# Differential Chemical Reactivities of Zinc in Horse Liver Alcohol Dehydrogenase*

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ABSTRACT: Even though highly purified horse liver alcohol dehydrogenase (LADH) may contain from 3.1 to 4.3 g-atoms of Zn per mole, only 2 g-atoms of Zn are catalytically active. Further, 2 moles of the competitive inhibitors 1,10-phenanthroline (OP) and 2,2′-bipyridine (BP) have been reported to bind to 1 mole of enzyme. This stoichiometry has been confirmed by titrations employing the chromophoric properties of these chelating agents, and the characteristic absorption, optical rotatory dispersion, and circular dichroic spectra of the LADH–Zn–OP(BP) complexes all yield this same value. In addition, spectral data confirm that OP and BP interact with the enzyme through coordination with Zn, not by nonspecific binding with amino acid side chains of the protein. The method of continuous variations was employed to resolve the statistical problem generated by the interaction of less than the total number of Zn atoms present in the enzyme. It also demonstrates that only 2 of the 3.5 g-atoms of Zn in the enzyme here employed interact with OP and BP. Moreover, both Zn atoms form 1:1 complexes with each agent. Since Zn complex ions containing both chemically reactive and unreactive Zn atoms are unknown, there are no model systems which could serve for comparison.

Hence, Job's curves, based on a series of hypothetical stoichiometries, were computer generated, and these show that other coordination species, *e.g.*, 1:2 or 2:1 complexes, are not compatible with the experimental data. Thus, liver alcohol dehydrogenase seems to be the first instance in which the use of Job's method demonstrates the existence of different classes of metal atoms, as gauged by their chemical reactivities. The method of continuous variations also provides indirect confirmation of the nonintegral Zn content, determined independently by direct analysis. These results are compared to those of chemical modification. Diethyldithiocarbamate inactivates the enzyme by selective removal of only the 2 active site Zn atoms. The resultant enzyme does not bind OP. In contrast, carboxymethylation labilizes only the structural Zn atoms while the catalytically active ones are retained and bind OP. Thus, the dual chemical reactivities of the Zn atoms as revealed by the Job's curves are also reflected in their biological properties.

The reversible inhibition of horse liver alcohol dehydrogenase, LADH,[1] by the chelating agent, 1,10-phenanthroline, OP (Vallee and Hoch, 1957), first suggested that zinc plays a role in the catalytic function of this enzyme. Subsequent kinetic (Vallee *et al.*, 1959; Plane and Theorell, 1961) and spectral studies (Vallee *et al.*, 1958; Vallee and Coombs, 1959; Yonetani, 1963a,b) indicated that two molecules of OP interact with zinc atoms in LADH and compete with NADH, the coenzyme. These data were consistent with the presence of two active enzymatic sites (Theorell and Bonnichsen, 1951) each containing a single essential zinc atom.

The more recent detection of zinc in excess of 2 g-atoms/mole of more highly purified LADH (Åkeson, 1964; Oppenheimer *et al.*, 1967; Drum *et al.*, 1969a) occasioned a reexamination of the interaction of 1,10-phenanthroline and of 2,2′-bipyridine with this enzyme. Spectrophotometric titration (Vallee and Coombs, 1959), optical rotatory dispersion titration (Ulmer *et al.*, 1961), and the present studies both of native and of chemically modified LADH indicate that only two of the zinc atoms of LADH interact with OP and BP. Thus the zinc atoms in this enzyme in excess of the two involved directly in catalysis do not react with either metal binding agent. Moreover, an extension of the method of continuous variations (Job, 1928) demonstrates its applicability to a system containing both reactive and unreactive metal atoms.

## Methods

LADH (Boehringer-Mannheim Corp., lots 607327, 625238, and 643228) was obtained as a suspension of crystals in 10% ethanol–0.02 M sodium phosphate (pH 7). Concentrated solutions of enzyme were prepared by suspending the crystals in 0.1 M $Na_2HPO_4$–0.01 M glycine (pH 9) and dialyzing 24 hr against 100 volumes of the buffer at 4°. After this preparation, enzymatic activity was unchanged, and the solution remained clear for 12 hr at 0°. For all spectral measurements the pH was adjusted with phosphate buffer (pH 6) to result in final buffer concentrations of 0.084 M $Na_2HPO_4$, 0.016 M $NaH_2PO_4$, and 0.0075 M glycine at pH 7.5.

The specific absorptivity of the preparations at 280 mμ was 0.43 $mg^{-1}$ $cm^2$ (Drum *et al.*, 1969a). Based on a molecular weight of $80 \times 10^3$ (Drum *et al.*, 1967), the enzyme samples, prepared as described above, contained 3.3–3.5 g-atoms of zinc/mole of protein, as measured both by atomic absorption

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[1] Abbreviations used in this paper are: LADH, horse liver alcohol dehydrogenase: OP, 1,10-phenanthroline; BP, 2,2′-bipyridine; $Zn_t'$ and $Zn_f'$, total and free (reactive) amounts of zinc in LADH; $Zn_f'$·OP and $Zn_f'$·BP, 1:1 complexes of a reactive zinc atom of LADH with OP and BP.

TABLE I: Absorption Maxima and Corresponding Molar Extinction Coefficients of 1,10-Phenanthroline, 2,2'-Bipyridine, and Their Zinc Complexes in 0.1 M Tris-Cl (pH 7.5).

| OP | | $Zn(OP)_1^{2+}$ | | $Zn_f' \cdot OP$ | |
|---|---|---|---|---|---|
| $\lambda_{max}$ (mμ) | $\epsilon \times 10^{-3}$ ($cm^{-1}$ $M^{-1}$) | $\lambda_{max}$ (mμ) | $\epsilon \times 10^{-3}$ ($cm^{-1}$ $M^{-1}$) | $\lambda_{max}$ (mμ) | $\epsilon \times 10^{-3}$ ($cm^{-1}$ $M^{-1}$) |
| 226 | 34.4 | 226 | 36.5 | | |
| 265 | 29.5 | 270 | 35.5 | 271 | 29.0 |
| 290 | 9.0 | 292 | 11.0 | 297 | 10.0 |
| 310 | 1.0 | 312 | 1.46 | 316 | 2.0 |
| 323 | 0.64 | 324 | 0.74 | 329 | 1.45 |
| | | 341 | 0.14 | 345 | 0.70 |
| BP | | $Zn(BP)_1^{2+}$ | | $Zn_f' \cdot BP$ | |
| 233 | 10.2 | 244 | 10.7 | 246 | 11.8 |
| 280 | 13.3 | 295 | 17.2 | 298 | 16.2 |
| | | 306 | 17.7 | 308 | 12.6 |

spectrometry (Fuwa *et al.*, 1964) and by diphenylthiocarbazone extraction (Vallee and Gibson, 1948). The specific activity of the enzyme samples was 13.0–13.6 $\Delta A_{340}$/min per mg, using the assay previously described (Drum *et al.*, 1969a).

2,2-Bipyridine-HCl and 1,10-phenanthroline-HCl (G. Frederick Smith Co., Columbus, Ohio) were dissolved in 0.1 M $NaH_2PO_4$ adjusted to pH 6.0 with NaOH. The absence of zinc contamination was verified analytically. Solutions of diethyldithiocarbamate were made by dissolving the sodium salt (Eastman) in oxygen-free Tris-Cl buffer (pH 7.5) at 4°. Carboxymethylation of LADH was accomplished as described previously (Li and Vallee, 1965).

Absorption spectra were obtained at 23° with a Cary Model 15 recording spectrophotometer using sample cells of 0.1–10.0-mm path length. To ensure reproducibility each solution and buffer was scanned successively three times with air as reference. Values for molar absorptivity of the $Zn_f' \cdot OP$ and $Zn_f' \cdot BP$ complexes were determined by addition of the chelating agents to excess enzyme, making appropriate corrections for the measured dissociation constants.

Optical rotatory dispersion and circular dichroism were measured with a Cary Model 60 spectropolarimeter and circular dichroism attachment at 27°, using 0.1–10.0-mm pathlength, fused quartz cells. The instrument was operated with a response time of 3 sec and scan speeds of 5–10 mμ/min. All measured rotations were the mean of three successive scans yielding a precision of ±0.0004° for optical rotatory dispersion and ±0.001° for circular dichroism, from 250 to 350 mμ. The results for the interaction of LADH with the two chelating agents are reported as specific rotation, $[\alpha]_\lambda^{27}$, based on the enzyme concentration. For comparison with values of molar extinction and molar ellipticity of the chromophores, molar rotation, [M], is employed, and $[M]_\lambda^{27} = (\alpha_{obsd})(l)(c)$, where $l$ = path length in centimeters and $c$ = molar concentration of the metal–chelate complex; $\alpha_{obsd}$ is measured after subtraction of the optical rotatory dispersion due to LADH. Similarly, circular dichroic spectra are reported as molar ellipticities, $[\theta]_\lambda^{27} = (\theta_{obsd})(l)(c)$, uncorrected for the refractive index of the buffer.

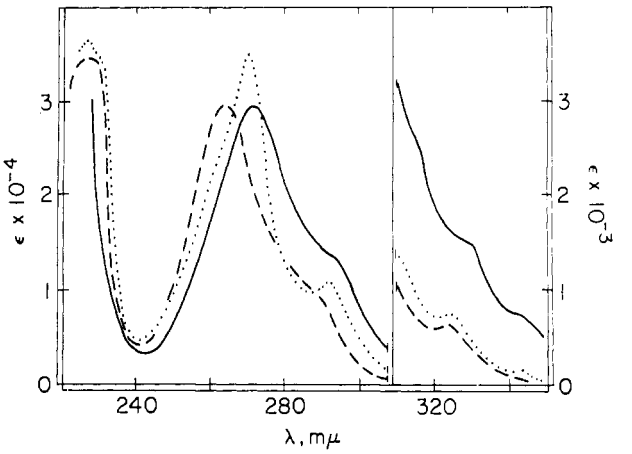


FIGURE 1: Absorption spectra of OP (– – – –), $Zn(OP)^{2+}$ (. . . . . ), and $Zn_f' \cdot OP$ (———). 0.1 M Tris-Cl (pH 7.5).

To establish the stoichiometry of the interaction of two species, the method of continuous variations (Job, 1928) was employed as follows: equimolar concentrations of the two components which form the complex are mixed in varying proportions while their *molar sum* is kept constant. On the assumption that both species present react to the same extent, a maximal change in absorbance is expected when the relative amounts of reacting zinc and OP correspond to the stoichiometry of the complex. For example, if the change in absorbance is plotted as a function of the mole per cent of zinc for an inorganic 1:1 $Zn(OP)_1^{2+}$ complex, the maximal change will occur at 50 mole % zinc; for a 1:2 $Zn(OP)_2^{2+}$ complex, at 33 mole % zinc.

In the present case one component, zinc in LADH, $Zn_t'$, was varied from 0 to 100 mole % whereas the second component, OP, was varied from 100 to 0 mole %. This was accomplished by mixing from 0 to 400 μl of $2.74 \times 10^{-3}$ M $Zn_t'$ with 400 to 0 μl of $2.74 \times 10^{-3}$ M OP such that the final volume was 400 μl and the molar concentration sum $(Zn_t') + (OP)$ was $2.74 \times 10^{-3}$ M. The spectra for the enzyme, OP, and for their mixture were recorded separately from 340 to 300 mμ using 1.0-mm path-length cells. From these data the difference absorbance ($A_{329} = A_{(LADH+OP)} - A_{LADH} - A_{OP}$) was calculated.

## Results

In either 0.1 M sodium phosphate or Tris-Cl (pH 7.5) the absorption spectrum of OP displays maxima at 226 mμ ($\epsilon$ $34.4 \times 10^3$ $cm^{-1}$ $M^{-1}$), 265 mμ ($\epsilon$ $29.5 \times 10^3$ $cm^{-1}$ $M^{-1}$), and 324 mμ ($\epsilon$ $0.64 \times 10^{-3}$ $cm^{-1}$ $M^{-1}$) and shoulders at 290 and 310 mμ (Table I). When $Zn^{2+}$ is in excess, the 1:1 $Zn(OP)_1^{2+}$ complex is formed: the maximum at 265 mμ shifts to 270 mμ ($\epsilon$ $35.5 \times 10^3$ $cm^{-1}$ $M^{-1}$), a second peak appears at 292 mμ, and additional smaller peaks occur at 312, 324, and 341 mμ (Figure 1). These spectra are identical with those previously obtained for the 1:1 complex in perchlorate at pH 5.5 (Sone *et al.*, 1955) and in ammonium acetate at pH 6.1 (McClure and Banks, 1951). On interaction of $3 \times 10^{-4}$ M OP with the zinc atoms of $4 \times 10^{-4}$ M LADH the absorption maximum of OP shifts from 265 to 271 mμ ($\epsilon$ $29 \times 10^{-3}$ $cm^{-1}$ $M^{-1}$) and smaller peaks occur at 297, 316, 329, and 345 mμ (Table I and Figure 1) (Vallee *et al.*, 1958).

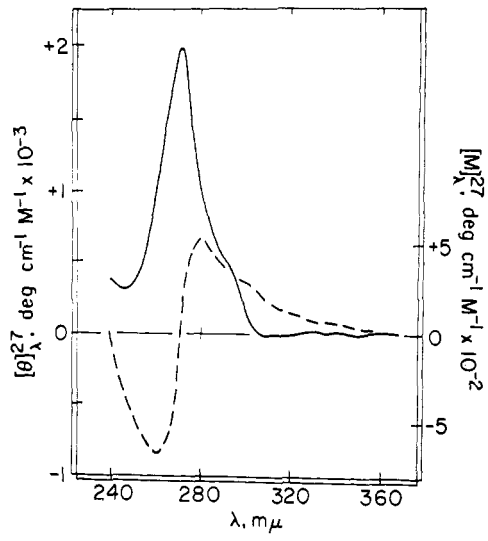


FIGURE 2: Optical rotatory dispersion and circular dichroism of the $Zn_f'$·OP complex. 0.1 M Tris-Cl (pH 7.5). The ellipticity (———) and rotatory dispersion (– – – –) curves are corrected for the dissociation constant of the $Zn_f'$·OP complex and expressed in terms of chromophore molarity as described under Methods.

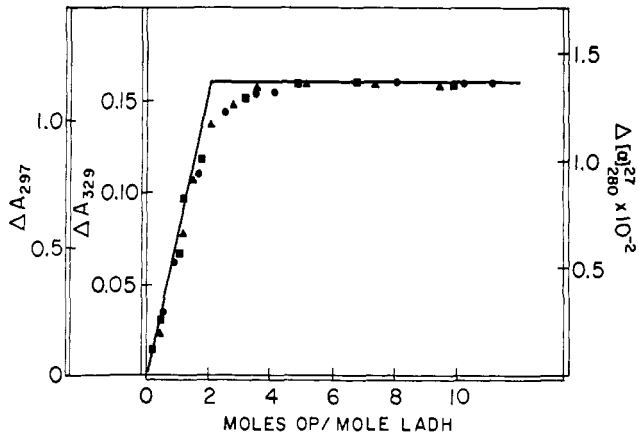


FIGURE 3: Spectrophotometric and rotatory dispersion titration of LADH with OP. The titrations were performed by mixing identical aliquots of LADH with increasing concentrations of OP, to yield the indicated ratios of OP to LADH. The final LADH concentration was $6.8 \times 10^{-4}$ M, in 0.1 M phosphate–0.0075 M glycine (pH 7.5), 27°. The difference absorbance, $\Delta A_\lambda = A_{(\text{LADH + OP})} - A_{\text{LADH}} - A_{\text{OP}}$, at 329 mμ (●) and at 297 mμ (■) and the difference rotation, $\Delta[\alpha]_{280}^{27} = [\alpha]_{280\,(\text{LADH + OP})} - [\alpha]_{280\,\text{LADH}}$ (▲), are plotted on the same scale in order to illustrate the identity of the titration curves.

The absorption bands of the $Zn_f'$·OP complex are optically active (Ulmer *et al.*, 1961; Li and Vallee, 1964). The resultant extrinsic Cotton effect, detected by optical rotatory dispersion, exhibits a positive peak at 280 mμ ($[M]_{280}^{27} = +5.2 \times 10^2$ deg cm$^{-1}$ M$^{-1}$) with a prominent shoulder at 300 mμ, a crossover point at 271 mμ, and a negative trough at 260 mμ ($[M]_{260}^{27} = -6.7 \times 10^2$ deg cm$^{-1}$ M$^{-1}$) (Figure 2).[2]

The corresponding measured circular dichroic spectrum for the $Zn_f'$·OP complex has a major positive band at 271 mμ ($[\theta]_{271}^{27} = 2.0 \times 10^3$ deg cm$^{-1}$ M$^{-1}$) and a shoulder at 295 mμ ($[\theta]_{295}^{27} = 4.5 \times 10^2$ deg cm$^{-1}$ M$^{-1}$). Small negative bands occur at 310, 319, and 349 mμ, and positive bands are seen at 329 and 342 mμ (Figure 2).

On titration of $6.8 \times 10^{-4}$ M LADH with successive additions of OP, a distinct break in the difference absorbance plot is apparent at a point corresponding to 2 moles of OP/mole of LADH (Figure 3). The results of titration at 329 mμ are identical with those at 297 mμ. At 4° over an interval of 24 hr after completion of the titration no change in the difference absorbance occurs. Thus, it appears that the interaction of OP with only two of the zinc atoms of LADH generates the absorption bands at 329 and 297 mμ, characteristic of the $Zn_f'$·OP complex (*vide infra*).[3]

The results of rotatory dispersion titration are identical with those of spectrophotometric titration at 329 and 297 mμ by successive additions of OP to LADH. The difference dispersion generated reaches a sharp end point on addition of 2.0 moles of OP/mole of LADH (Figure 3).

When the interaction of OP with $Zn_f'$ is studied by Job's method of continuous variations, the curve obtained by difference spectrophotometry is unlike that found in any conventional, inorganic system. Based on the *total* zinc content of the enzyme, *i.e.*, 3.5 g-atoms of zinc/mole of LADH in this instance, the lines drawn along the linear portions of the experimental data intersect at 64 mole % $Zn_t'$. The point of intersection would be expected to be at 50 mole % zinc if OP were to form a 1:1 complex with each zinc atom of LADH. If, however, *only two* of the enzyme zinc atoms were to react with OP at high concentrations of reactants, maximal formation of the 1:1 $Zn_f'$·OP complex should occur when the molar ratio of the OP to total enzyme zinc is 2.0/3.5, *i.e.*, at the point of 3.5/(3.5 + 2.0) = 64 mole % of $Zn_t'$, precisely that which is observed (Figure 4). Thus the method of continuous variations seemed capable of signaling the presence of nonreacting zinc atoms.

While verifying the extinction coefficient and dissociation constant of the complex (*vide infra*), Job's method should also yield information not readily obtainable from titrations employing the method of molar proportions, *e.g.*, more specific delineation of the precise stoichiometry at each zinc atom and detection of free zinc ions, when removed from the enzyme by the ligand.

Thus data indicating binding of 2.0 moles of OP to LADH containing 3.5 g-atoms of zinc/mole could denote the formation of two separate 1:1 $Zn_f'$·OP complexes, a single 1:2 $Zn_f'$·$(OP)_2$ complex, or 2:1 $(Zn_f')_2$·OP complexes, the latter, of course, being chemically unknown. To ascertain the presumable basis of the results, a computer program was designed which permits systematic examination of the experimental parameters affecting the ultimate results for Job's curves (see Appendix).

[2] The complexes of OP and $Zn^{2+}$ are racemic mixtures and are optically inactive. In Figures 2 and 7, the values for molar rotation and ellipticity refer to the molarity of the chromophore, since spectroscopic properties of the $Zn_f'$·OP and $Zn_f'$·BP are the object of major interest. The contributions of the enzyme alone have been subtracted from those obtained with the mixture.

[3] Spectrophotometric titration with OP at 329 mμ (Vallee and Coombs 1959) and with BP at 308 mμ (Sigman, 1967) avoids the large correction for background absorbance, due to the free chromophore, required at lower wavelengths. Thus, while titration at 271 mμ also yields a stoichiometry of 2 moles of OP/mole of LADH, the data are less precise, due to intense absorption both by the protein and chromophore.

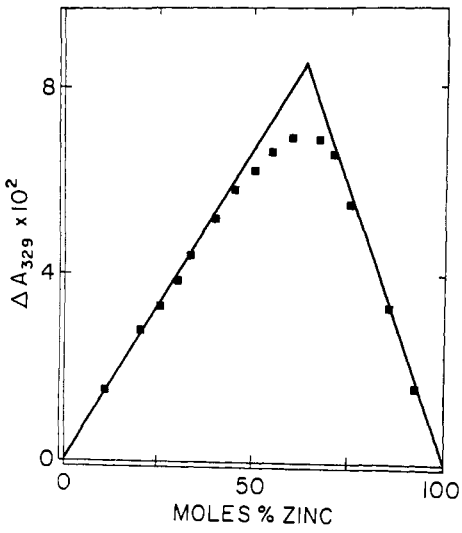


FIGURE 4: Job's method of continuous variations applied to the interaction of zinc in LADH with OP. LADH and OP were mixed in order to vary the moles per cent *total* Zn, $Zn_t'$, as indicated on the abscissa, and the difference absorbance, $A_{(LADH + OP)} - A_{(LADH)} - A_{(OP)}$, was measured at 329 mμ (■). The *total* molarity of $Zn_t'$ (3.5 g-atoms/mole) plus OP was kept constant at $2.74 \times 10^{-3}$ M. Conditions: 0.1 M phosphate–0.0075 M glycine (pH 7.5), 23°, 1-mm path-length cuvets.

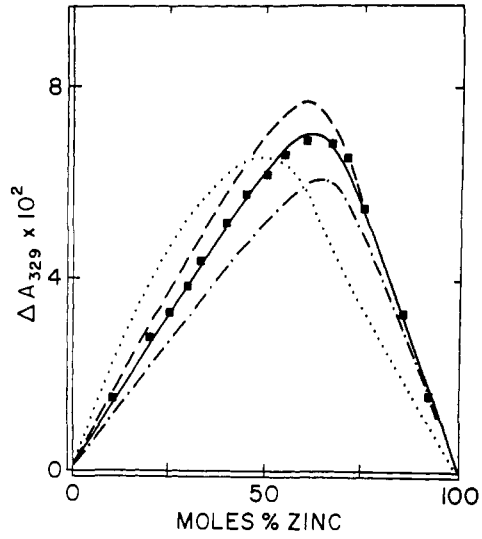


FIGURE 5: Comparisons of experimental and theoretical Job's curves for the interaction of zinc in LADH with OP. Job's curves are plotted for the following theoretical models: formation of a 1:2 $Zn_f' \cdot (OP)_2$ complex (– – – –); two $Zn_f' \cdot OP$ complexes, $s = 0.57$ (———); two $Zn_f' \cdot OP$ complexes, $s = 0.50$ (—·—); and two $(Zn_f')_2 \cdot OP$ complexes (· · · · · ·). Experimental points corresponding to Figure 4 are indicated by the closed squares (■). The application of curve parameters is outlined in the Results and Appendix sections.

The parameters required to generate Job's curves corresponding to each of the above postulates are known. The dissociation constant for the $Zn_f' \cdot OP$ complex at pH 7.5 is $3.3 \pm 0.5 \times 10^{-5}$ M (Vallee and Coombs, 1959). At pH 7.0 the values are slightly lower, $8.8 \times 10^{-6}$ (Sigman, 1967) and $8 \times 10^{-6}$ M (Yonetani, 1963a). The corresponding molar absorptivity varies from 1700 (Vallee and Coombs, 1959) to 1400 $cm^{-1}\ M^{-1}$ (*vide supra*), the latter calculated from values for difference absorptivity (Sigman, 1967; Yonetani, 1963a). Spectrophotometric titration of excess enzyme with OP (*vide supra*) gives an overall value of $\epsilon_{329}$ 2900 ± 100 $cm^{-1}\ M^{-1}$ for the LADH–$(OP)_2$ complex; this value fixes the *lower limit* for the molar absorptivity of a hypothetical $Zn_f' \cdot (OP)_2$ complex and was used in generating the corresponding curve (Figure 5).

Figure 5 shows the results of this analysis. The experimental values coincide with those generated by the computer program for formation of two separate 1:1 $Zn_f' \cdot OP$ complexes with $\epsilon_{329}$ 1450 ± 20 $cm^{-1}\ M^{-1}$ and $K = 3.0 \pm 0.2 \times 10^{-5}$ M. Formation of a 1:2 $Zn_f' \cdot (OP)_2$ complex is excluded because the Job's plot would exhibit both greater absorptivity and a maximum at too low a value of mole per cent of zinc. The shape of the curve and the position of the maximum for a 2:1 $(Zn_f')_2 \cdot OP$ complex also deviate significantly from the experimental points.

Finally a model was explored corresponding to the interaction of exactly 50% of $Zn_t'$ with equimolar amounts of OP resulting in the formation of a 1:1 complex. For this calculation $Zn_t'$ was assumed to be exactly 4.0 g-atoms rather than the actual value determined analytically, *i.e.*, 3.5 g-atoms of zinc/mole. This alternative when plotted in this manner also fails to fit the experimental data. Since the slope of the ascending limb of the curve at less than 50 mole % of $Zn_t'$ is far too low (Figure 5), the computer-generated Job's curves suggest that only two zinc atoms interact, one each with 1 molecule of OP, while the balance do not.

The structure of BP differs from that of the rigidly planar OP by virtue of the fact that free rotation about the carbon–carbon bond joining the two pyridine rings can occur; this causes significant changes in the spectral properties of its $Zn^{2+}$ complexes. In 0.1 M sodium phosphate or Tris-Cl buffer (pH 7.5) the absorption maxima of BP are at 233 mμ ($\epsilon$ 10.2 × $10^3$ $cm^{-1}\ M^{-1}$) and at 281 mμ ($\epsilon$ 13.3 × $10^3$ $cm^{-1}\ M^{-1}$).

Excess $Zn^{2+}$ shifts the maximum at 230–244 mμ (Table I) ($\epsilon$ 10.5 × $10^3$ $cm^{-1}\ M^{-1}$) and splits that at 281 mμ, giving rise to maxima at 295 mμ ($\epsilon$ 17.2 × $10^{-3}$ $cm^{-1}\ M^{-1}$) and 305 mμ ($\epsilon$ 17.7 × $10^3$ $cm^{-1}\ M^{-1}$) (Figure 6). These maxima and the absorptivities of the $Zn(BP)_1^{2+}$ complex are identical with those at pH 5.5 in perchlorate (Sone *et al.*, 1955). The absorption

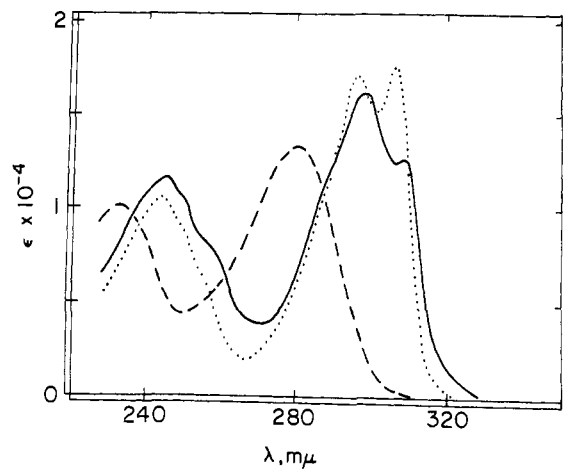


FIGURE 6: Absorption spectra of BP (– – – –), $Zn(BP)_1^{2+}$ (· · · · · ·), and $Zn_f' \cdot BP$ (———). 0.1 M Tris-Cl (pH 7.5).

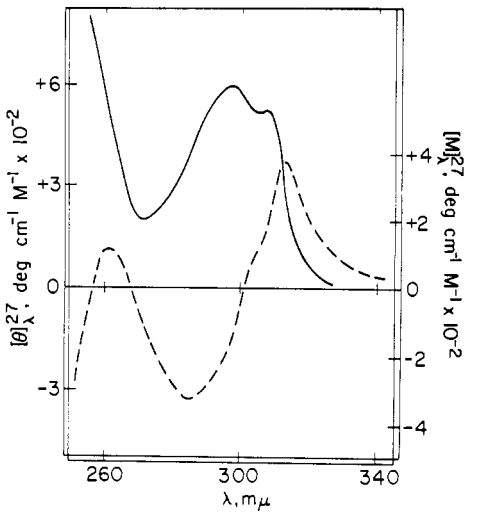


FIGURE 7: Optical rotatory dispersion and circular dichroism of the $Zn_f' \cdot BP$ complex. 0.1 M Tris-Cl (pH 7.5). The ellipticity (———) and optical rotatory dispersion (– – – –) curves are corrected for the dissociation constant of the $Zn_f' \cdot BP$ complex and expressed in terms of chromophore molarity as discussed under Methods.

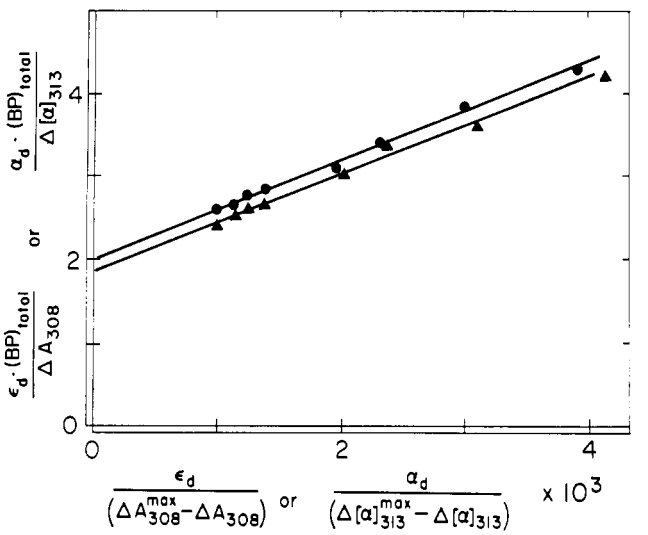


FIGURE 8: Spectrophotometric and rotatory dispersion titration of LADH with BP. The points from the spectrophotometric titration (●) and those from the rotatory dispersion titration (▲) are plotted according to the linear form of the mass law equation (eq 1c). The symbol $\epsilon_d$ denotes the molar difference absorptivity and $\alpha_d$ is the corresponding quantity for optical rotation, the molar difference dispersion. The numerical value of the stoichiometry for the interaction of BP with LADH, $n$, is given by the $y$ intercept; the slope of the curves is the dissociation constant, $K$. The final LADH concentration was $1.2 \times 10^{-3}$ M, in 0.1 M phosphate–0.0075 M glycine (pH 7.5), 27°.

spectrum of the $Zn_f' \cdot BP$ complex from 350 to 300 mμ has been described recently (Sigman, 1965, 1967), and the present data extend these observations. Interaction of $Zn_f'$ with BP causes hypochromic and bathochromic shifts in the absorption spectrum as compared to that of the $Zn(BP)_1^{2+}$ complex; maxima occur at 246 ($\epsilon$ $11.8 \times 10^3$ $cm^{-1}$ $M^{-1}$), 298 ($\epsilon$ $16.2 \times 10^3$ $cm^{-1}$ $M^{-1}$), and 308 mμ ($\epsilon$ $12.6 \times 10^3$ $cm^{-1}$ $M^{-1}$) (Figure 6). In the circular dichroic spectrum, similar maxima are observed at 298 mμ ($[\theta]_{297}^{27} = 6.0 \times 10^2$ deg $cm^{-1}$ $M^{-1}$) and 308 mμ ($[\theta]_{308}^{27} = 5.2 \times 10^2$ deg $cm^{-1}$ $M^{-1}$) (Figure 7). The optical rotatory dispersion of the complex exhibits a peak at 313 ($[M]_{313}^{27} = +3.7 \times 10^2$ deg $cm^{-1}$ $M^{-1}$), a trough at 285 ($[M]_{285}^{27} = -3.2 \times 10^2$ deg $cm^{-1}$ $M^{-1}$), and a second smaller peak at 260 mμ (Figure 7). In accord with the high dissociation constant for this complex, the spectrophotometric titration curve for $Zn_f' \cdot BP$ does not exhibit a sharp break (Sigman, 1967). In order to derive the stoichiometry by linear extrapolation the mass law expression for the $Zn_f' \cdot BP$ complex was rearranged (Klotz, 1946; Stockell, 1959).

For the reaction $Zn_f' \cdot BP = Zn_f' + BP_{free}$

$$K = \frac{(Zn_f')(BP_{free})}{(Zn_f' \cdot BP)} \quad (1)$$

$$= \frac{[2(LADH) - (Zn_f' \cdot BP)][(BP_{total}) - n(Zn_f' \cdot BP)]}{(Zn_f' \cdot BP)} \quad (1a)$$

where $n$ = moles of BP bound per mole of LADH.

Using the relations

$$\frac{\Delta A_{max}}{(Zn_f' \cdot BP)_{max}} = \frac{\Delta A}{(Zn_f' \cdot BP)} = \frac{(\Delta A_{max} - \Delta A)}{[2(LADH)] - (Zn_f' \cdot BP)} \quad (1b)$$

eq 1a may be rearranged to give

$$\frac{(BP_{total})}{\Delta A} \epsilon_d = \frac{K\epsilon_d}{(\Delta A_{max} - \Delta A)} + n \quad (1c)$$

The subscript, *max*, refers to the maximum possible value for absorbance, which is fixed by the concentrations of LADH and BP; $\epsilon_d$ is the difference absorptivity. Analogous expressions may be written for the difference *dispersion* measurements.

When plotted in this manner, the results of spectrophotometric and rotatory dispersion titration of the $Zn_f' \cdot BP$ complex correspond closely with each other (Figure 8). The data indicate that 1.9–2.0 moles of BP interacts with 2.0 moles of $Zn_f'$, and the dissociation constant is $6 \times 10^{-4}$ M. Based on this stoichiometry and dissociation constant the molar absorptivity, molar ellipticity, and molar rotation of the $Zn_f' \cdot BP$ complex can be computed over the wavelength range from 240 to 340 mμ (Figures 6 and 7). The absorptivities at the maxima are given in Table I.

The specific interaction of these chelating agents with only one of the two classes of zinc atoms in LADH (Drum *et al.*, 1967) was documented further. Diethyldithiocarbamate, a bidentate sulfur-containing chelating agent, selectively removes the two active site zinc atoms of LADH (Drum *et al.*, 1969a). This agent has a much higher affinity for zinc than either OP or BP. Addition of OP to LADH, modified in this manner, does not generate either the spectra observed when OP binds to native LADH or the Cotton effects of the mixed complex (Figure 9A). However, NADH still binds to LADH, inactivated by diethyldithiocarbamate, as evidenced by characteristic spectral (Theorell and Bonnichsen, 1951) and spectropolarimetric (Ulmer *et al.*, 1961) changes, but addition of excess OP does not affect these changes (Figure 9A). Thus, based on these criteria, OP does not bind to diethyldithiocarbamate-modified LADH.

Carboxymethylation with iodoacetate, previously shown to label two reactive cysteinyl residues of LADH at or near the two coenzyme binding sites of LADH (Li and Vallee, 1964),

affords further information in this regard. *S*-CM-cysteine–LADH contains 1.9–2.0 g-atoms of zinc/per mole of enzyme after dialysis against metal-free buffer (Drum *et al.*, 1967). When LADH is labeled selectively at the active sites with 2.0 g-atoms of $^{65}Zn$/mole (Drum *et al.*, 1969b), only the labeled but none of the unlabeled zinc atoms remain after carboxymethylation and dialysis against metal-free buffer; apparently, the labeled $^{65}Zn$ atoms are those which are catalytically active.

In contrast to diethyldithiocarbamate-inactivated LADH, *S*-CM-cysteine–LADH does bind OP, as measured by appearance of its distinctive, positive Cotton effects (Figure 9B). Furthermore, rotatory dispersion titration of *S*-CM-cysteine–LADH with OP indicates binding of 2 moles of OP/mole of *S*-CM-cysteine–LADH.

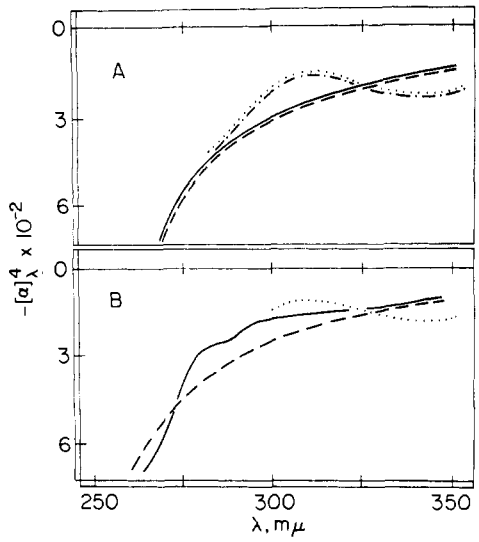


FIGURE 9: Cotton effects of modified LADH. (A) Optical rotatory dispersion of $5.7 \times 10^{-5}$ M diethyldithiocarbamate-inactivated LADH before (– – – –) and after addition of $3.1 \times 10^{-4}$ M OP (———) or NADH (· · · · · ·) or both (—·—·—·—). (B) Optical rotatory dispersion of $1.5 \times 10^{-4}$ M *S*-CM-cysteine–LADH before (– – – –) and after addition of $3.3 \times 10^{-4}$ M OP (———) or $3.0 \times 10^{-4}$ M NADH (· · · · · ·). Experiments were performed in 0.1 M Tris-Cl (pH 7.5), 4°.

## Discussion

The presence of metal atoms in enzymes may be detected directly, employing well-known analytical methods (Vallee, 1955; Drum *et al.*, 1969a), or indirectly, by measurement of properties arising from the metal–protein interaction, *e.g.*, enzymatic activity and its inhibition by chelating agents, and absorption, optical rotatory dispersion, circular dichroism, nuclear magnetic resonance, and electron paramagnetic resonance spectra (Vallee and Wacker, 1970). Considerations pertinent to the direct determination of the metal content of metalloenzymes have been discussed recently, with particular reference to zinc in LADH (Drum *et al.*, 1969a). LADH preparations currently available contain 3.1–4.2 g-atoms of zinc/mol wt $80 \times 10^3$ (Åkeson, 1964; Oppenheimer *et al.*, 1967; Drum *et al.*, 1969a); this stoichiometric variability appears to be related both to the different roles of the zinc atoms in LADH (*vide infra;* Drum *et al.*, 1969a,b) and to the existence of multiple isozyme forms of LADH (Pietruszko *et al.*, 1969).

The inhibition of LADH by 1,10-phenanthroline was one of the first indications, by indirect criteria, of the presence of zinc in LADH and of its functional role in the enzyme (Vallee and Hoch, 1957).[4] The interaction of LADH with OP results in spectral changes similar to, though not identical with, those of the $Zn(OP)_1^{2+}$ complex (Vallee *et al.*, 1958), and based on this property the stoichiometry and dissociation constant of the LADH–OP complex, $Zn_f' \cdot OP$, were determined (Vallee and Coombs, 1959). These and other studies indicated the existence of two active sites, data entirely in accord with those of physicochemical measurements on preparations of LADH then available. Indeed, since then evidence derived independently both from metal isotope exchange and chemical modification (*vide infra*) suggests that only two of the zinc atoms in LADH are catalytically active. This conclusion is also in accord with functional evidence (Drum *et al.*, 1969a,b) and with the properties of earlier preparations, now known to have been less active than those presently available and presumed, therefore, to have been impure.

Recognition of these circumstances prompted an examination of the stoichiometry of OP binding to highly purified enzyme by means of the method of continuous variations (Job, 1928). The results of the use of Job's method with LADH indicated two distinct advantages over the method of molar proportions for the study of this enzyme: the *shape* of the curve both excludes the possibility that zinc is removed from LADH by OP and shows that OP forms two separate 1:1 $Zn_f' \cdot OP$ complexes on the enzyme surface, rather than 2:1 $(Zn_f')_2 \cdot OP$ complexes with more than two zinc atoms or a 1:2 $Zn_f' \cdot (OP)_2$ complex with a single zinc atom. These points require further discussion.

The occurence of one or more maxima in the Job's curve at *less than 50 mole* % of zinc would suggest dissociation of zinc from the enzyme. A maximum at 25 mole % of zinc would confirm formation of the 1:3 $Zn(OP)_3^{2+}$ complex, as found by Felber *et al.* (1962) for the interaction of OP with carboxypeptidase A and indicating removal of the metal by the chelating agent.

When two *stable* complexes, *e.g.*, $Zn(OP)_1^{2+}$ and $Zn(OP)_2^{2+}$ are formed, $Zn(OP)_1^{2+}$ reaches its maximum concentration at a reagent ratio *less* than the formal ratio $(OP)/(Zn^{2+})$, and the concentration of $Zn(OP)_2^{2+}$ becomes maximum at a reagent ratio somewhat *above* the ratio $2(OP)/(Zn^{2+})$ (Katzin and Gebert, 1950). However, the measured spectroscopic property of a solution is the sum of those of the component species, and without information about their characteristics, *i.e.*, concentration, molar absorptivity, dissociation constants, the shape of the resulting Job's curve cannot be predicted.

In order to explore the effects of these parameters on the formation of the three hypothetical complexes (*vide supra*) and to extend this analysis to the LADH–OP interaction, the numerical computation of Job's curves was formulated to provide for the potential inaccessibility to a ligand of a fraction of the enzyme-bound zinc atoms (see Appendix).

If all zinc atoms were reactive, formation of the 1:1 $Zn_f' \cdot OP$ complex would be maximal at 50 mole % of zinc. Since the Job's plot is based on the presumption that the reactants are equimolar and their sum is constant, the presence of zinc atoms which do not react with OP of necessity shifts the maxi-

[4] Although subsequent kinetic studies with this and other metal binding agents further defined the catalytic role of the metal, they will not be discussed here.

mum of the curve toward higher values of mole per cent of zinc (Figure 4).

A formation curve for a hypothetical $(Zn_f')_2 \cdot OP$ complex exhibits a maximum at slightly over 50 mole % of ligand, and its descending limb is concave. Both these features are lacking in the experimental data, since the absorptivity of a $(Zn_f')_2 \cdot OP$ would have to be equal to or less than that of the $Zn_f' \cdot OP$ complex (Vallee *et al.*, 1958; Vosburgh and Cooper, 1941).

Past discussions of the theory underlying the method of continuous variations (Vosburgh and Cooper, 1941; Katzin and Gebert, 1950; Woldbye, 1955; Jones and Innes, 1958) have not considered its applicability to systems in which a fraction of the metal ion present will not react with a given ligand. Such a situation would be unusual in inorganic systems, although compounds such as the polynuclear cobalt acetate complexes might conceivably exhibit such behavior under certain conditions (Sharp and White, 1952).

The two classes of zinc atoms in LADH and their interactions with OP and BP provided the impetus for such an extension by incorporating a parameter, $s$, to account for the reactivity of a fraction of the metal atoms (see Appendix). When the Job's curve for formation of two distinct $Zn_f' \cdot OP$ complexes is generated employing a value for $s$ derived both from the total zinc content measured and the known stoichiometry of OP binding (Figure 3), it approximates the experimental data very closely (Figure 5). This is in marked contrast to other possible models which were examined.

The dependence both of the location and the absolute value of the maximum difference absorptivity on the fraction $s$ of the zinc in LADH which is reactive toward OP afforded additional confirmation of the measured zinc content of these LADH preparations. This value was found to be 3.5 g-atoms of zinc/mole of LADH, corresponding to a value of $s = 0.57$. Assuming arbitrarily a value of precisely 4.0 g-atoms/mole, then $s = 0.50$ when two molecules of OP bind to each molecule of LADH. However, the Job's curve generated for $s = 0.50$ does not then coincide with the experimental data, and the difference is sufficiently great to exclude an analytical error. Thus, when employed in this manner the method of continuous variations can serve parenthetically as a gauge of accuracy of analytical data, much as this is not the primary purpose for its use. The experimental data are compatible with the hypothesis that only two of the zinc atoms of this LADH preparation form separate 1:1 complexes with OP; the remaining zinc atoms of LADH neither react with OP to form a mixed complex nor does OP remove them from the enzyme.[5]

Spectrophotometric, circular dichroic, and rotatory dispersion titrations indicate the same stoichiometry of binding for OP and BP, *i.e.*, 2 moles/mole of LADH, suggesting that the same zinc atoms are responsible for both the Cotton effects and the changes in absorption.[6] The specificity implied both by these titrations and the Job's plot was confirmed independently by studies with LADH modified to selectively remove either the reactive, catalytically active zinc atoms or those which are unreactive or buried.

Diethyldithiocarbamate inactivates the enzyme while removing only the two functional zinc atoms of LADH (Drum *et al.*, 1969a,b). This modified enzyme binds neither OP nor BP (Figure 9A). The present data would further suggest that OP binds asymmetrically *only* to the catalytically active zinc atoms of LADH.

Carboxymethylation of LADH labilizes those zinc atoms of LADH which are *not* related directly to catalysis and which may then be removed by dialysis (Li and Vallee, 1962; Figure 9B). The zinc content of *S*-CM-cysteine–LADH (Drum *et al.*, 1967) is 2.0 g-atoms of zinc/mole and these metal atoms are those which exchange selectively with $^{65}Zn^{2+}$ in acetate buffer (Drum *et al.*, 1967, 1969b).

Though the characteristics of BP are similar to those of OP, it is less suitable than that agent for studies based on the method of molar proportions. The affinity of BP for $Zn^{2+}$ is lower than that of OP, but the absorption band of the $Zn_f' \cdot BP$ complex at 308 m$\mu$ is less subject to interference than the $Zn_f' \cdot OP$ bands at 271, 297, and 329 m$\mu$. These properties have been found advantageous when studying the binding of substrates, coenzyme analogs, or inhibitors to LADH in competition with BP. However, the low stability constant of the $Zn_f' \cdot BP$ complex renders it much less suitable for studies by means of the method of continuous variations since the maxima of the Job's curve are not well defined.

The molar absorptivities of the mixed complexes of LADH with OP and BP obtained here are in agreement with those of earlier studies but reported as *difference* absorptivities. Thus, Sigman (1965, 1967) determined the molar *difference* absorptivities for BP at 308 m$\mu$ and for OP at 297 to be $1.1 \times 10^{-4}$ and $8.0 \times 10^{-3}$ cm$^{-1}$ M$^{-1}$, respectively. The corresponding values from Table I are $1.26 \times 10^4$ and $10.0 \times 10^3$ cm$^{-1}$ M$^{-1}$, the difference reflecting contributions of the enzyme and of free chelating agent at the respective wavelengths. Similarly, the molar absorptivity of 1450 cm$^{-1}$ M$^{-1}$ at 329 m$\mu$ is close to that expected from the corresponding *difference* absorptivity at 329 m$\mu$ for the $Zn_f' \cdot OP$ complex reported previously (Vallee and Coombs, 1959; Yonetani, 1963a,b; Sigman, 1965, 1967).

The dissociation constant for the $Zn_f' \cdot BP$ complex, $6 \times 10^{-4}$ M, is in good agreement with the value $4 \times 10^{-4}$ M based on measurements in 0.045 M sodium phosphate (pH 7.0) in the presence of $10^{-3}$ M EDTA (Sigman, 1965, 1967). These values are also only slightly higher than those for the third stepwise dissociation constant of the ionic complex, where in 0.1 M $KNO_3$, $K_3 = 1.4 \times 10^{-4}$ M (Yasuda *et al.*, 1956) and in 0.1 M $NaNO_3$, $K_3 = 1.6 \times 10^{-4}$ M (Anderegg, 1963).

Diethyldithiocarbamate-inactivated LADH, which neither contains the "reactive," enzymatically active zinc atoms nor binds OP, exhibits characteristic changes both in absorption and optical rotatory dispersion on addition of NADH, both in the presence and absence of OP (Figure 9A). It has

[5] The linearized curve for titration of zinc in LADH with BP (Figure 8) also excludes formation of a 1:2 $Zn_f' \cdot (BP)_2$ complex. The experimental data are not compatible with the mass law expression for such a complex

$$K_2 = \frac{(Zn_f')(BP)^2}{(Zn_f' \cdot (BP)_2)}$$

[6] The circular dichroism of dissymmetric metal complexes containing OP and BP has recently been employed to verify a nonempirical method for establishing the absolute configuration of the bis and tris complexes in solution (Bosnich, 1968, 1969; Ferguson *et al.*, 1969). While interpretation of the optical activity generated in the mono complexes formed with $Zn_f'$ (Figures 2 and 7) is complicated by interactions with side-chain chromophores, studies now in progress may permit an empirical stereochemical assignment for $Zn_f' \cdot OP$ and $Zn_f' \cdot BP$ (Drum, 1970).

been shown previously that *S*-CM-cysteine-LADH lacks the structural, "buried" zinc atoms (Drum *et al.*, 1967), but binds NADH (Li and Vallee, 1965) (Figure 9B); hence, apparently coenzyme binding is not uniquely dependent either on the "free" or "buried" zinc atoms in LADH, in agreement with recent studies employing a spin-labeled analog of ADP-ribose: both electron paramagnetic resonance and proton relaxation measurements indicate displacement of the analog when NADH is added to zinc-free LADH (Weiner, 1969; Mildvan and Weiner, 1969). In each case NADH appears to bind slightly less firmly to modified than to native LADH. Thus, in contrast to binding of the chelating agents OP and BP, the binding of reduced coenzyme to LADH cannot wholly depend on interaction with the zinc atoms.

The interaction of chelating agents with metalloenzymes is generally thought to be dependent uniquely on interaction with their metal atoms. However, systematic examination of different possible modes of inhibition have not been conducted with physical-chemical characterization of such systems (Orgel, 1966; Vallee and Wacker, 1970).

Thus nonchelating polynuclear pyridine derivatives, such as *m*-phenanthroline, have been reported to inhibit and disaggregate glutamic dehydrogenase more effectively than does OP (Yielding and Tomkins, 1962). Similarly, both yeast alcohol dehydrogenase (Professor F. H. Westheimer, personal communication) and bacterial metapyrocatechase (Nozaki *et al.*, 1966) may, under certain conditions, be more susceptible to inhibition by *m*-phenanthroline than by OP.[7] The absorption maxima of both $Zn(OP)_1^{2+}$ in aqueous solution and OP base in benzene at 324 mμ, ε 740 $cm^{-1}$ $M^{-1}$, have led to the questionable conclusion that OP interacts *exclusively* with hydrophobic groups of *yeast* alcohol dehydrogenase, not with zinc of the enzyme (Anderson *et al.*, 1966).

Formation of the univalent ions of OP and BP by addition of $H^+$ to the bases in aqueous solution results in bathochromic shifts of their ultraviolet absorption maxima and slight enhancement of absorptivity (Krumholz, 1951). Similarly, the absorption maxima at 265 and 281 mμ, respectively, of uncharged OP and BP in benzene show bathochromic shifts of 3 mμ and absorption is increased as compared to aqueous solution (D. E. Drum, unpublished observations). In contrast, the changes accompanying the formation of the coordination complexes with $Zn^{2+}$ in aqueous solution are more marked. The maximum of OP at 265 mμ is shifted to 270 mμ. Importantly, a second peak at 292 mμ is not seen in benzene at all. The band of BP at 280 mμ is red shifted and split into discrete peaks at 296 and 306 mμ. This phenomenon is not observed either on protonation or when benzene serves as a solvent. When OP binds to $Zn_f'$ (Figure 1), the bathochromic shift is greater and absorbance at higher wavelengths is enhanced even more than in the $Zn(OP)_1^{2+}$ complex. Compared to $Zn(BP)_1^{2+}$, formation of the $Zn_f'\cdot BP$ complexes are characteristic of interaction with the metal and cannot be attributed to nonspecific effects.

The properties of the $Zn_f'\cdot OP$ and $Zn_f'\cdot BP$ complexes described here illustrate an approach to the characterization of a fraction of the zinc atoms in a metalloenzyme containing nonreactive metal atoms. The selective replacement of zinc atoms by a metal with more advantageous spectroscopic properties, such as cobalt, or with quantitatively different chemical properties, such as cadmium, represents an alternative approach (Drum, 1970; Drum and Vallee, 1970). Furthermore, preparation of "metal-hybrid" metalloenzymes, in which one or the other distinct class of native metal atom, *e.g.*, zinc, is replaced by a different metal, should facilitate more definitive evaluation of the functional roles of the separate classes of metal atoms.

[7] *m*-Phenanthroline is not optically active in the presence of horse LADH and is a much less effective inhibitor of LADH than is OP (D. E. Drum, unpublished observations).

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## Appendix

The equations for interaction of two chemical species were set up for computer analysis of the general method of continuous variations as follows.

Solutions of species A′ and B, each of molarity $M$ are mixed using $x$ fractional volumes of B and $1 - x$ volumes of A′. The possible complexes are formed by the reactions

$$A + nB = AB_n$$

$$AB_n + pB = AB_{n+p}$$

where $A = sA'$ and $s$ is the fraction of A′ which is specifically reactive toward B.

The increase in absorbance due to complex formation as the mole fraction, $x$, of ligand is varied from 0.0 to 1.0 in the case of the interaction of OP with LADH is

$$Y = l\{\epsilon_E c_E + \epsilon_1 c_1 + \epsilon_2 c_2 + \epsilon_0 c_0 - \epsilon_E sM(1 - x) - \epsilon_0 Mx\}$$

where $\epsilon$ denotes molar absorptivity and $l$ the absorption cell path length.

The following relations also apply

$$c_E = (A) = sM(1 - x) - c_1 - c_2 = (Zn_f')$$

$$c_0 = (B) = Mx - nc_1 - (n + p)c_2 = (OP)$$

$$c_1 = (AB_n) = \frac{c_E(c_0)^n}{K_1} = (Zn_f'\cdot OP)$$

$$c_2 = (AB_{n+p}) = \frac{c_1(c_0)^p}{K_2} = (Zn_f'\cdot (OP)_2)$$

This set of simultaneous equations can be solved to yield a polynomial in $c_0$

$$\frac{1}{K_1K_2}c_0^{n+p+1} + \frac{M}{K_1K_2}c_0^{n+p}\{s(n + p) - x[s(n + p) + 1]\} + \frac{1}{K_1}c_0^{n+1} + \frac{M}{K_1}c_0^{n}\{sn - x[sn + 1]\} + c_0 - Mx = 0$$

For a given set of parameters $s$, $p$, $n$, $M$, $K_1$, and $K_2$, this equation can be solved for any given value of $x$. The values of $c_1$, $c_2$, and $c_0$ are then obtained by insertion of the value obtained for $c_0$ in the previous relations, and $Y$ as a function of $x$ is then calculated, yielding a Job's curve for the given set of parameters. Solution of these equations was programmed in FORTRAN IV-H on a Sigma 7 time-sharing computer. The polynomial equation for $c_0$ was solved by successive approximations to the positive root in the range $0 \leq c_0 \leq Mx$ using Newton–Raphson iteration.

The accuracy of this program was checked by calculation of Job's curves for simple cases, *e.g.*, 1:1 AB complex formation ($s = 1, n = 1, p = 0, K_1 = 10^{-5}$ M, and $K_2 = 10^{+20}$ M) and 1:2 $AB_2$ complex formation ($s = 1, n = 1, p = 1, K_1 = K_2 = 10^{-5}$ M). These cases showed the expected maxima at 50 and at 33 mole % A, respectively. The values for difference absorbance were zero at $x = 0$ and 1.0 and positive for all intermediate values of $x$, both for these ideal cases and for those in which more complex relations prevailed. Figure 5 displays the theoretical curves obtained for formation of 2:1 $A_2B$, 1:1 AB ($s = 0.57$), 1:1 AB ($s = 0.50$), and 1:2 $AB_2$ complexes employing the best available estimates of the required parameters.

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