ACTA CHEMICA SCANDINAVICA 15 (1961) 1866–1874

Liver Alcohol Dehydrogenase

IV. Kinetics in the Presence of Zinc Binding Agents

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Measurements of the kinetics of both the oxidation of alcohol by DPN ** and the reduction of aldehyde by DPNH have been made in the presence of a variety of zinc binding agents. These substances show a varied pattern of behavior, which is summarized as follows. Binding of inhibitors to the enzyme parallels the binding of the third bidentate ligand to free zinc, indicating the zinc to be bound in the enzyme octahedrally at more than two of its coordination positions. Bidentate ligands compete with DPN and DPNH, thus indicating that at least one of their normal binding sites are at the zinc. In addition ternary complexes are formed between enzyme-substrate-inbibitor which show markedly increased affinity of inhibitor and substrate for the enzyme. Furthermore, monodentate ligands compete with substrate in forming ternary complexes with enzyme-coenzyme complexes. Both these results are taken to indicate that the substrates may also be bound to the zinc.

It has been shown that a number of species capable of binding to the zinc atoms of the enzyme liver alcohol dehydrogenase act as inhibitors for the enzyme [1-3]. In one case, that of 1,10-phenanthroline, the actual binding of the inhibitor was determined spectrophotometrically [4], and the kinetics of the inhibition studied in detail [3]. Studies of this type, if extended to cover a wide range of inhibitors, might provide considerable information as to the role played by zinc atoms in the enzyme. For this reason the present study was undertaken. Specifically, three topics were investigated. By studying in some detail the kinetics of both the forward (reduction of aldehyde by DPNH) and reverse (oxidation of alcohol by DPN) processes, it was hoped to obtain an indication as to the role of the zinc atom in binding the various reactants. By using as inhibitors species whose affinities for free Zn^{++} are known, it was hoped to confirm or deny the importance of Zn in the enzyme activity and furthermore

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** Abbreviations: DPNH and DPN, reduced and oxidized diphosphopyridine nucleotide, respectively, which are also abbreviated in dissociation constants as R and O.

to gain information concerning the state of the zinc in the enzyme. Finally, it was hoped that a study of several different anions would shed some light on the relatively large and apparently specific effects on the enzyme shown by negative ions [1].

For the present study, the following species were used as possible enzyme inhibitors: 1,10-phenanthroline, 2,2'-dipyridyl, ethylenediamine, oxalate ion, pyrophosphate ion, sulfate ion, thiosulfate ion, thiocyanate ion, fluoride ion, bromide ion, and perchlorate ion. For all, except perchlorate, the affinity for free zinc ion is known [5]. Perchlorate ion shows no measurable affinity for Zn^{++} or other positive ions in aqueous solution. Thus the series should be ideal for revealing any correlations between zinc binding and inhibitor action.

EXPERIMENTAL

Crystalline alcohol dehydrogenase was prepared according to the method of Bonnichsen and Brink [6]. After three recrystallizations, the assay of Dalziel [7] showed the purity to be 106 %. DPN and DPNH were obtained from Sigma Chemical Company and assayed spectrophotometrically. Acetaldehyde and ethanol were redistilled and assayed enzymatically. Ethylenediamine was redistilled just prior to its use and NaSCN was recrystallized from water. All other inhibitors and the buffer salts ($NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12\ H_2O$) were of the purest grade commercially available, used without further purification. All water was redistilled in an all glass system.

Initial rates were determined by following the change in DPNH concentration as reflected by the change of its fluorescence. Both the apparatus used [8] and the general technique have been described previously [1]. Rates were determined from tangents at the time of mixing and it was found that the values so obtained were precise, judging from duplicate experiments, to within a mean deviation of ± 5 %. For each inhibitor four sets of experiments were carried out. In the first, case 1, aldehyde concentration was relatively high and constant, while DPNH was varied from experiment to experiment. In the second, case 2, DPNH was constant while aldehyde was varied. Similarly, case 3 consisted of constant alcohol with varied DPN, and case 4 of constant DPN and varied alcohol. The temperature was 23.5° and the total ionic strength 0.1 μ. The pH was maintained at 7.1 in all experiments.

Table 1. Inhibitor effects.

Inhibitor	DPNH varied [Ald.] = 3.0 mM	Ald. varied [DPNH] = 9.8 μM	DPN varied [Alc.] = 8.0 mM	Alc. varied [DPN] = 200 μM
Ethylene-diamine	No effect I = 3.5 mM	Competitive $K_I > 3$ mM	No effect I = 3.4 mM	No effect I = 3.4 mM
Sodium oxalate	No effect I = 2.1 mM	Noncompetitive $K_I > 2$ mM	No effect I = 3.4 mM	Competitive $K_I = 30$ mM
Sodium pyrophosphate	No effect I = 2.7 mM	No effect I = 2.7 mM	No effect I = 3.0 mM	No effect I = 3.0 mM
Sodium sulfate	No effect I = 2.5 mM	No effect I = 2.5 mM	Competitive $K_I = 8$ mM	No effect I = 3.0 mM
Sodium thiosulfate	Competitive $K_I = 6$ mM	Noncompetitive $K_I > 20$ mM	Competitive $K_I = 4$ mM	No effect I = 3.5 mM

RESULTS

Figs. 1—4 show results obtained for the four sets of experiments run in the absence of inhibitors (lower lines, with actual points shown in Fig. 4) and in the presence of six of the inhibitors tested. Each set of experiments was duplicated with good agreement; however, for clarity, only one set of points is shown. The remaining five inhibitors showed very small effects and the results found with them are summarized in Table 1.

From the figures, or Table 1, it is seen that the various inhibitors display various patterns of behavior. Within the entire set, the most common is that shown, for example, by 1,10-phenantroline. The same pattern, competition with three of the reactants, but not with aldehyde, is shown by 2,2′-dipyridyl, Br^-, and ClO_4^-. On the other band, SCN^- and F^- seem to compete only with alcohol, although as will be discussed below, the apparent non-competition is probably illusory.

The inhibitor 1,10-phenanthroline has been studied in the greatest detail both in the present investigation and in a previous one [3]. Both investigations show strict competition with DPNH and DPN, non-competition with aldehyde

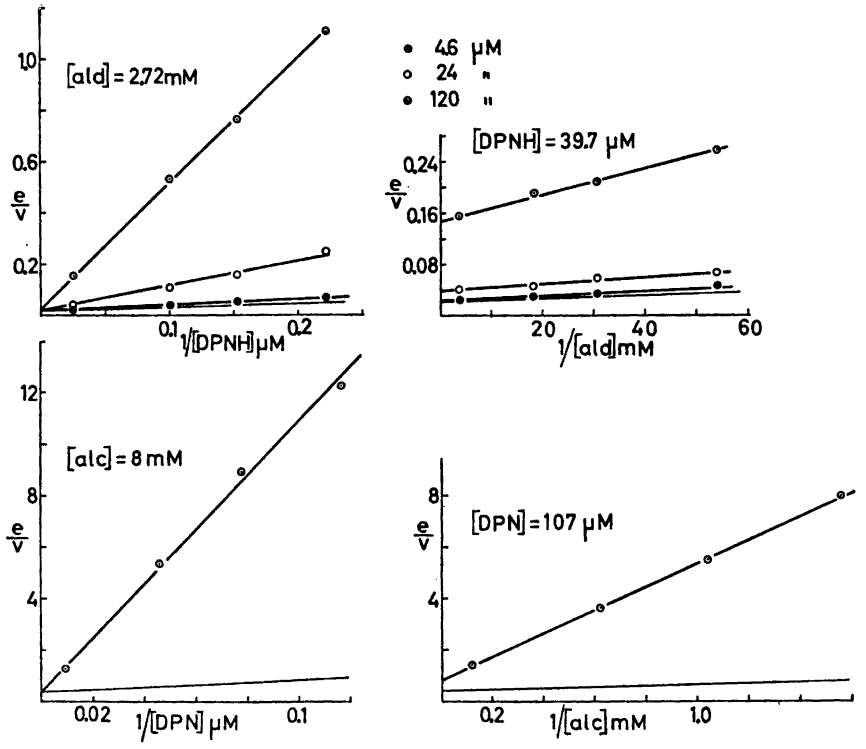


Fig. 1. Kinetic behavior with 1,10-phenanthroline. Closed circles refer to inhibitor = 4.6 μM, open circles, 24 μM, circles with dot, 120 μM. Enzyme concentrations are in terms of active sites.

and mixed inhibition with alcohol. The results for the latter case seem to differ somewhat between the two studies. However, the difference is not large and probably arises as follows. The results of Vallee *et al.* were obtained using alcohol in excess of 10 mM which causes some substrate inhibition [9] and thus biases the curves somewhat toward non-competitive behavior. The present results were obtained at lower DPN concentration, which because of the competition between DPN and the inhibitor emphasizes the competition between alcohol and 1,10-phenanthroline. For further discussion, see Part III, p. 1862.

In interpreting the 1,10-phenanthroline results, the simplest mechanism is to postulate the binding of free enzyme by the inhibitor thus preventing it from entering the reaction. This hypothesis can be tested by seeing whether the dissociation constants calculated from the various plots give the same value for the dissociation constant of the enzyme inhibitor complex ($K_{E,I}$). It should be noted that $K_{E,I}$ is not in general equal to the inhibitor constant, K_I (recorded in Table 1 for all competitive cases), which is merely the concentration of inhibitor necessary to halve the rate. The reason for the difference is that in most experiments only a small fraction of the total enzyme is present as free enzyme, the majority being present as binary complexes containing one of the four reactant species (alcohol, aldehyde, DPN, or DPNH).

It is thus necessary to calculate the fraction of free enzyme, prior to calculating $K_{E,I}$. To do so requires a knowledge of the dissociation constants of the various binary constants. These values have been determined (see Parts II and III) and the values used in calculating the present results are $K_{E,ald} = 10.2$ μM, $K_{E,R} = 0.31$ μM $K_{E,alc} = 4\,600$ μM, $K_{E,O} = 160$ μM[10]. Using these values, apparent values of $K_{E,I}$ can be calculated for 1,10-phenanthroline. These turn out to be 1.3×10^{-8} M (from the first series, high aldehyde), 3.0×10^{-6} (high alcohol), 1.1×10^{-5} (both from the series shown in Fig. 1, DPN = 107 μM, and from a separate experiment with DPN = 200 μM). Comparison of the three values obtained show that they vary widely and that only the last agrees at all reasonably with the value (3.3×10^{-5} M) determined directly by Vallee and Coombs [4]. Thus, we are forced to conclude that the situation is more complicated and that ternary complexes involving the inhibitor are indicated, *cf.* Parts II and III.

The data for the forward reaction (case 1) can be accounted for by assuming the presence of the ternary complex E ald I with dissociation constant $K_{E\,ald,\,I} = 4$ μM. The value seems of reasonable magnitude since the presence of aldehyde might be expected to stabilize the binding of 1,10-phenanthroline. The data for the reaction from the alcohol side (case 3) seems likewise to require the postulation of at least one ternary complex, this time between enzyme, alcohol and inhibitor. The value resulting for $K_{E\,alc,I}$ is 4.0 μM. This number is again of reasonable magnitude especially since it is the same as that for the ternary complex differing only by the substitution of aldehyde for alcohol.

K_I obtained from the slopes, in cases 2 and 4, is interesting, being 17 μM and 6.6 μM. The slopes reflect the situation when [ald] or [alc] are approaching zero, and thus competition between *o*-phenanthroline and the adenine moiety of DPNH or DPN (*cf.* Part III). The adenine is so weakly bound that the values are close to 14 μM, the third dissociation constant for *o*-phenanthroline and free zinc ions, the corresponding association constant k_3 being 4.85 [5]. This gives

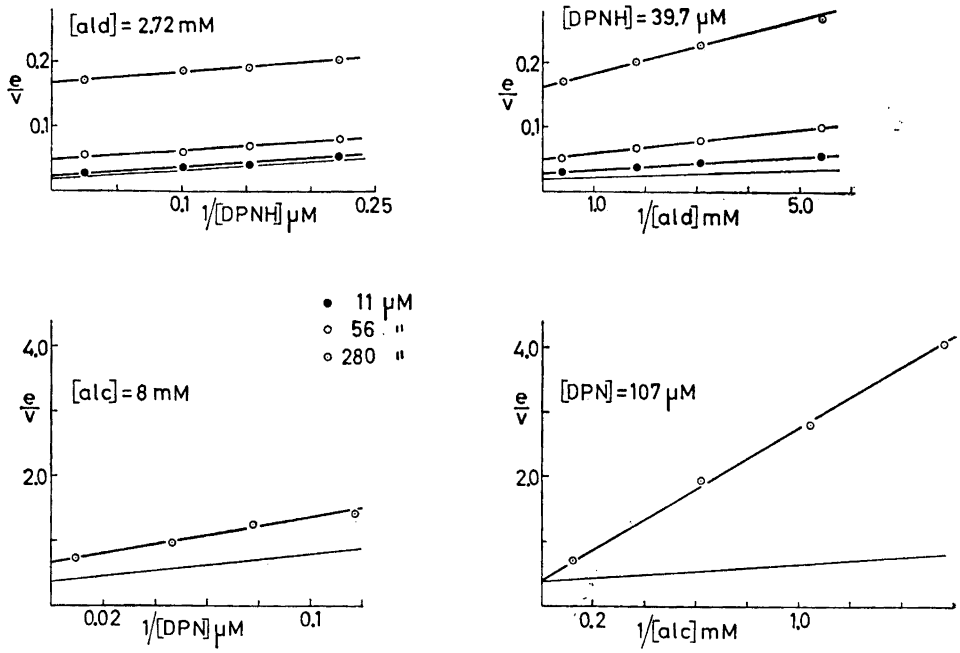
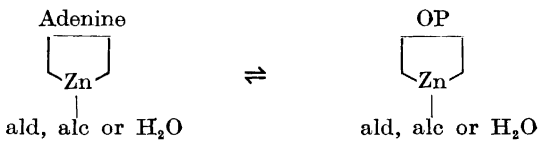


Fig. 2. Kinetic behavior with SCN^-. Closed circles refer to inhibitor = 1.1×10^{-5} M, open circles 5.6×10^{-5} M, circles with dots, 2.8×10^{-4} M.

additional evidence that the weak adenine-zinc bonds are essential for the activity.

As discussed in Part III the complexes could be pictured (omitting attachments of Zn to the enzyme) as follows:



The conclusion of Vallee [3] that the aldehyde and ethanol are not attached to the zinc rests upon his statement of non competition of OP and these substrates. However, this conclusion is clearly invalid. The three free valencies of the zinc allow ternary complexes with substrates and OP to be formed. The mixed competition occurring in case 4 is explained in Part III.

The results for 2,2'-dipyridyl are as follows. If no ternary complexes form, the apparent values for $K_{E,I}$ are 5.4×10^{-7} M (from competition with DPNH), 5.2×10^{-4} (from competition with DPN) and 6.3×10^{-4} M (from competition with alcohol). Agreement between the last two values is completely satisfactory and thus there is no reason to postulate ternary complexes for the reverse

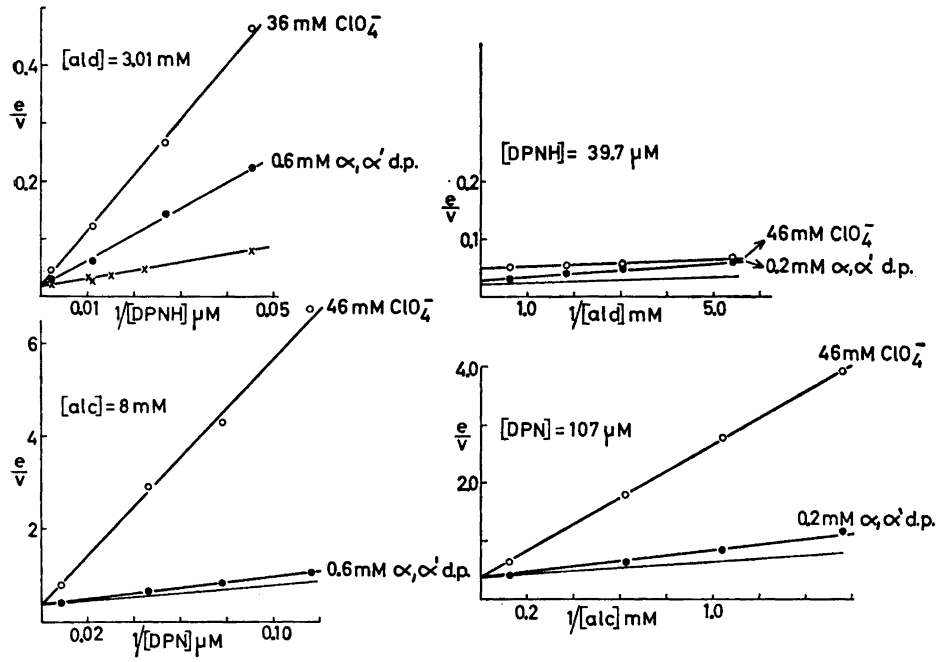


Fig. 3. Kinetic behavior with 2,2'-dipyridyl and ClO_4^-. Closed circles refer to 2,2'-dipyridyl = 6.0×10^{-4} M in cases 1 and 3, 2×10^{-4} M in others, open circles to ClO_4^- = 0.036 M in case 1 and 0.046 M in others.

reaction. However, the value 5.4×10^{-7} M is much too small and again the ternary complex enzyme-aldehyde-inhibitor is indicated. For this case, $K_{\text{Eald,I}} = 1.6 \times 10^{-4}$ M which represents a reasonable enhancement by aldehyde of the affiniy of the inhibitor.

For perchlorate inhibition the $K_{\text{E,I}}$ calculated from DPNH competition, 2.0×10^{-6} M, is again much the smallest, indicating a ternary complex with $K_{\text{Eald,I}} = 6.0 \times 10^{-3}$ M. The values obtained from the reverse reaction, 2.5×10^{-3} M and 9.1×10^{-3} M, do not clearly decide whether E alc I forms or not.

The case of Br^- (Fig. 4) is similar to that found earlier [1] for Cl^-, and differs from the cases just described in that at high concentration of alcohol and DPN, the reaction is accelerated. This is probably due to the presence of the ternary complex enzyme-DPNH-bromide whose decomposition is faster than that of enzyme-DPNH (under these conditions the rate limiting step). In addition Br^- seems to form another ternary complex with $K_{\text{E ald, Br}} = 2.3 \times 10^{-2}$ M. Apparent values for $K_{\text{E, Br}}$ obtained from the reverse reaction are 2.3×10^{-2} M and 6.9×10^{-2} M, sufficiently equal to make the decision as to additional ternary complexes difficult from the present data.

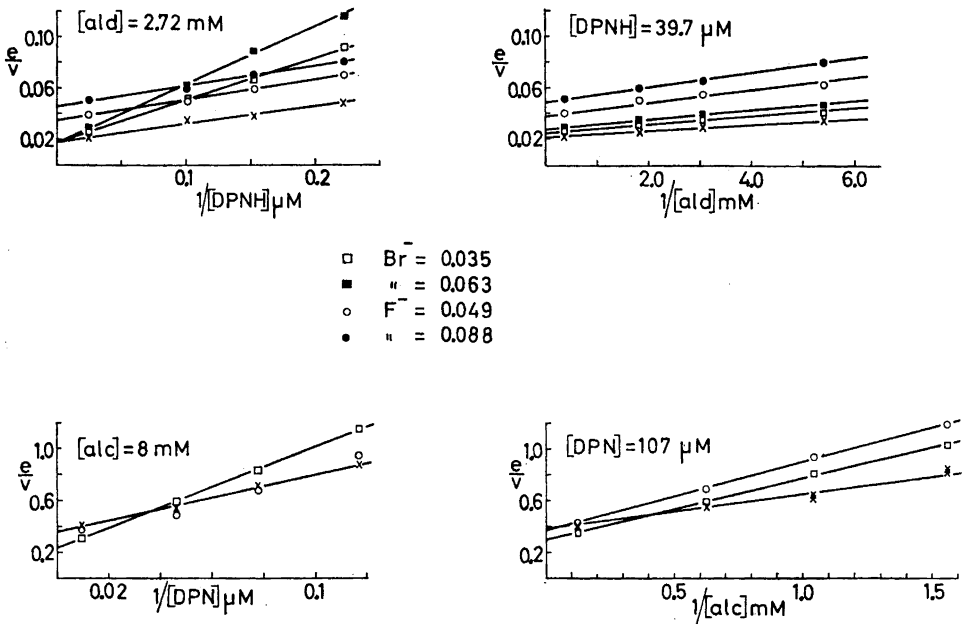


Fig. 4. Kinetic behavior with F^- and Br^-. Open squares refer to Br^- = 0.035 M closed squares, Br^- = 0.063 M; open circles, F^- = 0.049 M, closed circles, F^- = 0.088 M; × refers to no inhibitor.

The pattern of behavior shown by the thiocyanate ion, Fig. 2, is considerably different from that of the cases thus far discussed. Its uncompetitive behavior with DPNH suggests the formation of a ternary complex between enzyme, DPN, and SCN^-, which decomposes very much slower than the enzyme-DPN complex. On this assumption, the various concentrations of SCN^- are useful in calculating the dissociation constant $K_{EO,I} = 3.2 \times 10^{-5}$ M and its relative rate of decomposition, which is less than 1 % of that of EO. Finally, an independent check is given by the competition with alcohol which gives a value for $K_{EO,I}$ of 3.8×10^{-5}. This single species accounts for the entire behavior except for the apparent non-competitive behavior with aldehyde. Since the inhibition at infinite aldehyde is attributed to the last stage complex, enzyme-DPN-SCN, it seems this cannot be non-competitive inhibition in the early stage. It can, however, be due to a combination of a second phase effect with a first phase competitive effect. This first phase competition could either be between I and aldehyde for ER, with a resulting $K_{ER,I} = 3 \times 10^{-5}$ M, or competition for E, with $K_{E,I} = 2 \times 10^{-7}$ M. If the ternary complex, enzyme-DPNH-SCN^-, offers the correct explanation, it must decompose at the same rate as enzyme-DPNH, in order not to interfere with the kinetics of the reverse reaction. However, its dissociation constant seems of a more reasonable magnitude than that demanded for $K_{E,I}$, especially since its value is nearly the same as the similar constant $K_{EO,I}$. Finally, fluoride behaves in a manner quite

similar to SCN^-. From the case 1 type of experiments $K_{EO,I} = 5 \times 10^{-2}$ M with EOI decomposing less than 5 % as fast as EO. This value is checked by the fourth case (competition with alcohol) which gives $K_{EO,I} = 5.4 \times 10^{-2}$ M. As with SCN^-, the experiments with varied aldehyde can be accounted for either by assuming competition for free enzyme, with $K_{E,I} = 4 \times 10^{-4}$ M, or by competition for enzyme-DPNH, with $K_{ER,I} = 5 \times 10^{-2}$ M. Again $K_{ER,I} = K_{EO,I}$ which lends support to the existence of the ternary complex enzyme-DPNH-inhibitor for both F^- and SCN^-.

CONCLUSIONS

The results obtained with the inhibitor 1,10-phenanthroline show clearly that binding of the inhibitor blocks the binding of both DPN and DPNH. This gives strong indication, as Vallee, Williams, and Hoch have pointed out that coenzymes are bound to zinc [3]. However, the apparent lack of strict competition with the substrates need not indicate that they are not also bound to zinc. In fact the observed enhancement of the binding of inhibitor by the simultaneous presence of aldehyde or alcohol indicates that the substrate cannot be far removed from the zinc atom and quite likely is also bound to it. The ternary complexes, enzyme-coenzyme-inhibitor, indicated for SCN^-, F^-, and Br^- are significant in that no such complexes were formed with the larger, and bidentating ligands. If indeed the smaller, ionic inhibitors bind to zinc, these results mean that even when bound to coenzyme, zinc is able to coordinate small monodentate ligands as for example also imidazole (See Part II), although not large, bidentate ones. Furthermore, the competition shown between SCN^- or F^- and the substrates indicates that these ions are bound at the substrate site. However, a complete analysis of the results with ionic inhibitors is not possible from the present data. It should be noted that even in relatively dilute solutions, certain ions have significant effects and that these effects are remarkably specific. Thus, the enzyme system is affected differently by F- and Br-, which would not be at all true if the results were due merely to an ionic atmosphere, or general salt effects. The inhibition noted for perchlorate seems especially marked and this represents the anion least likely to coordinate to zinc (or any other metal) [5]. Quite possibly perchlorate affects a different portion of the enzyme and thus exerts its influence.

The very small inhibition shown by several of the zinc complexing agents, ethylene-diamine, oxalate, and pyrophosphate, should be noted. Judging from the affinity of each of these species for a free zinc atom, large inhibitions would be expected for the concentrations tested. However, each of these species seems to coordinate best to free zinc and in each case the affinity of zinc already bound to two (bidentate) ligands is extremely small [5]. Thus for ethylene diamine, for example, the first and second association constants are of the order of 10^6 and 10^5, respectively while the third is much smaller (about 10 [1]. Vallee and Coombs have noted, that the affinity of 1,10-phenanthroline for Zn in liver alcohol dehydrogenase most closely equals the third association constant of 1,10-phenathroline for aqueous zinc ion. This same correlation is noted throughout the present work, thus the $K_{E,I}$ found for 2,2'-dipyridyl (6×10^{-4}) agrees best with the dissociation constant of Zn (2,2'-dipyridyl)$^{++}$

which is 2×10^{-4}, rather than with the smaller values of the mono-[3] and di-complexes [5], as well as the poor inhibition caused by ligands whose third affinity constant for zinc is slight. The explanation for the correlation may be that the zinc in the enzyme is octahedrally coordinated to the enzyme and is bound at more than two coordination positions.

In conclusion, a consistent picture of the behavior indicated in the present study is that zinc in alcohol dehydrogenase binds both the coenzymes and substrates, while it is itself bound octahedrally to the enzyme at several points of attachment (for further discussion se Parts II and III).

Acknowledgements. Thanks are due to Mr. L. G. Falksveden for skilful technical assistance.

The authors are indebted for financial support given by *Statens Medicinska Forskningsråd, Statens Naturvetenskapliga Forskningsråd, Knut och Alice Wallenbergs Stiftelse, The Rockefeller Foundation* and *Institutet för Maltdrycksforskning.*

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Received March 28, 1961.