and hemoglobin, and mercury concentrations were estimated.

Incubation of liver homogenates. Appropriate dilutions were made from the 20% homogenates with Ringer solution. Exposure of 2.0-ml volumes of homogenate to mercury vapor was carried out as with human blood cells. After exposure the homogenate was washed out from the flasks with saline and the total mercury content was estimated.

Other incubations. Twice-crystallized bovine liver catalase (Sigma Chem. Co.), horseradish peroxidase (Sigma Chem. Co.), the solution of $FeCl_3$ and the suspension of MnO_2 were made in Ringer. Exposure to mercury vapor was carried out as described above.

Analytical methods. Hemoglobin was estimated by the method of Drabkin and Austin [11] and mercury by the method of Magos and Clarkson [12]. Catalase activity of unexposed blood or liver samples was estimated from the evolution of oxygen. The assay was performed at room temperature with a total volume of 5.5 ml (5.4 ml of 0.01 M phosphate buffer, pH 7, 50 µl of 0.22 M H_2O_2 and 50 µl of enzyme solution) contained in a closed and pressurized reaction vessel. The changes in O_2 concentration were measured with a membrane-covered micro oxygen electrode No. 17026 connected to a 125 A oxygen analyzer, both from Instrumentation Laboratory, Lexington, Ma. The output signal of the analyzer was fed into a strip-chart recorder. As described earlier [13], the increase in pO_2 within 15 sec can be used to calculate the first-order rate constant K [sec^{-1}] according to Chance and Maehly [14]. This is related to the enzyme–protein (g/ml) concentration in the assay to express specific activity in sec^{-1} g^{-1} ml for experiments with crystalline catalase. For the experiments with red cells the protein concentration was expressed in g hemoglobin/ml, whereas in the case of liver homogenates the organ wet weight/ml was used.

In experiments with cyanide the amount of inhibited catalase, i.e. the enzyme–CN complex, is dependent on its dissociation constant, $K_I = [FeOH] \times [HCN]/[FeCN]$. As shown by Chance [15], this constant does not change between 7 and 98% saturation with cyanide. Accordingly, to maintain a constant [FeOH]/[FeCN] ratio, we used the same inhibitor concentration and the same buffer as in the incubation mixtures.

RESULTS

The data in Table 1 indicate that, in human red blood cells, exogenous peroxide accelerates significantly the uptake of mercury vapor whereas in the liver no such effect was observed. In suspensions of red blood cells, the 3-hr uptake of mercury/mg of Hb increased from 3.2 ng in the absence of H_2O_2 to 23.7 ng in the presence of H_2O_2. The corresponding values for uptake by liver homogenate were 22.7 and 24.1 ng/mg of liver respectively.

The similarity of the rates of uptake by liver homogenates in the absence or presence of exogenous H_2O_2 suggested that the rate of diffusion of mercury vapor was probably rate determining. Observations of Hg vapor uptake by liver homogenates from control and aminotriazole-treated rats also support this conclusion (Fig. 1). The catalase activity was 44.2 ± 6.3 [sec^{-1} g^{-1} ml] (N = 4) in the case of control rats and 3.1 ± 0.7 [sec^{-1} g^{-1} ml] (N = 4) in the case of aminotriazole-pretreated rats. The large inhibition of catalase activity (93 per cent) caused no appreciable decrease in the mercury vapor uptake when a high concentration of homogenate

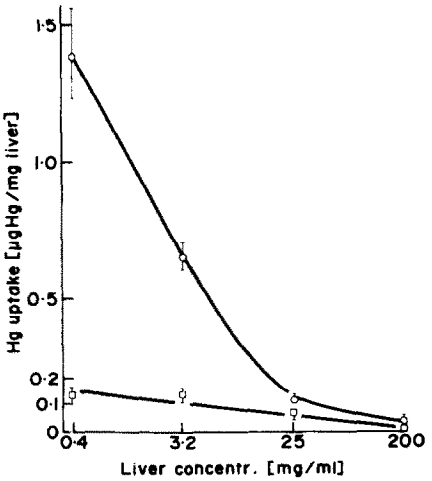

Fig. 1. Mercury uptake by liver homogenates of control rats (circles) and animals pretreated with 2.0 g/kg of aminotriazole (squares) 90 min before the animals were killed. Liver (200 mg) in 1 ml volume or higher dilutions was exposed for 90 min to mercury vapor at 37°. Every point represents the geometric mean ± S.E.M. of four estimations.

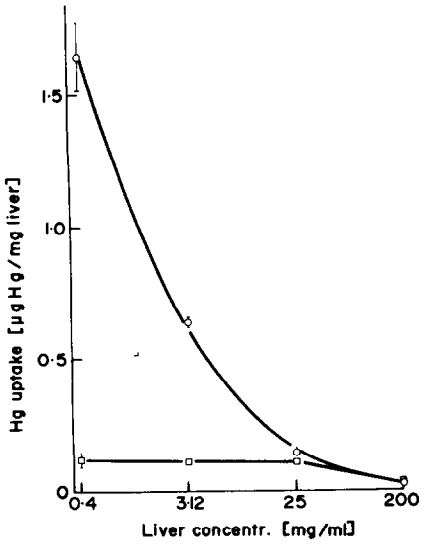

Fig. 2. Mercury uptake by liver homogenates of control (circles) and acatalasemic (squares) mice. Every point represents the geometric mean with the S.E.M. for three observations at 200 and 0.4 mg/ml of homogenate. At other homogenate concentrations, the points are the geometric means of two observations.

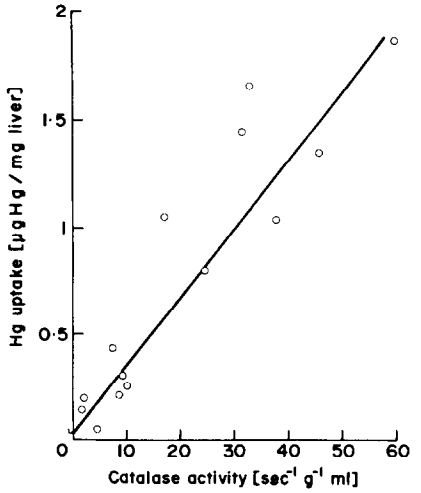

Fig. 3. Correlations between catalase activity (sec^{-1} g^{-1} ml) and mercury uptake (µg Hg/mg of liver) of liver homogenates obtained from controls and rats treated with 0.1 to 2.0 g/kg of 3-amino-1,2,4-triazole. Two ml of 0.04% liver homogenates was exposed to mercury vapor for 90 min at 37°. The line was drawn according to the equation $y = 0.11 + 0.031\,x$ calculated by least square linear regression analysis from the data points.

(200 mg/ml) was exposed to mercury vapor. However, the effect of enzyme inhibition became apparent with increasing dilutions of the homogenate, suggesting that catalase activity had now become the rate-determining factor in the uptake of mercury.

A similar pattern was seen in homogenates from wild type and acatalasemic mice (Fig. 2). The catalase activity of the pooled liver homogenates from wild type mice was 66.8 and that of acatalasemic mice was 41.2 [sec^{-1} g^{-1} ml]. No difference between the two groups in mercury uptake was found when the highest concentration of homogenate (200 mg/ml) was exposed to mercury. The mercury uptake/mg of liver from the wild-type mice, like that of liver from the control rats, increased with dilution. An increase in mercury vapor uptake/mg of liver was observed only with the first dilution in homogenates from acatalasemic mice.

When low concentrations of rat liver homogenates (0.4 mg/ml) were used, a close correlation was seen between catalase activity and the rate of uptake of mercury vapor ($r^2 = 0.85$, Fig. 3).

A close relationship between catalase activity and mercury vapor uptake was also seen in red cell suspensions without H_2O_2 in the presence of cyanide (Fig. 4). In these experiments catalase activity was reduced by treatment of the cells with KCN instead of aminotriazole since inhibition by the latter requires the availability of hydrogen peroxide. In the presence of added H_2O_2, the diffusion of mercury vapor was rate determining and the residual catalase activity was high enough to carry out the oxidation.*

The effect of horseradish peroxidase and bovine liver catalase on the oxidation of mercury is compared in Fig. 5. On a weight basis, the efficiency of peroxidase is very small and would even be smaller on a M basis. The difference between the two enzymes increases with increasing dilution.

The data in Table 2 also point to the importance of the catalase–hydrogen peroxide complex (complex I) in the oxidation of mercury vapor. Catalase without H_2O_2 produced only a slight increase in vapor uptake as compared to Ringer solution or to Ringer solution containing serum albumin. In the presence of H_2O_2, catalase produced a large acceleration of oxidation of the vapor.

Nascent oxygen released by the catalatic decomposition of H_2O_2 might be responsible for the rapid oxidation of the vapor. To test this possibility, two inorganic materials ($FeCl_3$ and MnO_2) that catalyze

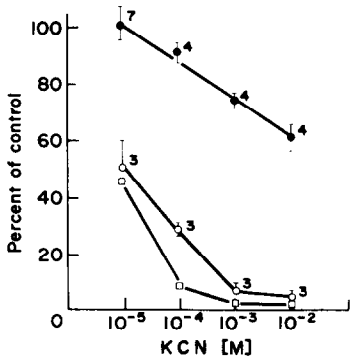

Fig. 4. Effects of KCN on catalase activity (squares) and on the uptake of mercury of human red blood cells in the presence (solid circles) and in the absence (empty circles) of exogenous hydrogen peroxide. Activities were calculated on the basis of hemoglobin concentration. Ordinate: specific activity and the uptake, each in per cent of controls without KCN. Incubation time: 90 min, temperature, 37°. Control values: catalase activity, 170 ± 1.6 [sec^{-1} g^{-1} ml]. Hg uptake without peroxide, 5.4 ± 0.42, with peroxide: 34.7 ± 0.41 ng Hg/mg of Hb.

* Since a constant H_2O_2 concentration (2 mM) is used in the catalase assay, a possible competition between H_2O_2 and CN for catalase [15] could not be excluded under these experimental conditions.

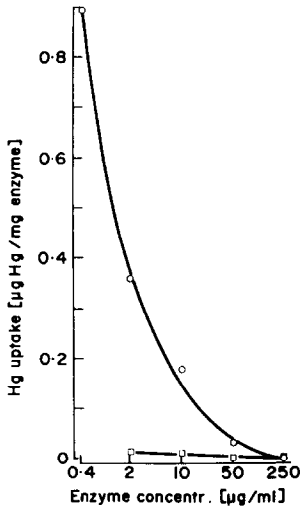

Fig. 5. Effects of bovine liver catalase (circles) and horseradish peroxidase (squares) on the uptake of mercury vapor. The enzymes were dissolved in 2 ml Ringer solution and exposed to mercury vapor for 90 min. After a 10-min incubation and at 10-min intervals, aliquots of 0.1 ml of 1% H_2O_2 (in 0.1 M phosphate buffer, pH 7.2) were injected into the incubation medium. Every point is the mean of two estimations.

the decomposition of H_2O_2 were tested. It can be seen that $FeCl_3$ was able to cause a considerable oxidation of the vapor in the absence of H_2O_2 though this effect was increased by H_2O_2. Thus, we cannot exclude the possibility that the Fe^{3+} was able to oxidize mercury and that H_2O_2 served to recycle the Fe^{2+} to Fe^{3+}. The results with MnO_2 were clear. Despite the evolution of O_2 elicited by MnO_2 from breakdown of H_2O_2, no significant increase in mercury vapor uptake was seen as compared to Ringer and H_2O_2 alone.

An apparent discrepancy should be noted between mercury uptake in liver homogenates from acatalasemic versus wild-type mice and normal rats. From the observed catalase activities, it should be expected that uptake by the most diluted suspension (0.4 mg/ml) from acatalasemic livers would be about 1.25 μg/mg but it was approximately ten times less (Fig. 2). The most likely reason for this discrepancy is the fact that catalase in acatalasemic mice is progressively inactivated when incubated at 37° [18, 19].

DISCUSSION

Our findings *in vitro* are in agreement with and extend those of Nielsen-Kudsk [7], indicating that the production of hydrogen peroxide is the rate-limiting step in the ability of red cells to oxidize mercury vapor. Nielsen-Kudsk found that stimulation of hydrogen peroxide production in red cells by treatment with methylene blue/glucose and/or with agents such as menadione greatly stimulated the uptake of mercury vapor. Earlier observations by Clarkson *et al.* [2] on the stimulatory effect of increased oxygen tension on mercury vapor uptake by red cells may also be interpreted as resulting in increased hydrogen peroxide production. Nielsen-Kudsk's findings [6] that methanol and ethanol, and our findings that potassium cyanide, inhibit mercury vapor uptake by red cells are compatible with complex I being the active catalase species responsible for the oxidation of mercury vapor. Our results also indicate that, in liver tissue, complex I is responsible for the oxidation of mercury vapor.

However, our findings suggest that the availability of mercury vapor may become the rate-determining step in the oxidation process in situations where hydrogen peroxide production is high, such as in concentrated liver homogenates or in situations where hydrogen peroxide is provided externally. Thus, in the interpretation of the role of catalase in the oxidation *in vivo* of inhaled mercury vapor, at least three factors must be taken into account: (1) the concentration of the enzyme in the tissue, (2) the endogenous production of hydrogen peroxide, and (3) the availability of mercury vapor to get to the site of oxidation. In the case of red blood cells *in vivo* it appears that the production of hydrogen peroxide is rate determining as alcohol can result in lower rates of deposition of inhaled mercury vapor in the red cells [20].

Table 2. Uptake of mercury vapor by ringer solution with different supplements*

Supplements to Ringer solution			Uptake of mercury vapor in 90 min† (mean)		
Catalyst	H_2O_2	No. of estimations	Hg (μg)	Hg (μg/μmoles protein)	Hg (μg/μmoles Fe)
Catalase (0.5 mg)	+	2	4.4	2200	733
	−	3	0.27	135	45
Peroxidase (0.5 mg)	+	2	0.6	48	48
FeCl (40 mg)	+	3	5.4		0.037
	−	2	1.8		0.012
MnO_2 (40 mg)	+	3	0.12		
Albumin (0.5 mg)	−	2	0.03	4.1	
None	+	3	0.18		
	−	4	0.05		

* Ringer solution 2 ml was exposed to Hg vapor as described in Materials and Methods. Incubation time was 90 min. H_2O_2 addition resulted in a final volume of 2.8 ml.

† The calculations assume a mol. wt of 240,000 and three hematin groups for catalase [16], a mol. wt of 40,000 and one hematin group for horseradish peroxidase [17], and a mol. wt of 60,000 for albumin.

In contrast, the liver has a plentiful supply of H_2O_2 and most of the catalase is in the form of complex I [21]. Thus, as we have demonstrated in liver homogenates, the catalase activity in the intact liver is probably so high that the delivery of mercury will be rate determining. Thus, treatment of animals with catalase inhibitors should not affect the rate of mercury oxidation. The increase in the proportion of the body burden deposited in the livers of aminotriazole-treated animals may therefore be understood as the result of the interplay of two factors: (1) liver catalase activity never falls so low as to be rate determining in the oxidation of vapor, and (2) the reduced rates of oxidation in extrahepatic tissues due to aminotriazole treatment allow more inhaled vapor to reach the oxidation sites in liver tissue.

A possible mechanism for oxidation of mercury vapor has already been mentioned, namely that nascent oxygen released from the decomposition of hydrogen peroxide oxidizes the vapor directly. However, the mechanism involving donations of an electron pair by metallic mercury to the catalase–H_2O_2 complex seems to explain the data reported in this paper better than other possible mechanisms that we have considered as explanation for the oxidation of metallic mercury by tissues.

Deisseroth and Dounce [22] have described the overall reaction between catalase and hydrogen peroxide, leading to the formation of oxygen and water in equations listed below:

$$Fe^{3+}OH + H_2O_2 = Fe^{3+}OOH + H_2O \quad (1)$$

$$Fe^{3+}OOH + H_2O_2 = Fe^{3+}OH + H_2O + O_2 \quad (2)$$

The reaction is believed to involve the transfer of a hydride ion from the second hydrogen peroxide molecule to catalase complex I and the fact that the oxygen molecule comes from this same molecule of hydrogen peroxide, thus involving the two-electron transfer. It seems reasonable that the oxidation of mercury vapor could be described by a reaction with catalase complex I involving a two-electron transfer as indicated in the reaction below.

$$Fe^{3+}\ OOH + Hg^0 = Fe^{3+}OH + HgO \quad (3)$$

The details of the molecular mechanism must be investigated in future research, since it is unclear how an uncharged mercury atom could attach itself to catalase complex I and then be capable of undergoing oxidation to Hg^{2+}.

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