The alcohol dehydrogenase (ADH) of yeast has twice the molecular weight of the ADH of horse liver (1), contains 4 gm. atoms of zinc (2), and binds 4 moles of DPN per mole of protein (1). The liver ADH binds 2 moles of coenzyme per mole (3). These facts prompted the prediction that the liver ADH should contain 2 gm. atoms of zinc per mole of protein (2). The presence of 2 atoms of zinc in liver ADH has been briefly reported (4, 5). This paper presents detailed spectrographic, microchemical, and polarographic analyses which demonstrate the presence of 2 atoms of zinc per molecule of liver ADH; all other metals are removed during purification. The activity of the enzyme can be modified in a manner analogous to but not identical with that observed for other zinc dehydrogenases.

Methods

Twice crystallized enzyme was prepared from horse liver (6). Analyses for the metal content of crystals and fractions were performed spectrographically (7), chemically (8), and polarographically (9). Protein was determined gravimetrically, after precipitation with trichloroacetic acid (10), or spectrophotometrically.

Enzymatic activity was estimated as previously described (2). The 3 ml. reaction mixture contained 50 μmoles of ethanol, 5 μmoles of DPN (Pabst Laboratories or Sigma Chemical Company), 0.5 ml. of 0.1 M pyrophosphate buffer, pH 8.8, and 20 or 40 γ of liver ADH. Activities, v, are expressed as change in optical density at 340 mμ per minute per mg. of enzyme or of protein.

1,10-Phenanthroline (G. Frederick Smith Company), 8-hydroxyquinoline, and sodium diethyldithiocarbamate (Eastman Organic Chemicals) were adjusted to the appropriate pH levels before use.

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1 The abbreviations used are ADH, alcohol dehydrogenase; YADH, yeast alcohol dehydrogenase; LADH, liver alcohol dehydrogenase; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; OP, 1,10-phenanthroline.
Results

Metal analyses of four preparations of crystalline liver ADH are shown in Table I. Zinc is the major constituent; by spectrographic analysis it varies in concentration from 1.72 to 2.31 gm. atoms per mole of protein (1 mole = 73,000 gm. (3)), averaging 2.04 for the four preparations, which corresponds to 1827 γ of zinc per gm. of liver ADH. By polarography, the average zinc content is 2.01 and, by chemical measurements, 2.28 gm. atoms of zinc per mole of liver ADH. Aluminum, barium, copper, and iron are detected in all preparations only in stoichiometrically insignificant quantities. Calcium, chromium, magnesium, and lead occur sporadically. Preparation III contains relatively high concentrations of metals other than zinc, among which are 1.18 gm. atoms of aluminum. Recrystallization of the enzyme from ethanol does not alter the zinc content markedly, but the concentrations of all other metals are lowered significantly. The activity of this recrystallized preparation is almost 4 times greater than that of other preparations (Table III, Column A, Fraction 10).

Variation of pH of the solvent removes zinc from the enzyme. Liver ADH was dissolved in buffers, the pH of which varied from 7.5 to 4.5; the enzyme was then dialyzed at 4° against the same buffer for 21 hours. Ac-

---

**Table I**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Preparation I</th>
<th>Preparation II</th>
<th>Preparation III</th>
<th>Preparation IV (recrystallized)</th>
<th>Average mole ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum</td>
<td>0.019</td>
<td>0.014</td>
<td>1.18</td>
<td>0.105</td>
<td>0.330</td>
</tr>
<tr>
<td>Barium</td>
<td>0.005</td>
<td>0.035</td>
<td>0.061</td>
<td>0.001</td>
<td>0.025</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.100</td>
<td>Trace</td>
<td>&lt;0.61</td>
<td>*</td>
<td>0.355</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.014</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>0.014</td>
</tr>
<tr>
<td>Copper</td>
<td>†</td>
<td>0.234</td>
<td>†</td>
<td>0.062</td>
<td>0.148</td>
</tr>
<tr>
<td>Iron</td>
<td>0.081</td>
<td>0.084</td>
<td>0.09</td>
<td>0.060</td>
<td>0.229</td>
</tr>
<tr>
<td>Lead</td>
<td>*</td>
<td>0.025</td>
<td>0.077</td>
<td>*</td>
<td>0.051</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.081</td>
<td>0.18</td>
<td>0.66</td>
<td>*</td>
<td>0.307</td>
</tr>
<tr>
<td>Zinc, spectrographic</td>
<td>1.72</td>
<td>1.91</td>
<td>2.31</td>
<td>2.20</td>
<td>2.04</td>
</tr>
<tr>
<td>&quot; polarographic</td>
<td>1.77</td>
<td>2.01</td>
<td>†</td>
<td>2.25</td>
<td>2.01</td>
</tr>
<tr>
<td>&quot; chemical</td>
<td>2.16</td>
<td>1.92</td>
<td>†</td>
<td>2.76</td>
<td>2.28</td>
</tr>
</tbody>
</table>

These preparations have not been dialyzed.

* Not detected; also beryllium, boron, cobalt, lithium, molybdenum, nickel, silver, and tin.
† Quantity not sufficient for analysis.
‡ Not done.
tivity and zinc content were measured before and after dialysis. The results are shown in Table II. The undialyzed liver ADH has an activity of 7.20 and contains 2.98 gm. atoms of zinc per mole of liver ADH, a zinc content higher than that of the purest crystals obtained. At pH 6.0 the activity of this preparation is increased to 8.00 and the zinc content is reduced to 2.39 gm. atoms per mole of protein. Buffers of lower pH inactivate the liver ADH completely, and zinc is progressively removed until almost none remains at pH 4.5.

The relationship of zinc to enzymatic activity is shown in Table III. The metal content and enzymatic activity of consecutive fractions obtained during a purification of ADH from horse liver are shown (Preparation II, Table I). Metal content is expressed in gm. atoms of metal per 73,000 gm. of protein. Zinc content is expressed also in micrograms per gm. of protein to allow for comparison of these data with those on other zinc enzymes (7). Specific activity rises progressively from 0.44 in the water extract of horse liver to 6.50 in the crystals obtained from ammonium sulfate, then to 24.8 in the material recrystallized from ethanol (Column A).

Zinc content increases from 0.252 gm. atom per 73,000 gm. of protein in the pulp of liver to 2.20 gm. atoms of zinc per 73,000 gm. of protein in the final crystals (Columns B and C). The activity to zinc ratio also rises progressively from 1.11 to 12.1 ΔE340 per minute per microgram of zinc, reflecting both the relationship between zinc and the activity in the more purified stages and the presence of extraneous zinc even in the first crystals (Column D). The concentration of all other metals, i.e. aluminum, barium, calcium, copper, iron, magnesium, molybdenum, and manganese, decreases during the course of purification from 4.27 gm. atoms total per 73,000 gm. of protein to 0.24 gm. atom in the final crystals (Column E). The sum of all these metals is highest in Fraction 4. Subsequent to Fractions

### Table II

**Dialysis of Horse Liver Alcohol Dehydrogenase at Various pH Values**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>(v_{\text{before}})</th>
<th>(v_{\text{after}})</th>
<th>Ratio, Zinc/Protein, gm. atoms per mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris,* 0.1 M</td>
<td>7.5</td>
<td>7.20</td>
<td></td>
<td>2.98</td>
</tr>
<tr>
<td>Phosphate, 0.1 M</td>
<td>6.0</td>
<td>7.20</td>
<td>8.00</td>
<td>2.39</td>
</tr>
<tr>
<td>&quot; 0.1 &quot;</td>
<td>5.5</td>
<td>3.74</td>
<td>0</td>
<td>0.56</td>
</tr>
<tr>
<td>Acetate, 0.1 M</td>
<td>4.5</td>
<td>0.50</td>
<td>0</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Liver ADH is dissolved in buffer, and the rate of DPN \(\rightarrow\) DPNH is measured \((v_{\text{before}})\). The solution is then dialyzed for 21 hours at 0° against the same buffer, and the activity \((v_{\text{after}})\) and zinc and protein content are measured.

* Tris(hydroxymethyl)aminomethane.
### Table III

**Activity and Metal Content of Fractions in Course of Purification of Horse Liver Alcohol Dehydrogenase**

The metal contents are in gm. atoms of metal per 73,000 gm. of protein; Column B in micrograms per gm. Activities are in units as defined in the text. $E(Me - Zn) =$ the sum of Al, Ba, Ca, Cu, Fe, Mg, Mo, and Mn.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Specific activity</th>
<th>Zinc Protein</th>
<th>Activity Zinc</th>
<th>$E(Me - Zn)$</th>
<th>Other metals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>1</td>
<td>Liver pulp</td>
<td>*</td>
<td>225</td>
<td>0.252</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>$H_2O$ extract</td>
<td>0.44</td>
<td>396</td>
<td>0.443</td>
<td>1.11</td>
</tr>
<tr>
<td>3</td>
<td>Supernatant, heat denaturation</td>
<td>0.70</td>
<td>675</td>
<td>0.755</td>
<td>1.04</td>
</tr>
<tr>
<td>4</td>
<td>Filtrate, 0.55 (NH$_4$)$_2$SO$_4$</td>
<td>2.87</td>
<td>896</td>
<td>1.00</td>
<td>3.20</td>
</tr>
<tr>
<td>5</td>
<td>Ppt., 0.8 (NH$_4$)$_2$SO$_4$</td>
<td>2.47</td>
<td>877</td>
<td>0.980</td>
<td>2.82</td>
</tr>
<tr>
<td>6</td>
<td>Fraction 5 dialyzed</td>
<td>2.52</td>
<td>829</td>
<td>0.827</td>
<td>3.28</td>
</tr>
<tr>
<td>7</td>
<td>Supernatant, ethanol-CHCl$_3$ condensed</td>
<td>5.15</td>
<td>1010</td>
<td>1.13</td>
<td>5.10</td>
</tr>
<tr>
<td>8</td>
<td>Fraction 7 dialyzed</td>
<td>0.33</td>
<td>1170</td>
<td>1.31</td>
<td>5.40</td>
</tr>
<tr>
<td>9</td>
<td>(NH$_4$)$_2$SO$_4$ crystals</td>
<td>6.50</td>
<td>1540</td>
<td>1.72</td>
<td>4.22</td>
</tr>
<tr>
<td>10</td>
<td>Ethanol crystals</td>
<td>24.8</td>
<td>2060</td>
<td>2.20</td>
<td>12.1</td>
</tr>
</tbody>
</table>

* Not measured.
† Not detected; also beryllium, boron, cobalt, lead, lithium, nickel, silver, and tin.
tion 5, however, the sum of metals other than zinc decreases with increasing purification.

The role of zinc in the enzymatic action of liver ADH was investigated by the use of chelating agents. Inhibition of enzymatic activity was observed with or without preincubation of the enzyme with these agents. Fig. 1 shows the effects of preincubation of liver ADH with $5 \times 10^{-3}$ M OP, $5 \times 10^{-3}$ M 8-hydroxyquinoline, 0.04 M sodium diethyldithiocarbamate, 0.45 M Versene, or 0.375 M ethylenediamine under the conditions stated.

**Fig. 1.** Effect of time of preincubation of liver ADH with chelating agents. Conditions of preincubation: pH 7.0, phosphate buffer, 0.05 M; OP, 8-hydroxyquinoline (8-OHQ), and sodium diethyldithiocarbamate (NaDDC) at 23°C; Versene (VER) and ethylenediamine (ED) at 37°C. Aliquots are removed at the times indicated and the rate of DPN $\rightarrow$ DPNH is measured; $v_o$ = activity of liver ADH alone, and $v_i$ = activity of liver ADH and chelating agent.

OP reduces activity to 54 per cent of its initial value during the shortest time in which the activity measurement could be made. Thereafter, the degree of inhibition does not increase during the period of observation. 8-Hydroxyquinoline, sodium diethyldithiocarbamate, Versene, and ethylenediamine reduce the enzymatic activity progressively; the initial rate of inactivation is first order, and equilibrium is reached after times which vary for each inhibitor and with the temperature of preincubation. These reagents also inactivate the enzyme up to 15 per cent within 1 minute.

Liver ADH activity is inhibited without preincubation when OP, 8-hydroxyquinoline, or sodium diethyldithiocarbamate is placed in the reaction
mixture and the enzyme is added to start the reaction. Similar results are obtained when the rate of DPNH oxidation is measured with acetaldehyde as the substrate. The partial activities, $v_i/v_o$, in the presence of varying concentrations of these reagents are shown in Fig. 2. 50 per cent inhibition results with $4.8 \times 10^{-4}$ M OP, $2.2 \times 10^{-3}$ M 8-hydroxyquinoline, and $4.3 \times 10^{-2}$ M sodium diethyldithiocarbamate. Fig. 2 also shows activity when varying concentrations of OP are preincubated with liver ADH for 1 minute. Similar results were obtained after 60 minutes of preincubation. These points fall closely on the line for inhibition by OP in the reaction mixture. The highest concentration of OP with which liver ADH could be preincubated at pH 8.8 was $9 \times 10^{-3}$ M, owing to limitations of solubility; thus concentrations of OP greater than $6 \times 10^{-4}$ M in the reaction mixture were not attainable under these conditions.

Crystalline liver ADH reduces DPN in the absence of added ethanol (11). The curves which relate the degree of inhibition of activity with concentration of OP are similar, whether ethanol is added as substrate or not. Preincubation of the enzyme with OP for up to 60 minutes does not affect the degree of inhibition of this "intrinsic" activity. Fractions obtained during the course of liver ADH purification prior to the addition of ethanol and chloroform (Table III, Fraction 7) also reduce DPN in the absence of added ethanol, and the activity of these fractions with and without added ethanol is inhibited by OP in a manner similar to that exhibited by crystalline enzyme.
Inhibition of liver ADH activity by \(10^{-3}\) M OP is decreased in the presence of Zn\(^{++}\) ions (Fig. 3). This concentration of OP in the reaction mixture produces 32 per cent of the control activity; when \(10^{-2}\) M ZnCl\(_2\) is also present, 67 per cent of activity is preserved, and with \(4 \times 10^{-3}\) M ZnCl\(_2\) the activity is 91 per cent of the control value, almost completely countering inhibitory effects of OP. Increase of activity is directly proportional to log (ZnCl\(_2\)), up to \(4 \times 10^{-3}\) M ZnCl\(_2\).

The inhibition established with OP can be reversed by addition of ZnCl\(_2\) (Fig. 4). When liver ADH is added to DPN and ethanol, the reaction
rate is 14. Addition of \(10^{-3} \text{ M} \) OP reduces the enzymatic rate to 2.6 immediately, and addition of \(4 \times 10^{-3} \text{ M} \) ZnCl\(_2\) then increases the rate to 8.1. All rates are corrected for decrease in the activity of the uninhibited enzyme. Addition of \(5 \times 10^{-3} \text{ M} \) CuCl\(_2\) also restores activity to approximately the same degree.

**DISCUSSION**

Emission spectrography, microchemistry, and polarography were employed to insure independent verification of the accuracy of the zinc analyses (7). The absolute differences observed in these analytical data are small. The microchemical data are higher than those obtained with the other two methods, which agree well with each other. This is expected, since the opportunities for contamination with the chemical procedure are somewhat greater. The spectrographic data may be accepted as representative, not only for zinc, but also for the other elements.

By spectrography (Table I), there is an average of 2.03 gm. atoms of zinc in 1 mole of liver ADH which is not removed either by recrystallization or by dialysis against 0.1 \(\text{M} \) phosphate buffer at pH 6.0 (Table II). Zinc, present in excess of 2 gm. atoms per mole of protein, is removed by dialysis under these conditions, but the intrinsic zinc content of liver ADH is not lowered. In some experiments, this removal has been accompanied by an increase in specific activity, suggesting that the excess zinc may be bound differently to the enzyme than is the zinc which is part of the molecule (Table II). Zinc in excess of this molar concentration may thus represent contamination. The apoenzyme has been given the empirical formula \([(\text{LADH})\text{Zn}_2]\) (7).

The functional significance of all metals other than zinc can be discounted, since their presence and concentrations are variable and recrystallization of the enzyme further eliminates them. Their removal not only does not lower activity, but is usually accompanied by an increase in activity.

Increase of \(\text{H}^+\) concentration above \(10^{-8} \text{ M}\) dissociates the zinc of \([(\text{LADH})\text{Zn}_2]\), which may then be removed by dialysis (Table II).

\[
\text{(1)} \\
[\text{(LADH)Zn}_2] + \text{(H}^+) \rightarrow (\text{LADH}) + 2\text{Zn}^{++}
\]

Activity is lost as zinc is removed, (LADH) being inactive. The degree of reversibility of Equation 1 is not yet determined. Thus, when liver ADH loses activity below pH 6 (6), this may be due to loss of zinc as in Equation 1.

A relationship between zinc and liver ADH activity is also demonstrated by their concomitant rise in the course of purification of the enzyme (Table III). The greater increase in activity may be accounted for in part by
the removal of metals extraneous to the enzyme. Conversely, the lesser rise in zinc concentration may be partially accounted for by the presence of non-ADH zinc in the impure fractions. This is not unexpected, since zinc glutamic dehydrogenase (12) has been identified in mammalian (beef) liver. Recent data (13) further imply that many other pyridine nucleotide-dependent dehydrogenases may also be metalloenzymes, possibly containing zinc. Thus the zinc content of impure fractions may reflect the presence of other zinc-containing proteins or the zinc porphyrin found in liver (14). Although the zinc to protein ratio and the specific activity do not increase proportionately with purification of liver ADH, they do rise in parallel fashion, achieving their maximal level in the highly purified crystals (Table III, Fraction 10).

The progressive diminution of extraneous metals (Table III, Column E) is in inverse proportion to the specific activity of the enzyme and its zinc content; this further emphasizes the specificity of the association of zinc in [(LADH)Zn₂]. These findings are analogous to those observed (2) in the purification and crystallization of the ADH of yeast [(YADH)Zn₄]. The considerations for using certain inhibitors of enzymatic activity to estimate the functional significance of the metal in metalloenzymes in conjunction with analytical data have been described (7). Three metal-binding agents, OP, 8-hydroxyquinoline, and sodium diethyldithiocarbamate, which inhibit other zinc metalloenzymes, were chosen to investigate the role of zinc in [(LADH)Zn₂]. These agents bind Zn²⁺, although, like other similar agents, they are not specific in their capacity to form complexes with this metal. All these reagents inhibit liver ADH activity. The mechanisms involved in the inhibition of liver ADH and other pyridine nucleotide-dependent metallodehydrogenases will be discussed in separate communications.

Comparison of the inhibitions produced by 8-hydroxyquinoline and by sodium diethyldithiocarbamate with preincubation (Fig. 1) and without preincubation (Fig. 2) indicates that these agents behave differently from OP. Preincubation with 8-hydroxyquinoline and sodium diethyldithiocarbamate produces a progressive inhibition which proceeds initially according to first order kinetics. These agents also inhibit liver ADH activity immediately when they are included in the reaction mixture; the same final concentration is less inhibiting than under the conditions of preincubation.

The inhibition of [(LADH)Zn₂] by OP is immediate and reversible upon dilution. Preincubation does not enhance the degree of inhibition, and the effect of OP depends only upon its concentration in the reaction mixture (Fig. 2). All of the [(LADH)Zn₂] (OP)₂ enzyme-inhibitor complex is ap-

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1. In preparation.

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ZINC AND LIVER ADH

parently formed as instantaneously as are the \([\text{Zn}(\text{OP})_n]^{++}\) complexes in solution (15). Therefore, the effect of OP on \([(\text{LADH})\text{Zn}_2]\) and on the ADH of yeast (2) differs.

Preincubation with Versene in concentrations of 0.45 \(m\) inhibits \([(\text{LADH})\text{Zn}_2]\) progressively, initially according to first order kinetics (Fig. 1), while the yeast enzyme is not inhibited under similar conditions (2). The presence of 0.45 \(m\) Versene in the reaction mixture does not produce inhibition of liver ADH, confirming the observations of others (4).

Crystalline horse liver ADH is capable of reducing DPN in the absence of added ethanol, while yeast ADH is not. This "intrinsic" activity has been ascribed to firm binding of ethanol added during the course of purification of the enzyme (11). Fractions obtained early during purification, which have not been exposed to ethanol during fractionation, also reduce DPN. The activity of liver ADH, in the presence or in the absence of added ethanol as substrate, is inhibited by OP, indicating that zinc is involved in the mechanism of enzymatic action of both processes.

The inhibition of \([(\text{LADH})\text{Zn}_2]\) activity by OP is partly prevented when the chelating sites of OP are occupied by \(\text{Zn}^{++}\) ions, indicating these sites to be essential to the inhibitory effect of OP. The incompleteness of the prevention and reversal of inhibition under these conditions may be due to either the inhibitory effects of free OP or of free \(\text{Zn}^{++}\) ions in equilibrium with the \([\text{Zn}(\text{OP})_n]^{++}\) complex, or to the incomplete dissociation of the enzyme-inhibitor complex. Assuming 1 molecule of OP to combine with each zinc atom of the enzyme, the equilibria may be formulated as follows:

\[
\begin{align*}
(2) & \quad n\text{OP} + \text{Zn}^{++} \rightleftharpoons [\text{Zn}(\text{OP})_n]^{++} \\
(3) & \quad [(\text{LADH})\text{Zn}_2] + 2\text{OP} \rightleftharpoons [(\text{LADH})\text{Zn}_2](\text{OP})_2
\end{align*}
\]

where \(n = 1, 2, 3\), and

\[
[(\text{LADH})\text{Zn}_2] + 2[\text{Zn}(\text{OP})_n]^{++} \rightleftharpoons [(\text{LADH})\text{Zn}_2](\text{OP})_2 + 2\text{Zn}^{++} + n\text{OP}
\]

The over-all reaction in these experiments upon prevention or reversal of OP inhibition is

\[
(4) & \quad [(\text{LADH})\text{Zn}_2] + 2[\text{Zn}(\text{OP})_n]^{++} \rightleftharpoons [(\text{LADH})\text{Zn}_2](\text{OP})_2 + 2\text{Zn}^{++} + n\text{OP}
\]

As the ratio of \([\text{Zn}^{++}]:[\text{OP}]\) increases to 4, OP inhibition is progressively diminished. Incomplete formation of \([\text{Zn}(\text{OP})_n]^{++}\) (Equation 2) at pH 8.8 in the presence of pyrophosphate may account for the remaining 9 per cent inhibition. These results are analogous to those obtained with yeast ADH (2). \(\text{Cu}^{++}\) ions are also effective in reversing established inhibition by OP.

SUMMARY

Horse liver alcohol dehydrogenase is a zinc metallodehydrogenase containing 2 gm. atoms of zinc per mole of protein. Zinc content rises to this
value during the course of purification of the enzyme; concomitantly, the concentration of aluminum, barium, calcium, copper, iron, lead, magnesium, manganese, and molybdenum fall to stoichiometrically insignificant levels. The enzyme is given the empirical formula [(LADH)Zn2]. Zinc is dissociated and may be removed by dialysis below pH 6. Certain metal-binding agents inhibit enzymatic activity. The inhibition with 1,10-phenanthroline is reversible. It is concluded that zinc is an enzymatically active component of [(LADH)Zn2].

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Bert L. Vallee and Frederic L. Hoch


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