Effect of Mercury on Nadh and the Protective Role of Oxalacetate

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The many biochemical roles of heavy metals include formation of the active sites of enzymes, enzyme activation, and stabilization of the macromolecular structure of proteins and nucleic acids. Compounds of mercury and other heavy metals are known to initiate configurational changes and to inhibit enzymes as well as many metabolic processes (WEBB 1966), particularly those requiring reduced sulfhydryl groups for activity. Mercuric ions also react readily with linear and cyclic, aryl and alkyl disulfides and solid reaction products were isolated and characterized when the coenzyme α-lipoic acid (1,2-dithiolane-3-valeric acid) and di-n-butyl disulfide served as substrates (BROWN 1969). Enzymes may be protected against mercurials by substrates, coenzymes (TAKENAKA and SCHWERT 1956), reversible inhibitors, thiols, other mercuric complexes (WEBB 1966), or by dietary selenium (MYKKANEN and GANTHER 1974). During studies on the effects of mercurials on enzymes, it was noted that lactate dehydrogenase (LDH) was inhibited by mercuric chloride ($HgCl_2$) to a much greater extent than was malate dehydrogenase (MDH). Also, addition of $HgCl_2$ to all components of the reaction mixture, in absence of the enzyme, caused a rapid decrease in absorbance at 340 nm. Therefore, we carried out further experiments to examine the nature of the interaction between NADH and $HgCl_2$.

MATERIALS AND METHODS

Stock solutions. Fresh solutions of all mercury compounds were made before each experiment (NOYES et al. 1975). Methylmercury chloride (MM) and phenylmercuric acetate (PMA) were dissolved in 20% aqueous ethanol; $HgCl_2$, $CdSO_4$, $ZnSO_4$, and oxaloacetate (OAA) were prepared in saline. The NADH, NAD, and adenine (6-amino purine) were each dissolved in sodium-phosphate buffer (0.05 M, pH 7.4).

The reaction mixture for the LDH assay consisted of 3 ml Na-phosphate buffer (0.05 M, pH 7.4) containing 1.0×10^{-6} M Na-pyruvate, 0.2×10^{-6} M NADH, varying concentrations of MM or $HgCl_2$, and the LDH enzyme. The

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order of addition of reactants, unless stated otherwise, was: Na-phosphate buffer, NADH, Na-pyruvate, MM or $HgCl_2$, and LDH enzyme. A 2 min period was allowed for substrate, coenzyme, and inhibitor to equilibrate, the absorbance at 340 nm was adjusted to 0.4 and then LDH was added to initiate the reaction. The MDH assay reaction mixture consisted of 3 ml Na-phosphate buffer (0.05 M, pH 7.4) containing 0.2×10^{-6} M NADH, 0.6×10^{-6} M OAA, various levels of $HgCl_2$ and the MDH enzyme. Unless stated otherwise the order of addition of reactants was: Na-phosphate buffer, OAA, NADH, $HgCl_2$, and MDH. Again, 2 min were allowed for the OAA, NADH and $HgCl_2$ to equilibrate, the absorbance (340 nm) was adjusted to 0.4 and the MDH enzyme was added. Control experiments were conducted in a similar manner except for omitting the mercury compounds from the reaction mixtures.

Enzyme assays. LDH and MDH activities were determined (25 C) using a Perkin-Elmer, model 124, double beam spectrophotometer following the methods of KORNBERG (1955) and OCHOA (1955), respectively. A unit of activity for LDH or MDH is that which causes an initial rate of oxidation of one micromole of NADH/min in a 3 ml reaction mixture. Specific activities are reported as enzyme units/mg enzyme protein.

Interaction of mercurials with NADH. The NADH, NAD, or adenine was dissolved in 3 ml Na-phosphate buffer (0.05 M, pH 7.4). The order of addition of reactants was altered to: 0.2×10^{-6} M NADH (NAD, adenine), Na-phosphate buffer, 1×10^{-6} M Na-pyruvate, 0.2×10^{-6} M $HgCl_2$ and LDH. The spectral characteristics of the NADH, NAD or adenine, alone and after interaction with various concentrations of mercurials ($HgCl_2$, MM, or PMA) were determined at 25 C. The effects of other compounds ($CdSO_4$, $ZnSO_4$, Na-pyruvate, succinate, tartarate, L-malate and OAA) on their spectral characteristics were also examined.

RESULTS

Enzyme inhibition by mercurials. The comparative effects of $HgCl_2$ and MM on beef heart LDH were studied spectrophotometrically following the oxidation of NADH by pyruvate. When 0.003×10^{-6} M MM was present in the 3 ml reaction mixture LDH activity was not inhibited. However, when the mixture contained 0.03 or 0.3×10^{-6} M MM, the enzyme activity was inhibited 11.7 and 41.7% respectively, compared to the control. When 0.0003×10^{-6} M $HgCl_2$ was added to the reaction mixture in place of MM there was no inhibition of LDH activity (Table 1). The enzyme was completely inhibited by 0.15×10^{-6} M $HgCl_2$. Inhibition of LDH by $HgCl_2$ was non-competitive

TABLE 1

Comparative Effects[a] of Differents Levels of $HgCl_2$ on the Specific Activities[b] of LDH and MDH Enzymes.

Concentration (10^{-6} M/3 ml)	Specific activities		% Inhibition	
	LDH	MDH	LDH	MDH
none (control)	6.0	92.89	0.0	0.0
0.0003	6.0	92.89	0.0	0.0
0.0015	5.5	87.53	8.4	5.8
0.0030	5.3	82.17	11.7	11.5
0.0150	3.0	67.88	49.7	27.0
0.0300	2.0	48.21	66.7	48.1
0.1500	0.0	0.0	100.0	100.0

[a]Specific activity, reported as enzyme units/mg protein.

[b]The reaction mixture (3 ml phosphate buffer, 0.05 M, pH 7.4) contained 0.2 x 10^{-6} M NADH, 1 x 10^{-6} M Na-pyruvate, $HgCl_2$, and LDH. For MDH, 0.6 x 10^{-6} M OAA replaced the pyruvate.

and K_i values of 4.2 x 10^{-7} M and 1.61 x 10^{-8} M were established in the presence of MM and $HgCl_2$, respectively.

The data on the inhibition of pig heart MDH by $HgCl_2$ is also summarized in Table 1. In the presence of 0.0003 x 10^{-6} M $HgCl_2$, MDH activity was not inhibited. At 0.003 and 0.03 x 10^{-6} M $HgCl_2$, however, the inhibition increased to 11.5 and 48.1%, respectively, compared to the control. There was complete enzyme inhibition by 0.15 x 10^{-6} M $HgCl_2$. Compared to LDH at the same concentrations of $HgCl_2$, MDH activity was much less inhibited.

Interaction of mercurials with NADH. The addition of 0.2 x 10^{-6} M $HgCl_2$ to Na-phosphate buffer containing 0.2 x 10^{-6} M NAD had no effect on the normal NAD spectra. However, when the NADH-$HgCl_2$ reaction mixture was incubated 2 min prior to the addition of LDH enzyme, it was noted that the absorbance at 340 nm decreased rapidly due to interaction between NADH and $HgCl_2$ (Fig. 1a). The normal absorption spectra of 0.2 x 10^{-6} M NADH (in 3 ml Na-phosphate buffer) exhibited two maxima, one at 259 and the other at 340 nm. Upon addition of $HgCl_2$ there was a sharp decrease in absorbance at 340 nm from 0.39 to 0.05 accompanied by a shift of peak at 259 nm to a larger one at 269 nm. This experiment was repeated using 0.1 and 0.2 x 10^{-6} M $CdSO_4$ or $ZnSO_4$ and 0.2 x 10^{-6} M of either MM or PMA in lieu of $HgCl_2$, but the results revealed no apparent deviation from the normal spectra of NADH. Various levels of other intermediate metabolites

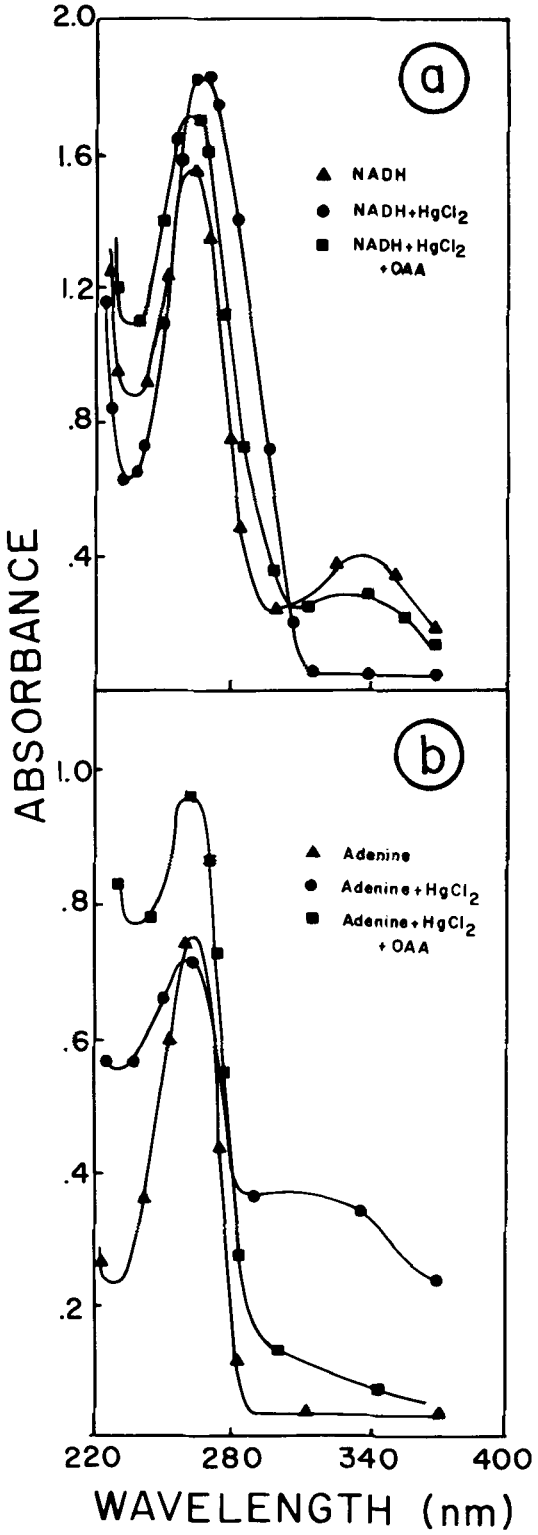


Figure 1. Spectral characteristics of 0.2 x 10^{-6} M NADH (a) and 0.2 x 10^{-6} M adenine (b) as affected by equimolar concentrations of $HgCl_2$ and upon addition of 0.6 x 10^{-6} M OAA. All reactants were prepared in 3.0 ml Na-phosphate buffer (0.05 M, pH 7.4).

such as L-malate, succinate, tartarate and OAA (0.2 to 0.6 x 10^{-6} M) or pyruvate (0.2 to 1 x 10^{-6} M) were added to the NADH-$HgCl_2$ complex in the reaction mixture. Only OAA restored, in part, the absorbance at 340 nm (0.05 to 0.20) and regenerated the peak at 259 nm (1.5 to 1.75) as compared to the NADH spectrum.

This prompted further investigation into the effects $HgCl_2$ on adenine in presence and absence of OAA. Figure 1b depicts the spectra of 0.2 x 10^{-6} M adenine alone in 3 ml Na-phosphate buffer, after interaction with 0.2 x 10^{-6} M $HgCl_2$, and following the addition of 0.6 x 10^{-6} M OAA to the adenine-$HgCl_2$ complex. Adenine exhibited a single maxima at 259 nm as well as other spectral characteristics which were modified following interaction with Hg^{2+}, including formation of a shoulder at 320 nm. The addition of OAA restored most of this shoulder and caused an increase in absorbance at 259 nm from 0.76 to 0.96.

Protective effect of OAA. Figure 2a shows the data obtained when 0.2 x 10^{-6} M OAA was added to the NADH-$HgCl_2$ complex. NADH (0.2 x 10^{-6} M) was preincubated 5 min with different levels of $HgCl_2$ (0.02 to 0.2 x 10^{-6} M) in Na-phosphate buffer. OAA (0.2 x 10^{-6} M) was then added and the absorbance followed for an additional 10 min. An immediate decrease in absorbance was noted which was $HgCl_2$-concentration dependent. The presence of OAA reversed this effect in a manner indicating a dependent ratio of OAA to $HgCl_2$.

Figure 2b depicts results obtained when two levels of OAA (0.2 and 0.4) x 10^{-6} M were preincubated with 0.2 x 10^{-6} M $HgCl_2$ for either 10 or 30 min prior to the addition of NADH. When the OAA:NADH ratio was 1 and a 10 min preincubation was used, the absorbance at 340 nm decreased from 0.4 to 0.18 within 3 min after addition of NADH. This was followed by a slight increase to 0.195 after an additional 20 min incubation. After a 30 min preincubation the absorbance decreased to 0.195 within 10 min with no changes thereafter. When the OAA:NADH ratio was increased to 2 and the experiments repeated, the decrease in absorbance was about half that noted when the ratio was 1.

The protective effect of OAA on NADH against the action of $HgCl_2$ was examined using higher molar ratios of OAA to NADH (10, 3.3) and decreased incubation time. NADH (0.2 x 10^{-6} M) in phosphate buffer was preincubated 5 min with 0.2 x 10^{-6} M $HgCl_2$ followed by the addition of various levels of OAA (0.2 to 2.0) x 10^{-6} M. The OAA:NADH ratio was plotted as a function of incubation time and the results (Fig. 2c) clearly show the rapid restoration at 340 nm upon addition of OAA. Maximum

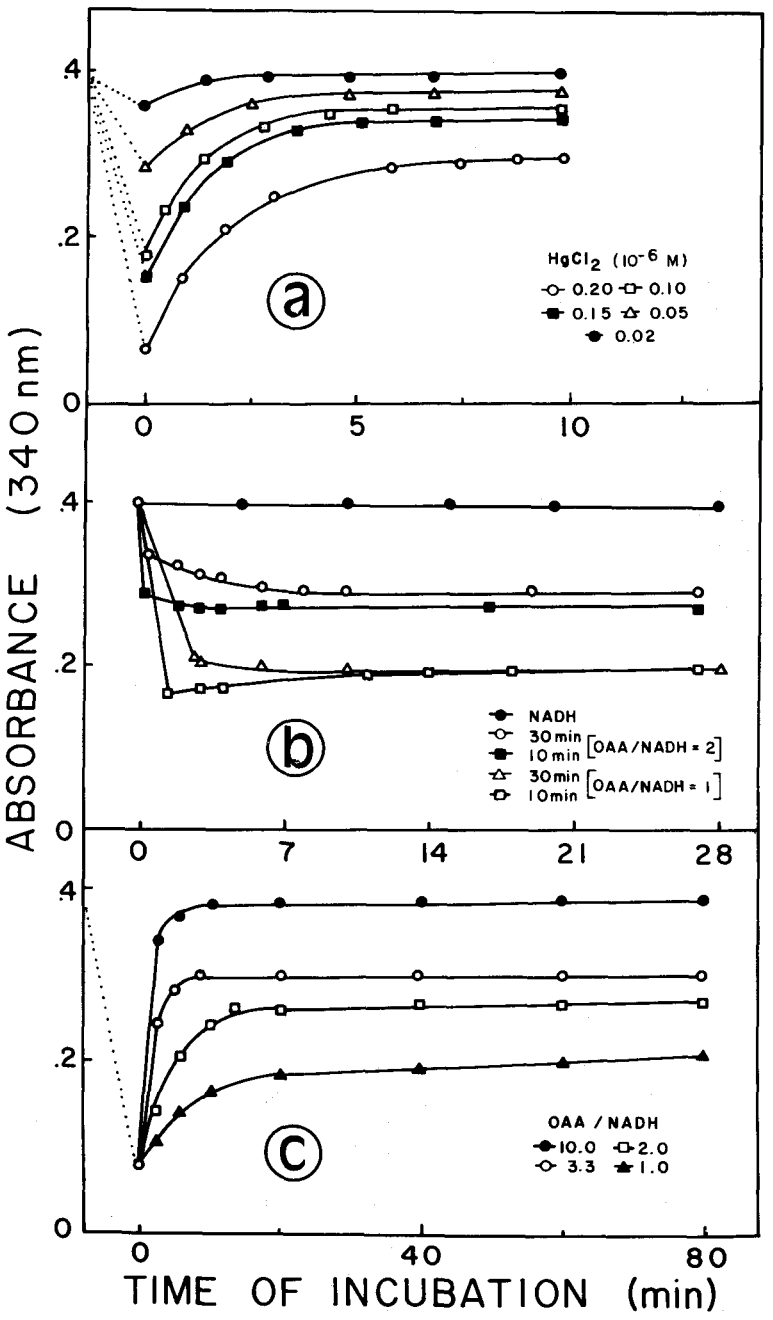


Figure 2. Protective effect of OAA on NADH. (a) 5 min incubation of various levels of $HgCl_2$ with 0.2×10^{-6} M NADH, then 0.2×10^{-6} M OAA was added. (b) Preincubation of two levels of OAA with $HgCl_2$, then NADH was added, leading to OAA/NADH ratio of 1 and 2. (c) 5 min preincubation of NADH with equimolar concentrations of $HgCl_2$, then various levels of OAA were added giving different ratios of OAA/NADH.

absorbance was reached within 4-8 min and remained unchanged throughout the post-incubation period. As the OAA:NADH ratio increased the absorbance increased, and at a ratio of 10 the absorbance was almost completely restored to its original value.

DISCUSSION

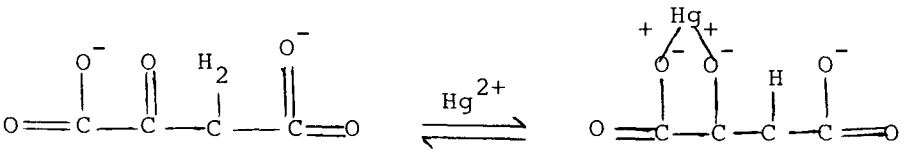
The data obtained in this investigation revealed that $HgCl_2$ inhibited LDH more than did MM, probably due to the monofunctional nature of MM, which forms one ligand (R-Hg-L), whereas $HgCl_2$ is bifunctional and can react with two ligands (L-Hg-L). The difference in inhibition between these two mercurials may also be due to their configuration and molecular size. WEBB (1966) suggested that the effects of Hg^{2+} on enzyme systems are not necessarily directed only toward the apoenzyme but could also affect either the substrate or the co-enzyme. VALLEE and ULMER (1972) demonstrated this by showing that Hg^{2+} complexes with sulfhydryl groups of lipoic acid and co-enzyme A. It is also possible that substrates could protect enzymes against Hg^{2+} by forming complexes at the active sites which may diminish the deleterious effects of the Hg^{2+}. Such protection could be achieved through reaction of the protector with Hg^{2+} leading to inactivation of the mercurial. NADH is known to protect dehydrogenases against mercurials (STOPPANI and MILSTEIN 1957, WITTER 1960, YONETANI and THEORELL 1962) and HILL (1956) stated that the formation of an NADH-$HgCl_2$ complex, in a molar ratio of 1, was less of an inhibitor on LDH than either $HgCl_2$ or p-chloromercuribenzoate. It is clear that we are dealing not only with $HgCl_2$ binding to the protein moiety of LDH and MDH but also with their reduced co-enzyme.

The addition of Hg^{2+} to NADH caused the band at 340 nm to decrease in a manner similar to the oxidation of NADH to NAD. There are three possible reactions when NADH and $HgCl_2$ are incubated together; the first is the complexing of Hg^{2+} with NADH at one or more sites; the second represents the oxidation of NADH to NAD with the simultaneous reduction of Hg^{2+} to Hg^{o}; and the third is when Hg^{2+} oxidizes the NADH to NAD leading to the release of one proton (H^+) and the formation of Hg^+ ion. The latter forms a weak bond with the pyridine ring in the para position to form the NAD-Hg^+ complex that could be reversed with OAA. The loss of absorbance at 340 nm and our failure to detect Hg^{o} experimentally eliminate both the first and second reactions, however, the presence of NAD was confirmed. Therefore, the oxidation of NADH to NAD and formation of the NAD-Hg^+ complex is the most logical reaction. The reaction of Hg^{2+} with

adenine resulted in an increase in absorbance at 340 and 259 nm and no shift in peak at 259 nm suggesting that the adenine moiety of NADH is not the only site of interaction with $HgCl_2$. FERREIRA et al. (1961) reported that a shift in maxima from 255 to 265 nm occurred upon addition of Hg^{2+} to the adenine cation.

The presence of Zn^{2+} or Cd^{2+}, replacing Hg^{2+} in the reaction mixture, had no effect on the NADH spectrum. The half-cell oxidation potential of NADH is -0.430 and the oxidation potentials of the above cations in this reaction are: Hg^{2+}, +0.905, Zn^{2+} and Cd^{2+}, -0.763 and -0.403, respectively (WEAST 1967). Thus, it is possible for Hg^{2+}, but not Zn^{2+}, to react spontaneously with NADH. Cd^{2+} may react, but at a very slow rate.

The results indicate that OAA protects NADH by reaction with Hg^{2+} and there appear to be three means of interaction. One is the formation of a Hg^{2+} salt upon reaction with the dicarboxylic acid groups of OAA. However, malate, succinate, and tartarate, all having similar groups, offered no protection. It is also possible that Hg^{2+} formed a coordinate complex with at least two molecules of OAA involving both the beta-keto and the carboxylic acid groups. However, this mechanism does not completely explain the 10:1 OAA/NADH ratio required for complete protection. We believe the third possibility, that the enol structure of OAA is the active form which combines with Hg^{2+} and initiates the return of absorbance at 340 and 259 nm, leads to the protection noted in these experiments. KOSICKI (1962) reported that OAA in aqueous solution, between pH 5.0 and 10.0, exists as a tautomeric mixture of the enol and keto forms and that at this pH range the enol form constitutes about 10% of the mixture (KUMLER 1962). The proposed reaction of OAA with Hg^{2+} is as follows:



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