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# TOXIC EFFECTS OF SEVERAL MERCURY COMPOUNDS ON SH- AND NON-SH ENZYMES

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SUMMARY

The toxic effects of several mercury compounds on the activities of horse liver alcohol dehydrogenase (an SH-enzyme) and bovine pancreatic trypsin (a non-SH enzyme) have been studied.

Non-competitive type inhibition of the alcohol dehydrogenase activity was observed, and the $K_i$ values were calculated to be of the order of $10^{-7}$ M, $10^{-6}$ M and $10^{-5}$ M for mercuric chloride, monosubstituted and disubstituted mercury compounds, respectively.

On the other hand, competitive type inhibition of the activity of trypsin was found, and the $K_i$ values were of the order of $10^{-6}$ M and $10^{-4}$-$10^{-5}$ M for mercuric chloride and monosubstituted mercury compounds, respectively (the disubstituted compound could not be tested for technical reasons).

These results indicate that the enzymatic activities depend on the level of substitution of mercury compounds, and the SH-enzyme was found to be more sensitive to every mercury compound tested than the non-SH enzyme. These findings may reflect the degrees of toxicity of these mercury compounds in mammalian tissues.

## INTRODUCTION

Mercury compounds are dangerous environmental pollutants [1, 2] and the effect of mercury intake on health has become a matter of concern. For example, methyl mercuric chloride was found to be a cause of Minamata disease [1], and PMA has been used as an agricultural chemical in Japan [3].

Studies of the effects of these mercury compounds on various enzymes

Abbreviations: EMC, ethyl mercuric chloride; EPM, ethyl phenethynyl mercury; PCMB, *p*-chloromercuribenzoic acid; PMA, phenyl mercuric acetate; PMC, phenyl mercuric chloride.

showed them to be effective SH-enzyme inhibitors [4, 5] and they also inhibited non-SH enzymes [6, 7]. Furthermore, the mode of the inhibition of these two types of enzymes has been studied [8], and it appeared that competitive type inhibition was observed in non-SH enzyme reactions and non-competitive type inhibition in SH-enzyme reactions. However, no detailed kinetic studies of the effects of inorganic, monosubstituted and disubstituted mercury compounds on SH- and non-SH enzymes have been reported.

In this study, the effects of these three types of mercury compounds on an SH enzyme (horse liver alcohol dehydrogenase) and a non-SH enzyme (bovine pancreatic trypsin) were studied and the modes of inhibition and $K_i$ values were determined.

## EXPERIMENTAL

Horse liver alcohol dehydrogenase (1 × crystallized) and bovine pancreatic trypsin (2 × crystallized) were purchased from Nutritional Biochemical Co. (Cleveland, Ohio). NAD from yeast was purchased from Sigma Chemical Co. (Saint Louis, MO). Mercuric chloride ($HgCl_2$) and PCMB were of reagent grade. PMA, EMC, PMC and EPM were the kind gifts of Sankyo Co. (Tokyo). All organic mercury compounds were confirmed to be free from inorganic mercury contamination by alumina column chromatography of the dithizonates [9].

Alcohol dehydrogenase activity was estimated by measuring spectrophotometrically the initial rate of reduction of NAD in the presence of excess ethanol [10]. In this procedure, 0.1 ml of a solution containing 8 mg per ml of NAD in 0.1 M glycine-sodium hydroxide buffer, pH 9.6, and 3.03 ml of semicarbazide solution (0.75 mg per ml) in the same buffer were added to the cuvette of a Shimadzu spectrophotometer. Just before the measurement, ethanol and 0.04 ml of water containing 15 μg of alcohol dehydrogenase were added. The contents were mixed and the absorbance at 340 nm was estimated at intervals of 1 min from zero time. When mercury inhibition was to be estimated, 2.03 ml of semicarbazide solution (1.12 mg per ml) and 1 ml of the solution of mercury compound were added to the cuvette instead of 3.03 ml of semicarbazide solution.

Trypsin activity was determined by measuring spectrophotometrically the initial rate of hydrolysis of benzoylarginine ethyl ester (Sigma Chemical Co.) [11]. In this procedure, 0.2 ml of trypsin solution (15 μg/ml) in 0.01 M acetic acid and 3 ml of benzoyl arginine ethyl ester at various concentrations in 0.05 M phosphate buffer, pH 8.0 were added to a cuvette of the spectrophotometer. The contents were mixed and the absorbance at 253 nm was estimated at intervals of 1 min. When the mercury compounds (1 ml of solution) were added, only 2.0 ml of benzoylarginine ethyl ester solution was added.

Since EPM is poorly soluble in water, Tween 80 (1.6 mg/3.03 ml) was used; it was confirmed to have no inhibitory effect on the alcohol dehydrogenase activity within the concentration range of 0.1—5.0 mg/4 ml.

RESULTS

The oxidation rates of ethanol by alcohol dehydrogenase with and without PMA as an inhibitor are shown in Fig. 1. The reaction was found to be linear during the first 5 min. The degree of inhibition was calculated from the decrease of the tangent of the slope compared to the control.

The inhibitions of alcohol dehydrogenase activity by several mercury compounds are shown in Fig. 2. The mercury compounds used as inhibitors can be classified into three groups, i.e., inorganic, monosubstituted (PCMB, EMC, PMC and PMA) and disubstituted (EPM). As shown in Fig. 2, the strongest inhibition was produced by inorganic mercury, while monosubstituted mercury showed moderate inhibition and disubstituted mercury showed the weakest inhibition.

To determine the modes of inhibition of alcohol dehydrogenase by various types of mercury compounds, Lineweaver-Burk plots were prepared. The tested compounds included mercuric chloride, PMA, PCMB, PMC and EPM. It was found that non-competitive type inhibition occurred in each case. The results obtained for mercuric chloride, PCMB and EPM as examples of the three groups are shown in Fig, 3, a, b, c. The $K_i$ values calculated for the inhibitors are summarized in Table I. It can be seen that the different groups of mercury compounds have different $K_i$ values, i.e., inorganic mercury has a $K_i$ of the order of $10^{-7}$ M, while monosubstituted and disubstituted mercury compounds have $K_i$ values of the order of $10^{-6}$ and $10^{-5}$ M, respectively.

Fig. 4 shows the rates of hydrolysis of benzoylarginine ethyl ester by trypsin in the presence and absence of inhibitor. The rates of hydrolysis were linear as a function of time during the first 6 min.

To study the modes of inhibition of trypsin by various types of mercury

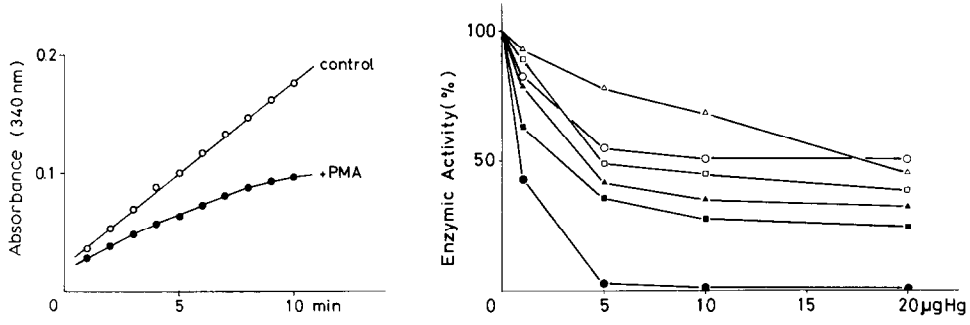


Fig. 1. Oxidation rates of ethanol by alcohol dehydrogenase in the presence and absence of PMA as an inhibitor. The conditions of enzyme assay are described in EXPERIMENTAL.

Fig. 2. Inhibition of alcohol dehydrogenase be several mercury compounds. The enzyme assay method is described in EXPERIMENTAL. △———△, EPM; ○———○, PCMB; □———□, EMC; ▲———▲, PMC; ■———■, PMA; ●———●, $HgCl_2$.

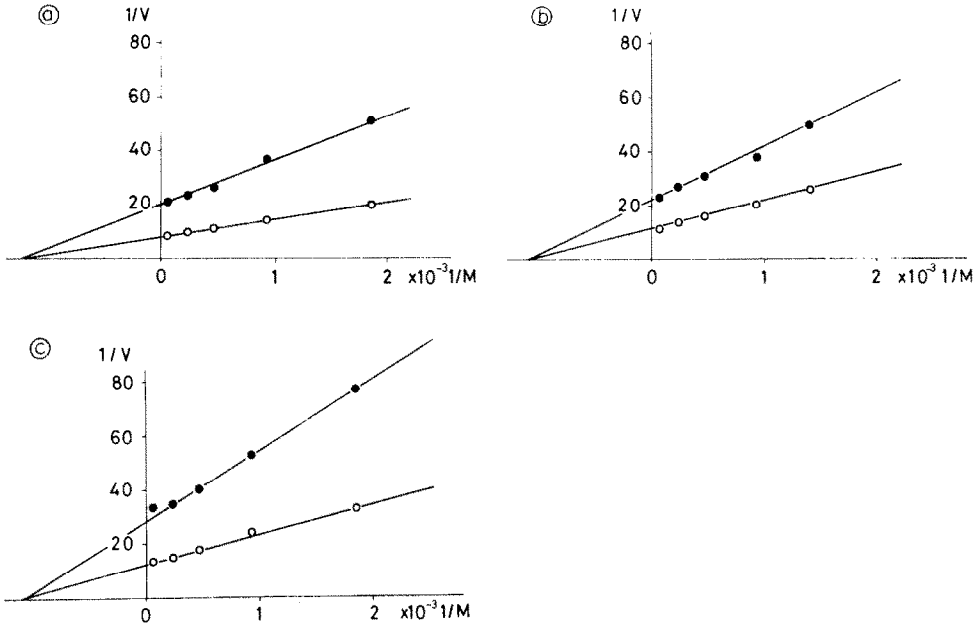


Fig. 3. Lineweaver-Burk plots for alcohol dehydrogenase activity. (a) $HgCl_2$ ; (b) PCMB; (c) EPM. The vertical line indicates 1/absorbance/3 min. The enzyme assay method is described in EXPERIMENTAL.

TABLE I

$K_i$ VALUES OF SEVERAL MERCURY COMPOUNDS WITH HORSE LIVER ALCOHOL DEHYDROGENASE AND BOVINE PANCREATIC TRYPSIN

| Mercury compound | Number of substitution | $K_i$ value (M) |
|---|---|---|
| | alcohol dehydrogenase | |
| $HgCl_2$ | 0 | $5.7 \cdot 10^{-7}$ |
| PMA | 1 | $1.1 \cdot 10^{-6}$ |
| PMC | 1 | $4.3 \cdot 10^{-6}$ |
| PCMB | 1 | $8.0 \cdot 10^{-6}$ |
| EPM | 2 | $1.5 \cdot 10^{-5}$ |
| | trypsin | |
| $HgCl_2$ | 0 | $3.3 \cdot 10^{-6}$ |
| PMA | 1 | $1.2 \cdot 10^{-4}$ |
| EMC | 1 | $8.7 \cdot 10^{-5}$ |

compounds, the effects of mercuric chloride, PMA and EMC were examined by means of Lineweaver-Burk plots. EPM could not be tested because of its strong absorbance at 253 nm. As shown in Fig. 5 a, b, c, all the mercury compounds tested acted as competitive inhibitors, in contrast with the case of alcohol dehydrogenase. The $K_i$ values calculated for each inhibitor are shown in Table I; these values show the same tendency as those for alcohol

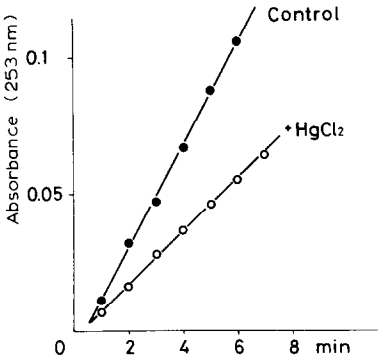


Fig. 4. Measurement of trypsin activity in the presence and absence of mercuric chloride as an inhibitor. The procedure for estimation of the enzyme activity is described in detail in EXPERIMENTAL.

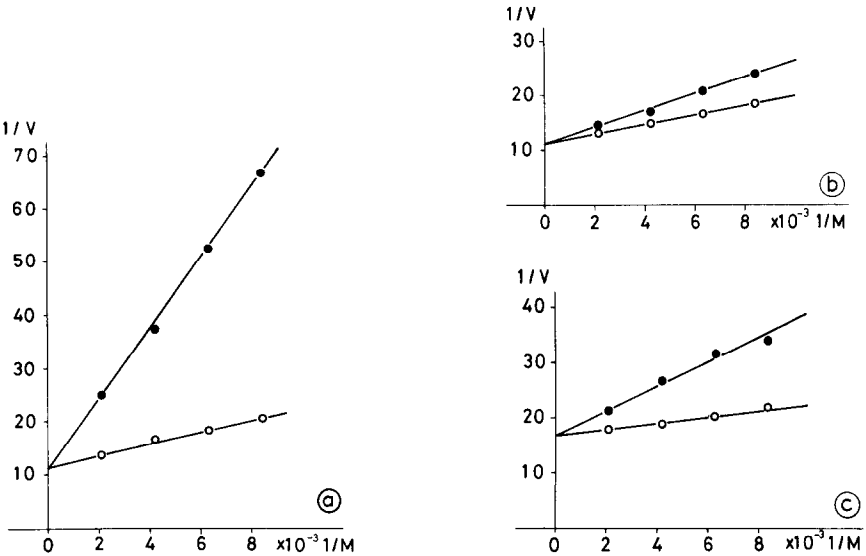


Fig. 5. Lineweaver-Burk plots for trypsin activity. (a) $HgCl_2$ ; (b) PMA; (c) EMC. The vertical line indicates 1/absorbance/4 min. The enzyme assay method is described in EXPERIMENTAL.

dehydrogenase, i.e., inorganic mercury and monosubstituted mercury have $K_i$ values of the order of $10^{-6}$ M and $10^{-4}$ -$10^{-5}$ M, respectively.

## DISCUSSION

The effects of mercury compounds on various enzymatic activities are of considerable interest. Our results show that inorganic mercury has the strongest affinity for both alcohol dehydrogenase (an SH enzyme) and tryp-

sin (a non-SH enzyme), while monoalkyl mercury has a weaker affintiy for the enzymes and dialkyl mercury interacts very weakly with alcohol dehydrogenase (its interaction with trypsin was not studied for technical reasons). The dissociation constants of mercuric chloride are 6.74 ($pK_1$) and 6.48 ($pK_2$), and that of methyl mercuric chloride is 5.45 [12, 13]. These data suggest that the strong inhibitory effect of inorganic mercury on the enzymes may be due to the strong ionic interaction, while the weak interaction of the dialkyl compound may be due to the non-ionizability of this compound.

As regards the possible steric effects of alkyl residues of monoalkyl mercury compounds on the enzymes, several kinds of alkyl residues on monoalkyl compounds have been investigated and no marked differences in $K_i$ values were found. It thus appears that steric hinderance does not have a significant effect in the interaction of the enzymes and the inhibitors.

When the $K_i$ values of these two enzymes for several mercury compounds are compared, the $K_i$ value of alcohol dehydrogenase for a given mercury compound is about ten times lower than that of trypsin. Thus, mercury compounds are able to interact one order of magnitude more strongly with the SH residue than with the active center of trypsin. The association constant between cysteine and mercury compounds is known to be extremely strong [13].

The observation that mercury compounds inhibit alcohol dehydrogenase non-competitively and trypsin competitively is interesting. The non-competitive inhibition might be explained as follows. First, mercury compounds were found to cause dissociation into subunits or denaturation of the enzyme in studies on the interaction between mercury compounds and yeast alcohol dehydrogenase [8]. Second, since Cys 46 and Cys 174 are considered to be located in the active center of the enzyme [14], the interaction of mercury compounds with SH residues might be so strong that the ES complex cannot dissociate smoothly into enzyme + product.

On the other hand, mercury compounds may interact only with the active center of trypsin, because these compounds inhibit the activity of trypsin competitively. Histidine 46 and serine 183 may participate in the active center of trypsin [15] and the dissociation constants ($p\beta_2$) for mercuric chloride with serine and histidine are 17.5 [16] and 21.2 [17], respectively, indicating very high affinities. Thus, mercury compounds could interact with the amino acids at the active center of trypsin.

The present results indicate that when mercury compounds penetrate into living cells, the SH enzymes will be selectively inhibited or denatured in a manner that cannot be reversed by the substrate, while though non-SH enzymes may interact with mercury compounds if the concentration is high enough, the resulting inhibition can be overcome by the presence of sufficient substrate.

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