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*ZINC, A COMPONENT OF YEAST ALCOHOL DEHYDROGENASE**

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The ADH of yeast, first crystallized by Negelein and Wulff in 1937,[1] depends upon the presence of DPN for activity.[2] The mechanisms of its enzymatic actions have been studied.[3, 4]

The present work extends previous observations concerning the chemical composition,[1] physical-chemical interactions, and enzymological behavior of yeast ADH. The apoenzyme has been shown to contain uniformly large amounts of zinc firmly bound to the protein. The metal is a functional component of the molecule in its enzymatic activity.

Methods—Enzyme activity of ADH was determined by measuring the rate of formation or disappearance of DPNH.[5] A Cary recording spectrophotometer was employed to record the change in optical density at 340 mμ. The slope of the initial linear part of the reaction was measured and expressed as activity units V: change in absorbance ($\Delta\epsilon$) at 340 mμ per minute. These values are presented as specific activity units V per milligram of ADH. For the measurement of the rate of formation of DPNH, the following 3.0-ml. reaction mixture was employed at 23° C.: DPN, 5 μmoles; ethanol, 1,000 μmoles; 1.0 ml. of 0.1 *M* pyrophosphate buffer, pH 8.8; approximately 5μgm. of ADH in 0.2 ml. The 3.0-ml. mixture for the reaction DPNH → DPN at 23° C. contained approximately 3 μmoles DPNH;[6] 25 μmoles acetaldehyde; 1.0 ml. of 0.1 *M* phosphate buffer, pH 6.5; ADH as above. Inhibition of ADH activity was expressed as percentage of the uninhibited control reaction: $(V_i/V_0) \times 100$.

Zinc: Two dithizone methods differing in the mode of sample preparation were employed.[7]

Emission spectrography: Samples were dry-ashed in a thermostatically controlled electric muffle furnace. Internal standards were added to the ash. Aliquots were sparked in porous cup electrodes. Jarrell-Ash "Varisources" were employed to generate and control the spark. The two spectrographs were 21′ Wadsworth mountings but differed in the characteristics of their gratings. One instrument was provided with a 15,000-line-per-inch grating reflecting maximally in the first-order green, while the other 15,000-line-per-inch grating reflected maximally in the first-order violet. The reciprocal linear dispersions were 5.18 and 5.25 A/mm, respectively. Eastman Kodak 103-O photographic plates were em-

ployed throughout. Lines and backgrounds were measured densitometrically. Working curves were prepared with spectroscopically pure chemicals. Zinc was determined both chemically and spectrographically to insure accuracy.

All analytical procedures were carried out with precautions against metal contamination. Water was obtained by slow passage through a mixed ion exchange resin bed containing IR 120 and IRA 420 (Rohm and Haas). The effluent had a specific resistance of at least 1.5×10^6 ohms. Reagents were freed of metals when necessary and stored in acid-cleaned polyethylene bottles throughout.

Results.—Table 1 shows analyses on eight different crystalline preparations of highly purified yeast ADH. Preparations Nos. 6 and 7 were dialyzed against large volumes of metal-free distilled water for 24 hours at 0° C.; No. 8 was recrystallized four times.

TABLE 1

METAL CONTENT OF DIFFERENT CRYSTALLINE YEAST ADH PREPARATIONS
(All Values in Micrograms of Metal per Gram of Protein)

Metal	Exp. 8-225 (1)	Exp. 26-156 (2)	Exp. 8-200 (3)	Exp. 3-178 (4)	Exp. 23-91 (5)	Exp. 23-135 (6) (Dialyzed)	Exp. 23-148 (7)	Exp. 26-165 (8) (Recrystallized)
Zinc	1,440	1,600	1,660	1,910	1,800	2,050	1,900	1,800
Magnesium	296	130	1,180	95	630	28	25	2.4
Calcium	105	0*	39	<1	679	<1	<1	0
Aluminum	48	500	79	53	34	0	0	0
Barium	20	28	11	27	0	0	0	9.1
Strontium	2	0	4	0	0	0	0	0
Lead	0	0	45	0	0	0	0	0
Cadmium	0	0	13	0	0	0	0	0
Chromium	0	0	8	0	0	0	0	0
Iron	81	0	80	0	0	0	0	0
Copper	†	†	165	†	†	†	†	†
Moles zinc/mole protein	3.3	3.7	3.8	4.4	4.1	4.7	4.4	4.1
Moles magnesium/mole protein	1.8	0.80	7.3	0.59	3.9	0.17	0.15	0.015

* 0 = Not detectable. † Not done.

Zinc, the major metallic constituent, varies in concentration from 1,440 to 2,050 μgm. of zinc per gram of protein. The molar ratios of zinc to protein, shown in Table 1, line 12, range from 3.3 to 4.7, averaging 4.1 moles of zinc per mole of ADH, based on a molecular weight of 150,000.[3] The zinc content of one crystalline preparation was lowered from 2,210 to 1,720 μgm. of zinc per gram of protein by dialysis against OP, corresponding to 3.9 moles per mole of protein. The concentrations of magnesium, the only other element consistently present, vary widely in different preparations.

Table 2 shows data on metal content and activity obtained on consecutive fractions during a purification of ADH from yeast.[3, 5] Specific activity (Table 2, col. A) rises from 17.2×10^6 in the materials initially extracted from yeast (line 1) to 550×10^6 V per gram of protein in the fourth crystals (line 11). Concomitantly, zinc content (col. C) rises from 362 (line 1) to 1,800 (line 11) μgm. per gram of protein.

The activity:zinc ratio (col. B) reflects the relationship of the activity to the metal, rising from 47.5×10^3 in the extract (line 1) to 305×10^3 V per microgram of zinc in the fourth crystals (line 11).

Magnesium, the element present in largest concentration in the extract (line 1), falls to 2.4 μgm. per gram of protein in the fourth crystals (line 11), a 10,000-fold decrease. Activity and magnesium content vary inversely in the course of purification (cols. A and D). All other elements are also either reduced in concentration or remain at their initially insignificant levels (cols. E–H). The second crystals are contaminated with aluminum and barium (line 10).

Figure 1 shows the effects of the preincubation of 5×10^{-3} *M* OP with ADH for one hour at 0° C. at the indicated pH levels. Below pH 7.5, the enzyme is completely inhibited by OP and less so with rising pH of the incubation medium. Figure 1 also shows spectrophotometric data on the effect of pH on the formation of Zn-OP complexes for comparison.[8]

TABLE 2

ACTIVITY AND METAL CONTENT OF FRACTIONS IN THE COURSE OF PURIFICATION OF ADH FROM YEAST

	Fraction	A Specific Activity (V/Gm P + 10^6)	B Activity: Zinc (V/μgm × 10^3)	C Zinc (μgm/ Gm P)	D Magnesium (μgm/ Gm P)	E Calcium (μgm/ Gm P)	F Barium (μgm/ Gm P)	G Alumi-num (μgm/ Gm P)	H Iron (μgm) Gm P/
1.	Extraction + 55° precipitation; supernatant	17.2	47.5	362	29,000	2,800	10	63	530
2.	Acetone precipitation; supernatant 1	14.0	24.1	582	2,200	0*	39	17	200
3.	† Acetone precipitation; supernatant 2	0	0	7,695	2,100	0	0	0	0
4.	Dialysis supernatant	24.8	91.5	271	4,100	0	10	12	86
5.	† $(NH_4)_2SO_4$ precipitation; supernatant 1	1.5	6.6	227	0	150	2.2	5.3	130
6.	$(NH_4)_2SO_4$ precipitation; precipitate 1	110	199	554	161	15	0.9	1.1	24
7.	$(NH_4)_2SO_4$ precipitation; supernatant 2	74.0	128	580	620	33	1.7	1.0	32
8.	† Crystals 1; supernatant	44.1	126	351	370	0	3.5	3.0	0
9.	Crystals 1	242	266	910	270	0	0	0	0
10.	Crystals 2	555	290	1,910	95	<1	27	53	0
11.	Crystals 4‡	550	305	1,800	2.4	0	9.1	0	0

Present: K, Na, P; absent: Ag, B, Cd, Co, Cr, Li, Mn, Mo, Ni, Pb, Sr, Ti.
* 0 = Not detectable.
† Discard.
‡ The fourth crystals were more than 85 per cent pure with respect to protein. We are indebted to Dr. Hans Neurath, Department of Biochemistry, University of Washington, for ultracentrifuge and electrophoresis analyses.

The effects of various concentrations of OP, 8-OHQ, 8-OHQ5SA, αα-D, DZ, and TU on the oxidation of ethanol are shown in Figure 2; the conditions of preincubation are indicated.

Figure 2*a* shows similar data on the reduction of acetaldehyde. The enzyme was preincubated with varying concentrations of OP at pH 6.5. The concentration curve is very similar in shape and close in absolute values to that obtained for the effect of OP on the oxidation of ethanol (see Fig. 2).

With the agents studied, inhibition does not occur without preincubation (*V* at 23° C.). Figure 3 shows ADH activity as a function of time in the presence and absence of 2×10^{-3} *M* OP at 0° C., pH 7.5. In five hours, activity of the uninhibited enzyme falls slowly to 80 per cent of the original value, while in the presence of OP it falls to 17.5 per cent during the oxidation of ethanol (DPN → DPNH). During the same period, activity of the uninhibited enzyme falls to 72.5 per cent,

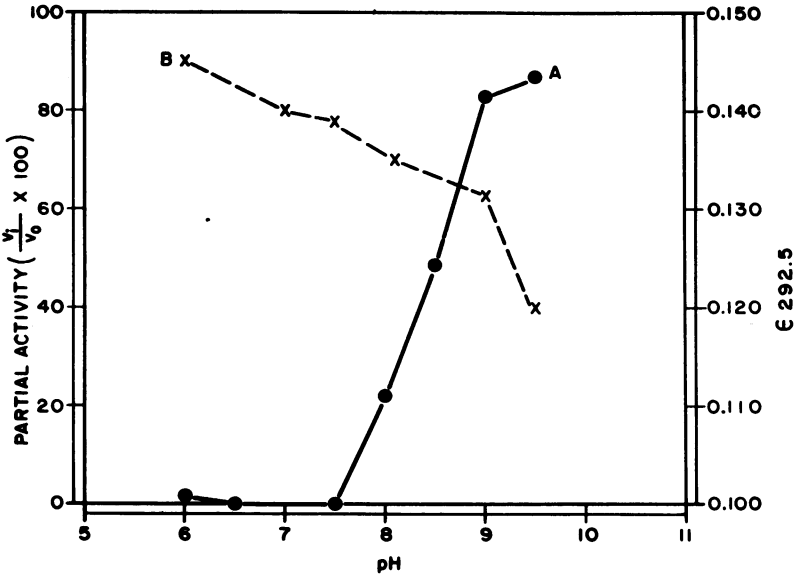

FIG. 1.—*A*, left ordinate: Effect of pH of preincubation with OP on inhibition of ADH activity. Preincubation conditions: ADH ± 5×10^{-3} *M* OP, 0° C., 60 minutes. Activity measurements: The rate of DPN → DPNH, pH 8.8, 23° C., is measured. Each point represents an activity measurement after preincubation with the inhibitors (V_i) as a percentage of the uninhibited control (V_0).

B, right ordinate: Effect of pH on the formation of $[Zn(OP)_n]^{++}$ complexes. The absorption at 292.5 mμ measures the formation of $[Zn(OP)_n]^{++}$ complexes; points are calculated from the data of McClure and Banks, U.S. Atomic Energy Commission Bull. ISC-164, 1951.

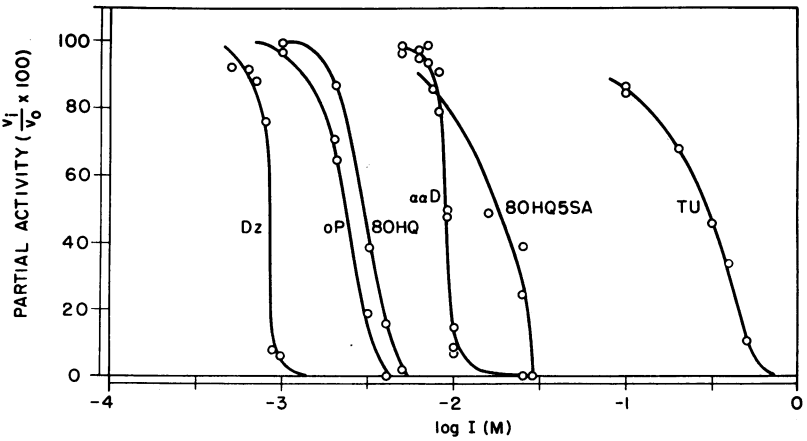

FIG. 2.—Effect of concentration of inhibitors on ADH activity, following preincubation. Preincubation conditions: ADH ± inhibitor, pH 7.5, 0° C., 60 minutes. Activity measurements: The rate of DPN → DPNH, pH 8.8, 23° C., is measured. The partial activity after inhibition, $(V_i/V_0) \times 100$, is plotted as a function of the logarithm of the molar inhibitor concentration ($\log I$).

and in the presence of OP to 18.2 per cent, of the original value during the reduction of acetaldehyde (DPNH → DPN). In the presence of OP the activity of ADH falls much more rapidly during the first few hours than without it. Thereafter, the rates of inactivation of control and inhibited enzyme proceed nearly parallel. Sixty minutes at 0° C. was chosen as a standard condition for studies of inhibition of the enzyme.

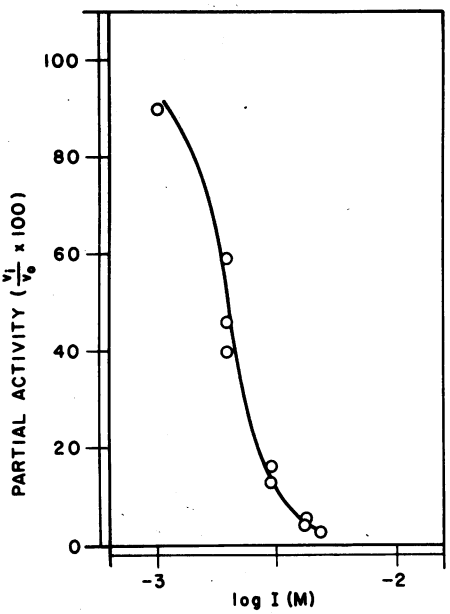

FIG. 2a.—Effect of concentration of OP on ADH activity. Preincubation conditions: ADH ± OP, pH 6.5, 60 minutes. Activity measurements: The rate of DPNH → DPN, pH 6.5, 23° C., is measured.

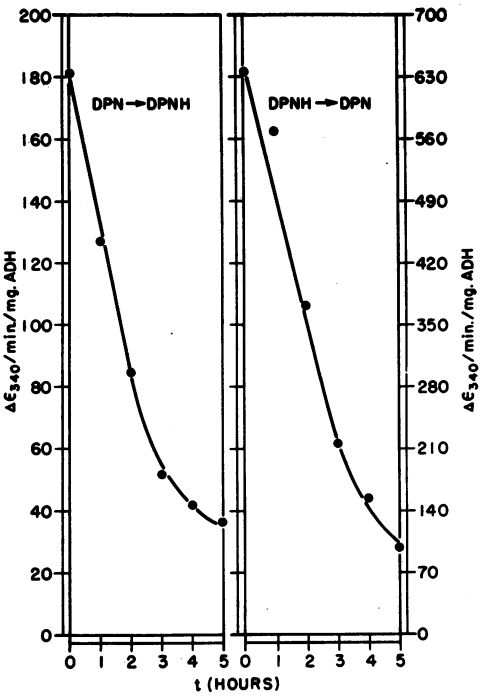

FIG. 3.—Effect of time of preincubation on inhibition of ADH activity by OP. Preincubation conditions: ADH ± 2 × 10^{-3} *M* OP, pH 7.5, 0° C. Activity measurements: Aliquots are removed from the same preincubation mixture at the times indicated. The rate of DPN → DPNH at pH 8.8, 23° C., or the rate of DPNH → DPN at pH 6.5, 23° C., is measured.

The higher the temperature, the greater the rate of inactivation of both the uninhibited and inhibited enzyme (Fig. 4). The rates of inactivation at 37° and 25° C. are very much higher than at 0° C.

When 2 × 10^{-3} *M* Zn^{++} (always as $ZnCl_2$) is added at the time at which the OP-inhibited enzyme still exhibits 18 per cent of initial activity, an immediate return to 59 per cent of the uninhibited enzymatic activity is observed (Fig. 5); 5 × 10^{-3} *M* Zn^{++} does not reverse the inhibition further. The addition of 2 × 10^{-3} *M* Zn^{++} to ADH produces a decrease to 26 per cent of control activity in 18 minutes.

Inactivation can be *prevented* completely when the ferroine group of OP is occupied by Zn^{++} or Cu^{++} ions before the enzyme is exposed to the inhibitor. Addi-

tion to the enzyme of 5×10^{-3} *M* OP exposed for 5 minutes to the same concentration of Zn^{++} results in 96 per cent of normal activity, while 5×10^{-3} *M* OP inhibits ADH to 30 per cent of normal activity.

Discussion.—The average molar ratio of zinc to protein in ADH is 4.1. It appears that four atoms of zinc are present in one molecule of ADH apoenzyme. The zinc in these crystalline preparations is firmly bound: the zinc-protein bond is maintained against competitive physical-chemical factors involved in fractionation, potentially capable of dissociating it. Recrystallization or dialysis against water fails to remove zinc. In some preparations more than 0.2 per cent of zinc was found. In these instances small amounts of zinc can be removed by relatively mild procedures, such as dialysis against OP. Such dialyses have not, thus far, lowered the zinc content below four moles of zinc per mole of protein.

It is thought that four moles of zinc are bound firmly to the protein, constituting an integral part of the molecule in the "natural state".[9] This complex has been assigned the empirical formula $[(ADH)Zn_4]$, in accord with the theoretical considerations and nomenclature to be presented elsewhere.[10] Zinc, present in excess of this concentration, represents contamination extraneous to $[(ADH)Zn_4]$ and does not increase enzymatic activity, nor does the removal of such zinc decrease it.

The data in Table 2 extend the conclusions reached on the basis of analysis of crystals. The concomitant increases of specific activity (activity:protein) and zinc content (zinc:protein) imply an interdependence typical of a metalloenzyme.[11, 12]

The discard fractions during purification contain zinc. The presence of substantial concentrations of zinc in the absence of activity (Table 2, fractions 3 and 5) indicates that zinc extraneous to $[(ADH)Zn_4]$ is removed. The activity and zinc of fraction 8 probably reflect incomplete removal of crystals from this fraction by centrifugation.

The magnesium concentration of the yeast extract (fraction 1) is almost one hundred times as high as the corresponding zinc concentration. Magnesium concentrations in the same fraction of other preparations varied from about 10 to 30 mgm. per gram of protein, giving a basis for the variable concentrations encountered in crystals. During purification the magnesium concentration decreases 10,000-fold, while specific activity increases about 30-fold. Magnesium, calcium, aluminum, barium, and iron are neither structurally nor functionally associated with ADH.

Preincubation of ADH with certain chelating agents produces marked inhibition of enzymatic activity. Since data on the physical chemistry of zinc 1,10-phenanthrolinates, $[Zn(OP)_n]^{++}$, are available,[8, 13, 14] OP was studied most intensively. The association constants, obtained by spectrophotometry, are shown in Table 3. Similar data have been obtained by potentiometric titrations (unpublished experiments).

OP has been employed for the analytical determination of zinc in the absence of iron, the only metal with a known association constant for this ligand higher than that of zinc. The absence of iron from purified ADH (Tables 1 and 2) and the inhibition of ADH activity of OP support the analytical data. Addition of $[Zn(OP)_n]^{++}$ or $[Cu(OP)_n]^{++}$ to the enzyme does not inhibit it, indicating that the

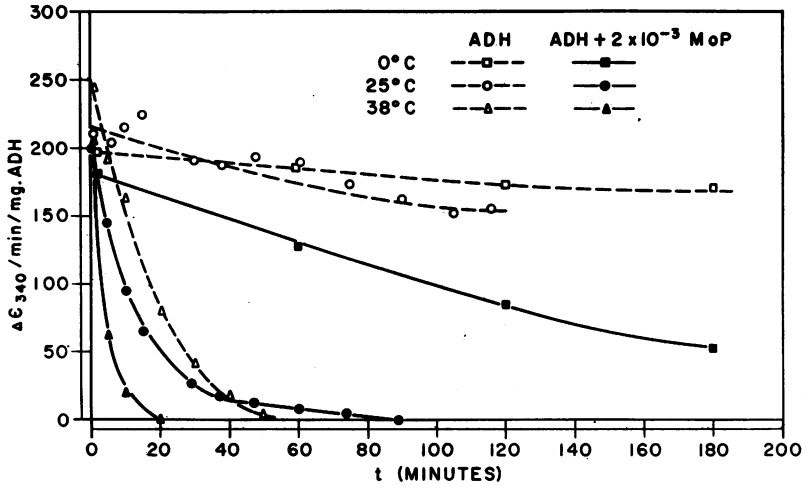

FIG. 4.—Effect of temperature and time of preincubation on inhibition of ADH activity by OP. All open symbols and broken lines represent rates of the control reaction in the absence of OP; all solid symbols and solid lines represent rates of the reaction in the presence of OP. Different temperatures are represented by the shape of symbols as indicated. Preincubation conditions: ADH ± 2 × 10^{-3} *M* OP, pH 7.5. Activity measurements: Aliquots are removed from each preincubation mixture at the times indicated, and the rate of DPN → DPNH, pH 8.8, 23° C., is determined.

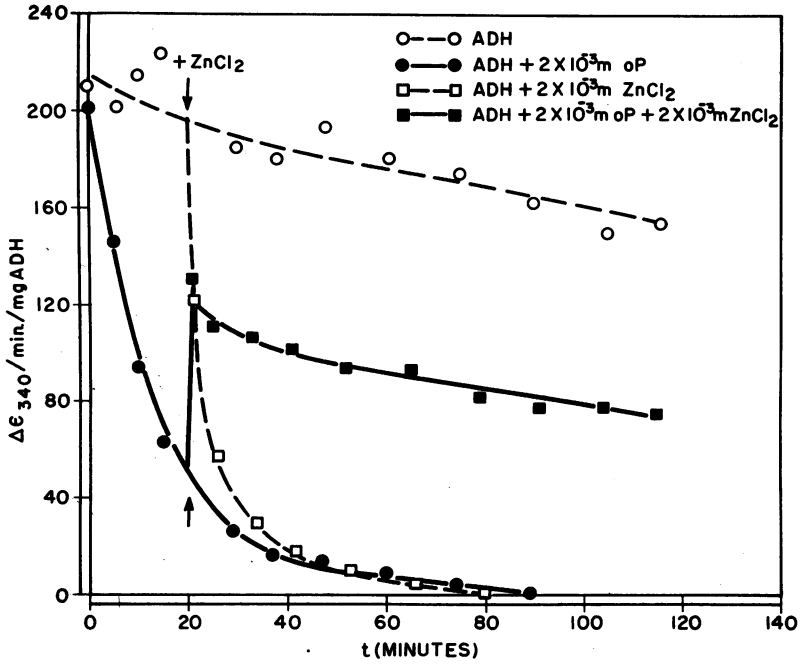

FIG. 5.—Reversal of inhibition of ADH activity by OP on addition of Zn^{++} (as $ZnCl_2$). All broken lines represent rates of the control reaction in the absence of OP, all solid lines represent rates of the reaction in the presence of OP. The addition of Zn^{++} is marked by arrows, and rates in the presence of Zn^{++} are shown as square symbols. Preincubation conditions: ADH ± 2 × 10^{-3} *M* OP, pH 7.5, 25° C. Activity measurements: Aliquots are removed from each preincubation mixture at the times indicated, and the rate of DPN → DPNH, pH 8.8, 23° C., is ascertained.

—N=C—C=N-group of OP[15] is responsible for the inhibitory action of this compound. The effect of pH on the inhibition of [(ADH)Zn_4] by OP is similar in manner to its effect on the formation of $[Zn(OP)_n]^{++}$ (Fig. 1).[8]

The rates of both the oxidation of ethanol and the reduction of acetaldehyde fall on prolonged contact of [(ADH)Zn_4] with OP at 0° C. Since aliquots of the same enzyme-OP mixture were used for these experiments, it would appear that OP acts upon [(ADH)Zn_4] and not upon other components of the reaction mixture. This is further supported by the data shown in Figure 2*a*. The quantitative aspects and the kinetics of inhibition will be the subjects of a separate communication.

At 0° C., activity of the OP-inhibited enzyme decreases slowly and linearly for 180 minutes, while there is little change in the activity of the control (Fig. 4). At 25° or at 38° C. the rates of decrease in activity are so rapid that their accurate measurement is difficult, and, particularly at 38° C., any comparison with the rapidly deteriorating controls becomes difficult. Accurate measurements could be made conveniently with a one-hour preincubation period at 0° C. The concentration of agents required to produce 50 per cent inhibition is in the order DZ < OP < 8-OHQ < $\alpha\alpha$-D < 8-OHQ5SA < TU, under these conditions.

TABLE 3

Reagent	Symbol Used in Text	pk_1	pk_1k_2	$pk_1k_2k_3$	Reference
1,10-Phenanthroline	OP	6.4	12.2	17.0	1
8-Hydroxyquinoline	8-OHQ	10.9	20.8	...	2
8-Hydroxyquinoline-5-sulfonic acid	8-OHQ5SA	8.4	15.1	...	3
Dithizone	DZ	..	20.1	...	4

1. I. M. Kolthoff, D. L. Leussing, and T. S. Lee, *J. Am. Chem. Soc.*, **73**, 390, 1951.
2. A. E. Martell and M. Calvin, *Chemistry of the Metal Chelate Compounds* (New York, 1952), p. 557.
3. A. Albert, *Biochem. J.*, **54**, 646, 1953.
4. A. K. Babko and A. T. Philipenko, *Zhur. Anal. Khim.*, **2**, 33, 1947; *Chem. Abstr.*, **43**, 5344, 1949.

The degree of inhibition may be related to the stability of the zinc complex formed. The association constants of some of these compounds with Zn^{++} in Table 3 have been used as a first approximation to gauge enzyme inhibition and to relate it to stability of the expected zinc-inhibitor complex. The inhibitory effects, while consistent with the stability of the zinc complexes, are no direct function of this parameter and present the difficulties commonly encountered in the interpretation of data on mixed complexes.[16] The geometric arrangement of the zinc atoms with respect to protein and ligand molecules, the steric and chemical factors contributed by the reactive, polar groups of protein and ligand, and their respective charges would significantly alter the constants derived in simpler systems. These analytical and enzymological data lead to the conclusion that zinc is a structural and functional component of ADH and participates in the mechanism of its enzymatic action. The four metal atoms are firmly bound to the protein apoenzyme. Pending the results of investigations in progress, this discussion will assume that the four zinc atoms are bound to the protein in an equivalent manner and that each atom of zinc acts independently of the other three in the catalytic action of alcohol dehydrogenase.

One molecule of yeast ADH has been shown to bind four molecules of DPN.[3] Potentiometric titrations of DPN and $Zn(NO_3)_2$ demonstrate the formation of a Zn++(DPN) complex (unpublished data). It is thought, therefore, that each zinc atom serves as a locus of reversible attachment for a molecule of the coenzyme, DPN. The general interaction is formulated as follows, under certain experimental conditions:

$$[(ADH)Zn_4] + 4(DPN) \rightleftarrows [(ADH)Zn_4](DPN)_4. \quad (1)$$

$[(ADH)Zn_4](DPN)_4$ is the active complex composed of the apoenzyme, zinc, and the coenzyme, the brackets denoting structural association.[10]

Enzymatic activity is decreased by incubation of the metalloenzyme with an inhibitor, I:

$$[(ADH)Zn_4] + 4I \rightleftarrows [(ADH)Zn_4]I_4. \quad (2)$$

n moles of I are bound through covalent and/or ionic bonds to each zinc atom. For the purpose of the presentation of the reactions, n has been set equal to 1. This reaction should result in an inactive, reversibly dissociable ZnI complex. Combination of equations (1) and (2) depicts the complete reaction in the presence of an inhibitor:

$$2[(ADH)Zn_4] + 4(DPN) + 4I \rightleftarrows [(ADH)Zn_4](DPN)_4 + [(ADH)Zn_4]I_4. \quad (3)$$

The existence of the two equilibria should lead to competitive inhibition between DPN and I. Preliminary work has, indeed, shown that the inhibition of ADH by OP is competitive with DPN (unpublished data), lending support to the formulation of the reaction as shown in equations (1)–(3). Apparently, chelate ligands exert their effects and are bound to $[(ADH)Zn_4]$ in a manner similar to that employed for DPN.

These considerations imply that the association between the zinc moiety of ADH and an inhibitor is reversible. This hypothesis is based on the thought that the zinc-protein bond is much stronger than any formed between zinc and an inhibitor. If it were postulated that the zinc-protein bond is broken by a ligand of high affinity for zinc, the reaction should result in the removal of zinc from the protein, and activity would be lost:

$$[(ADH)Zn_4] + 4I \underset{?}{\rightleftarrows} (ADH) + 4ZnI. \quad (4)$$

Activity could only be restored by addition of Zn^{++} to (ADH) to reconstitute the original compound. Experience with most other metalloenzymes[10, 12] makes this appear improbable. The mechanism of inhibition, therefore, depends upon the equilibrium constants of equations (2) and (4) for any given inhibitor.

The inhibition of alcohol dehydrogenase activity by OP has been reversible under experimental conditions employed thus far. With OP as the inhibitor, equation (2) apparently prevails. In view of the high association constants of the $[Zn(OP)_n]^{++}$ complexes,[13] the corresponding constants of the $[(ADH)Zn_4]$ complex must be very high indeed.

Since zinc is bound so firmly to the protein but the groups to which it is bound have not been verified, one can only speculate at this time concerning their identity.

It has been postulated that sulfhydryl groups bind DPN to liver ADH.[17] The existence of some 22 free -SH groups in yeast ADH has been reported.[18] The zinc atom itself may be bound to ADH through a thiol bond. The present findings do not rule out the existence of two linkages between ADH and DPN: one is definitely through a zinc atom of $[(ADH)Zn_4]$.

The experiment described in Figure 5 was designed to test further the formulations of the reactions given in equations (1)–(4). Zinc and OP form complexes in solution:

$$Zn^{++} + n(OP) \rightleftarrows [Zn(OP)_n]^{++} \quad (5)$$

Such complexes should form when Zn^{++} is added to $[(ADH)Zn_4](OP)_4$ (eq. [2]), competing for OP bound to the metalloenzyme. The competition of the equilibrium shown in equation (5) with that of equation (2) should result in restoration of activity:

$$[(ADH)Zn_4](OP)_4 + 4Zn^{++} \rightleftarrows [(ADH)Zn_4] + 4[Zn(OP)_n]^{++} \quad (6)$$

Figure 5 demonstrates the operation of this mechanism. The addition of Zn^{++} rapidly restores and maintains activity at 59 per cent of the control level. Enzymatic activity is not restored completely, however, under the conditions of the experiment. The following possibilities have been considered to explain the incomplete restoration of activity: (*a*) The inactive metalloenzyme-inhibitor complex may be dissociated incompletely (eq. [6]). (*b*) Zinc may be removed from the apoenzyme (eq. [4]). (*c*) OP may first remove some zinc from the metalloenzyme. Zinc ions, thereafter, only partially reconstitute the $[(ADH)Zn_4]$ complex. (*d*) Zinc ions may combine with DPN, making it unavailable. (*e*) Zinc ions may interact with enzymatically reactive end groups of the apoenzyme. The irreversible removal of zinc (possibility *b*) cannot be ruled out, even though experiments specifically designed to remove zinc from the apoenzyme have not been successful thus far (possibilities *b* and *c*). For this reason the reactivation of zinc-free (ADH) by Zn^{++} has not lent itself to experimental verification. The incomplete dissociation of $[(ADH)Zn_4](OP)_4$ (possibility *a*) is at present hypothesized to be the cause for the incomplete restoration of activity. Since zinc ions themselves inhibit the enzymatic reaction (Fig. 5), the incomplete restoration of activity may be accounted for in part by the mechanisms proposed under *d* and *e*, which are believed to be responsible for the effect of Zn^{++} on $[(ADH)Zn_4]$.

Zinc ions and complexes do not absorb light in the visible region of the spectrum and therefore do not draw attention to themselves, as do iron and copper, for instance. The absence of changes in its valence coupled with the stability of its complexes may be a clue to the function of zinc in this and similar systems.

It has been reported that *Neurospora crassa*, grown on zinc-deficient media, was devoid of ADH activity,[19] not restored by the addition of Zn^{++}. The *structural* association of zinc with the ADH apoenzyme was not considered as a possible explanation for the resultant functional derangements,[19] which were attributed to "an indirect influence on the synthesis of the apoenzyme."[20] In the absence of zinc, the present data imply that this organism is unable to form the functional metalloenzyme molecule $[(ADH)Zn_4]$. Apparently, the ADH of *Neurospora*, like the ADH of yeast, is a zinc metalloenzyme.

The molecular weight of ADH of horse liver has been reported to be 73,000.[21] Two DPN molecules per molecule of protein are bound.[21] The characteristics of the two enzymes differ in many respects.[22] One can only speculate as to their cause. Solely on the basis of molar proportions of protein to cofactor, the presence of two moles of zinc per mole of protein might be expected. The known high zinc content of liver,[23] of which ADH has been stated to be 1 per cent of the total protein weight,[24] lends support to this assumption.

It has been reported[25] and confirmed[26] that retina contains high concentrations of zinc. Horse liver ADH oxidizes vitamin A_1 and reduces retinene, probably being identical with retinene reductase.[27] If mammalian ADH is found to be a zinc enzyme, enzymological and analytical observations on liver and retina, and those on yeast ADH here reported, may be shown to have an analogous structural denominator.

Summary.—The ADH of yeast is a zinc metalloenzyme containing four moles of zinc firmly bound to one mole of protein. The activity of the enzyme is directly dependent on zinc. Enzymatic action is inhibited by dithizone, 1,10-phenanthroline, 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, α,α'-dipyridyl, and thiourea. The inhibition is reversible and probably is competitive with DPN. The mechanisms of the enzymatic reaction, inactivation, and reversal of inhibition have been formulated in terms of the existence of a structural metalloenzyme entity. This has been assigned the empirical formula $[(ADH)Zn_4]$ in conformity with a general scheme of notation of metalloenzyme structure. The active metalloenzyme-coenzyme complex is represented by $[(ADH)Zn_4](DPN)_4$.

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The following abbreviations will be used: ADH, alcohol dehydrogenase; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; OP, 1,10-phenanthroline; 8-OHQ, 8-hydroxyquinoline; $\alpha\alpha$-D, α,α'-dipyridyl; 8-OHQ5SA, 8-hydroxyquinoline-5-sulfonic acid; TU, thiourea.

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PRODUCTION OF ABNORMAL (PSYCHOTIC?) BEHAVIOR IN MICE WITH LYSERGIC ACID DIETHYLAMIDE, AND ITS PARTIAL PREVENTION WITH CHOLINERGIC DRUGS AND SEROTONIN

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The production of hallucinations in men by the feeding of ergot alkaloids, and especially of the derivative lysergic acid diethylamide (LSD-25), has been known for many years.[1] The hallucinogenic action of LSD-25, particularly, has been much studied recently because such minute doses (1 μgm. per kilogram) are effective and because the mental aberration called forth by this compound is said to resemble schizophrenia. Our demonstration in 1953[2, 3] and that of Gaddum[4] that the ergot alkaloids are naturally occurring antimetabolites of the hormone serotonin, capable of antagonizing the action of this hormone on smooth muscle preparations, suggested that these alkaloids, and especially LSD-25, acted on the brain to antagonize the normal functioning of serotonin just as they did on muscles.[5, 6] The deficiency of serotonin thus induced in the brain by the drug was considered as a possible cause of the hallucinations, and it was suggested that some of the naturally occurring mental diseases might similarly arise from a lack of serotonin in the brain. This lack would result not from drug action but rather from a failure to form enough serotonin in this organ.

In order to test such a hypothesis, it was necessary to induce a recognizable mental disorder with LSD-25 and to overcome it with serotonin. Because it was already quite clear that serotonin administered peripherally did not enter the brain readily,[5] various means of direct introduction into the central nervous system seemed a prerequisite of the experiment, and for this reason, as well as for others, a test system involving animals rather than men was essential. Intracerebral injections into men for experimental purposes would be out of the question.