sodium methoxide in methanol using thymol blue as indicator.[19]

Poly-O-p-tolylsulfonylhydroxy-L-proline (XII).—O-p-tolylsulfonyl-N-carboxyhydroxy-L-proline anhydride (XI) (2.0 g.) was dissolved in dry pyridine (20 ml.) and left to polymerize for two days at room temperature. The polymer formed was precipitated with ether (100 ml.), collected, washed with ether and dried *in vacuo;* yield 1.0 g. (58%). An average degree of polymerization $n = 30$, was calculated from end group analysis[14]; $[\alpha]^{25}D$ 0.0° (*c* 0.5, in glacial acetic acid) immediately after dissolution, changing to the final value of $[\alpha]^{25}D$ −120° (*c* 0.5, in glacial acetic acid) within three hours.

Anal. Calcd. for $(C_{12}H_{13}O_4NS)_n$: C, 53.9; H, 4.9; N, 5.2; S, 12.0. Found: C, 54.1; H, 4.5; N, 5.2; S, 11.9.

Poly-O-p-tolylsulfonylhydroxy-L-proline, $n = 30$, is soluble in glacial acetic acid, dimethylformamide and pyridine. It is insoluble in water and ethanol.

N-Carboxy-L-proline Anhydride.—Dry phosgene was passed at room temperature through a suspension of L-proline (3.0 g.) in dry dioxane (60 ml.) until a clear solution was obtained (one hour). Excess of phosgene was removed with dry carbon dioxide and the solution was concentrated *in vacuo* at 40°. The oily residue was dissolved in dry acetone (150 ml.) and the resulting solution was mechanically stirred with silver oxide[11] (3.0 g.) until free of chloride (6 hours at room temperature). The solution was decanted from the precipitate, evaporated to dryness *in vacuo* and the oily residue washed twice with petroleum ether. The oily material was dissolved in ethyl acetate (5 ml.), and petroleum ether (75 ml.) was added. The oil which separated out crystallized in long colorless needles in the refrigerator; yield 2.5 g. (68%), m.p. 45° dec. The m.p. did not change on recrystallization from ethyl acetate–petroleum ether. Molecular weight 143 (calcd. 141), determined by titration with 0.1 N sodium methoxide in methanol using thymol blue as indicator.[19]

Acknowledgment.—This investigation was supported by a research grant (PHS H-2279) from U.S.A. National Institutes of Health, Public Health Service.

REHOVOTH, ISRAEL

(19) A. Berger, M. Sela and E. Katchalski, *Anal. Chem.*, **25**, 1554 (1953).

[CONTRIBUTION FROM THE BIOPHYSICS RESEARCH LABORATORY OF THE DEPARTMENT OF MEDICINE, HARVARD MEDICAL SCHOOL AND THE PETER BENT BRIGHAM HOSPITAL]

Spectrophotometric Evidence for Enzyme Inhibitor Complexation[1,2]

BY BERT L. VALLEE, THOMAS L. COOMBS AND ROBERT J. P. WILLIAMS[3]

RECEIVED JULY 29, 1957

A mixture of ionic zinc and 1,10-phenanthroline demonstrates absorption maxima at 3125, 3275 and 3425 Å. in a special 5 cm. absorption cell; these maxima have not been previously described. Addition of 1,10-phenanthroline or of 8-hydroxyquinoline-5-sulfonic acid to the zinc metalloenzymes, carboxypeptidase, yeast alcohol dehydrogenase or liver alcohol dehydrogenase, or to the zinc protein complex, insulin, produces ultraviolet absorption spectra, which are entirely analogous to those observed with the ionic zinc–1,10-phenanthroline or 8-hydroxyquinoline-5-sulfonic acid system. These spectra therefore demonstrate the existence of protein–zinc–chelate mixed complexes. Spectral evidence indicates these complexes to be completely dissociable upon dialysis. 1,10-Phenanthroline and 8-hydroxyquinoline-5-sulfonic acid, previously shown to inhibit carboxypeptidase, yeast ADH and liver ADH, had been postulated to exert these inhibitory effects through interaction with the zinc atoms of the enzymes. The spectral data provide direct evidence for this proposed chemical interaction resulting in the formation of enzymatically inactive enzyme–zinc-chelator complexes. These findings offer additional evidence that zinc is an active site for these metalloenzymes and substantiate the previously proposed mechanisms for inhibition by metal chelators.

Introduction

While until recently carbonic anhydrase was the only enzyme known to contain zinc as an integral and functional part of the molecule,[4a] detection of functional zinc atoms in a number of other enzymes[4b] has greatly extended the hitherto unsuspected biochemical role of this element.

The firm binding of zinc to the apoenzyme and its stoichiometry in enzyme–coenzyme complexes have been discussed. Metal binding agents have been employed to inhibit the catalytic activity of such systems.[4b] These inhibition data *imply* that loss in activity occurs through complexing of the agent with the zinc of the metalloenzyme, but no *direct* evidence for the mechanism of this action is at hand.

Zinc ions in aqueous solution form complexes with either 1,10-phenanthroline (OP) or 8-hydroxyquinoline-5-sulfonic acid (8OHQ5SA) which exhibit characteristic absorption spectra. The present data demonstrate similar absorption spectra for zinc containing proteins with these agents, constituting direct evidence for the postulated mechanism of inhibition.

Materials and Methods

Standard zinc solutions were prepared from weighed amounts of spectrographically pure zinc metal (Johnson Matthey Co.) dissolved in dilute metal-free hydrochloric acid.

Liver Alcohol Dehydrogenase (LADH) and Yeast Alcohol Dehydrogenase (YADH).—Commercial crystalline preparations were obtained from the Worthington Biochemical Corporation and used without further purification; the LADH had a zinc-to-protein ratio of 1650 μg. Zn/g. (1.9 g. atoms Zn/mole protein); the YADH had a zinc-to-protein ratio of 1760 μg. Zn/g. (4.1 g. atoms Zn/mole protein) and insignificant amounts of all other metals. Both enzymes, when ultracentrifuged in a Spinco Model E Ultracentrifuge, were more than 90% monodisperse and had turnover numbers of 8.2 and 240 moles DPNH per sec. per mole enzyme, respectively.

Carboxypeptidase (Cp).—A Worthington Biochemical Corporation three times crystallized preparation was twice recrystallized further by the method of Neurath, *et al.*[5] The

(1) These studies were aided in part by a contract between the Office of Naval Research, Department of the Navy and Harvard University Contract No. Nonr-1866(04), a Grant-in-Aid from the National Institutes of Health of the Department of Health, Education and Welfare and the Howard Hughes Medical Institute.

(2) Presented in part before a meeting of the Federated Societies of Experimental Biology, March, 1957.

(3) Fellow of Wadham College, Oxford, England.

(4) (a) D. Keilin and T. Mann, *Biochem. J.*, **34**, 1163 (1940); (b) B. L. Vallee. *Advances in Protein Chem.*, **10**, 317 (1955).

(5) H. Neurath, E. Elkins and S. Kaufman, *J. Biol. Chem.*, **170**, 221 (1947).

final product was 95% pure by ultracentrifugation and had a zinc-to-protein ratio of 1830 μg. Zn/g. (1.0 g. atom Zn/mole of protein), insignificant amounts of all other metals, and a proteolytic coefficient of 16.2.[5]

Insulin.—A two-times crystallized preparation was kindly donated by Dr. D. Waugh of the Massachusetts Institute of Technology. It had a zinc-to-protein ratio of 4600 μg./g.

"Zinc-free" Insulin.—This preparation was kindly donated by Dr. Eric Ball of the Harvard Medical School. The zinc content was 16 μg./g.

1,10-Phenanthroline (OP).—The crystalline dihydrochloride (G. F. Smith Co.) was used without further purification. A solution of this compound gave a molar extinction coefficient E_{2925} of 6800 cm.^{2}/mole/liter, in agreement with the value given by Banks and McClure.[6]

8-Hydroxyquinoline-5-sulfonic Acid (8OHQ5SA).—The Eastman Kodak reagent grade product was used without further purification.

Tris(Tris-hydroxymethylaminomethane).—Reagent grade crystals (Sigma Chemical Company) were used without further purification. TRIS buffers were prepared by dissolving the weighed crystals in metal-free water. The solution was then neutralized to the desired *p*H with metal-free hydrochloric acid and diluted to volume. All *p*H's were measured at 23° using a Beckman Model G *p*H meter with standard glass and calomel electrodes.

Dialysis Tubing.—Visking-Nojax tubing was prepared and cleaned according to the method of Klotz.[7]

Metal-free hydrochloric acid was prepared by saturating metal-free water with hydrochloric acid gas and stored in polyethylene containers.[8]

Metal-free Water.—All water was purified by passage over a mixed anion-cation resin bed and stored in polyethylene containers.[9]

Glassware was cleaned of contaminating metals by soaking in nitric acid followed by rinsing thoroughly with metal-free water.

Metal Determinations.—All zinc determinations were performed using a zinc dithizonate extraction method,[10] and all other metals were measured spectrographically.[4b]

Protein Dry Weight Determinations.—The protein was precipitated by trichloroacetic acid and dried to constant weight at 104° in an air oven.[11]

Spectrophotometry.—Spectra of aqueous solutions of Zn^{++}, insulin, liver alcohol dehydrogenase, 1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid and their complexes were obtained at 23° and of yeast alcohol dehydrogenase, carboxypeptidase and their complexes with these chelating agents at 4° using a Cary recording spectrophotometer, the absorption chamber of which was held constant at these temperatures. A difference spectrum, characteristic of the zinc complexes, was obtained by placing zinc ions or zinc containing proteins together with complexing agent and buffer into the sample absorption cell. The reference cell contained complexing agent and buffer only, compensating for the absorption of free complexing agent. Solutions were measured either in standard 1 cm. path length quartz cells or in special 5 cm. path length, small volume, rectangular quartz cells[12] adapted to the Cary cell-housing unit. The Cary spectrophotometer was calibrated with a mercury ultraviolet arc source, and the wave length scale was accurate to ±1 Å., in the wave length ranges under consideration.

Experimental

Studies with 1,10-Phenanthroline. Ionic Zinc.—Difference spectra between a mixture of aqueous Zn^{++} and 1,10-phenanthroline in buffer and 1,10-phenanthroline alone in buffer over the wave length range 2800–3600 Å. obtained with a 1 cm. path length cell showed the single sharp absorption maximum at 2950 Å. previously observed,[6] and no other absorption maxima at longer wave lengths were detectable. With the small-volume 5 cm. cells, however, three new maxima were resolved, as a shoulder at 3125 Å. and as bands at 3275 and 3425 Å., the intensity of the latter band being about one half that of the former (Fig. 1A). When increasing amounts of Zn^{++} were added to a constant amount of OP, absorbance at 3275 or at 3425 Å. increased linearly and levelled off when the molar ratio of Zn:OP was 1:3; greater amounts of Zn^{++} eventually produced decreased absorbance (Fig. 2).

Zinc Proteins.—Using the small-volume 5 cm. cells over the range 3000–4000 Å., difference spectra for the zinc proteins, liver alcohol dehydrogenase, yeast alcohol dehydrogenase, carboxypeptidase and insulin were obtained before and after the addition of concentrations of 1,10-phenanthroline in excess of the protein-bound zinc concentration. In all cases the spectrum for the protein alone showed high absorption at 3000 Å. decreasing rapidly and smoothly, levelling off to low absorption at longer wave lengths. The presence of 1,10-phenanthroline, in addition to a rise in absorption over the whole wave length range, produced two new maxima at 3275 and 3425 Å. for YADH, Cp and insulin and at 3287 and 3450 Å. for LADH (Fig. 1B, D, E, F). The addition of 1,10-phenanthroline to "zinc-free" insulin gave no evidence of increased absorption at either 3275 or 3425 Å. (Fig. 1C).

To test the characteristics of the interaction, the spectra of the LADH Zn–OP complexes were compared before and after dialysis against 0.1 *M* Tris buffer at *p*H 7.5. The spectrum of the dialysate was compared to that of 1,10-phenanthroline alone. The LADH Zn–OP complex exhibited the characteristic absorption maxima at 3287 and 3450 Å. (Fig. 3A, curve I) which were removed by dialysis (Fig. 3A, curve II). The spectrum of the dialysate (Fig. 3B, curve III) was identical with that of 1,10-phenanthroline alone (Fig. 3B, curve IV), and the zinc–1,10-phenanthrolinate spectrum was not discernible in the dialysate.

8-Hydroxyquinoline-5-sulfonic Acid. Ionic Zinc.—The difference spectrum of a mixture of buffered zinc ions and 8-hydroxyquinoline-5-sulfonic acid was obtained over the range 3000–4000 Å. with 8-hydroxyquinoline-5-sulfonic acid in the reference solution. A broad band with a maximum at 3700 Å. was obtained (Fig. 4A). Increasing concentrations of zinc added to one concentration of 8-hydroxyquinoline-5-sulfonic acid between *p*H's 5.0–8.5 resulted in a concomitant linear rise of absorption at 3700 Å., becoming constant in the presence of excess zinc (Figs. 5 and 6).

Zinc Proteins.—Similar spectra for liver alcohol dehydrogenase, carboxypeptidase and insulin were obtained with 8-hydroxyquinoline-5-sulfonic acid. In each instance, the absorption band at 3700 Å., characteristic of the Zn^{++}–8OHQ5SA complex, could be demonstrated (Fig. 4B, C and D).

Discussion

The reactions of 1,10-phenanthroline with ionic zinc confirm the formation of stable $ZnOP^{++}$ complexes reported by Kolthoff, Leussing and Lee[13] and by McClure and Banks[6] and stable Zn–8-hydroxyquinoline-5-sulfonic acid complexes reported by Nasanen, *et al.*[14] McClure and Banks demonstrated an absorption maximum at 2925 Å. for $[ZnOP]^{++}$ complexes at *p*H 4.0 which we find shifted to 2950 Å. at *p*H 7.5. The present investigation additionally discerns absorption maxima at 3125, 3275 and 3425 Å. not hitherto reported. In the presence of excess zinc, the complexes are stable between *p*H 2.5 and 8.5 and in the presence of excess OP, the complexes are stable between *p*H 4.5 and 8.5.[6] The dissociation constant of the $[ZnOP]^{++}$, $[ZnOP_2]^{++}$ and $[ZnOP_3]^{++}$ complexes have been found to be $K_1 = 3.7 \times 10^{-7}$, $K_2 = 7 \times 10^{-13}$ and $K_3 = 1 \times 10^{-17}$, respectively.[13] A

(6) J. H. McClure and C. V. Banks, U. S. Atomic Energy Commission Publication ISC-164, 1951.

(7) I. M. Klotz and T. R. Hughes, "Methods of Biochemical Analysis," Vol. III, Interscience Publ. Inc., New York, N. Y., 1956, p. 265.

(8) R. E. Thiers, "Methods of Biochemical Analysis," Vol. V, Interscience Publ. Inc., New York, N. Y., 1957.

(9) B. L. Vallee, *Anal. Chem.*, **26**, 194 (1954).

(10) F. L. Hoch and B. L. Vallee, *J. Biol. Chem.*, **181**, 295 (1949).

(11) F. L. Hoch and B. L. Vallee, *Anal. Chem.*, **25**, 317 (1953).

(12) B. L. Vallee, *ibid.*, **25**, 985 (1953).

(13) I. M. Kolthoff, D. L. Leussing and T. S. Lee, THIS JOURNAL, **73**, 390 (1951).

(14) (a) R. Nasanen and U. Penttinen, *Acta Chem. Scand.*, **6**, 837 (1952); (b) R. Nasanen and E. Uusitalo, *ibid.*, **8**, 835 (1954).

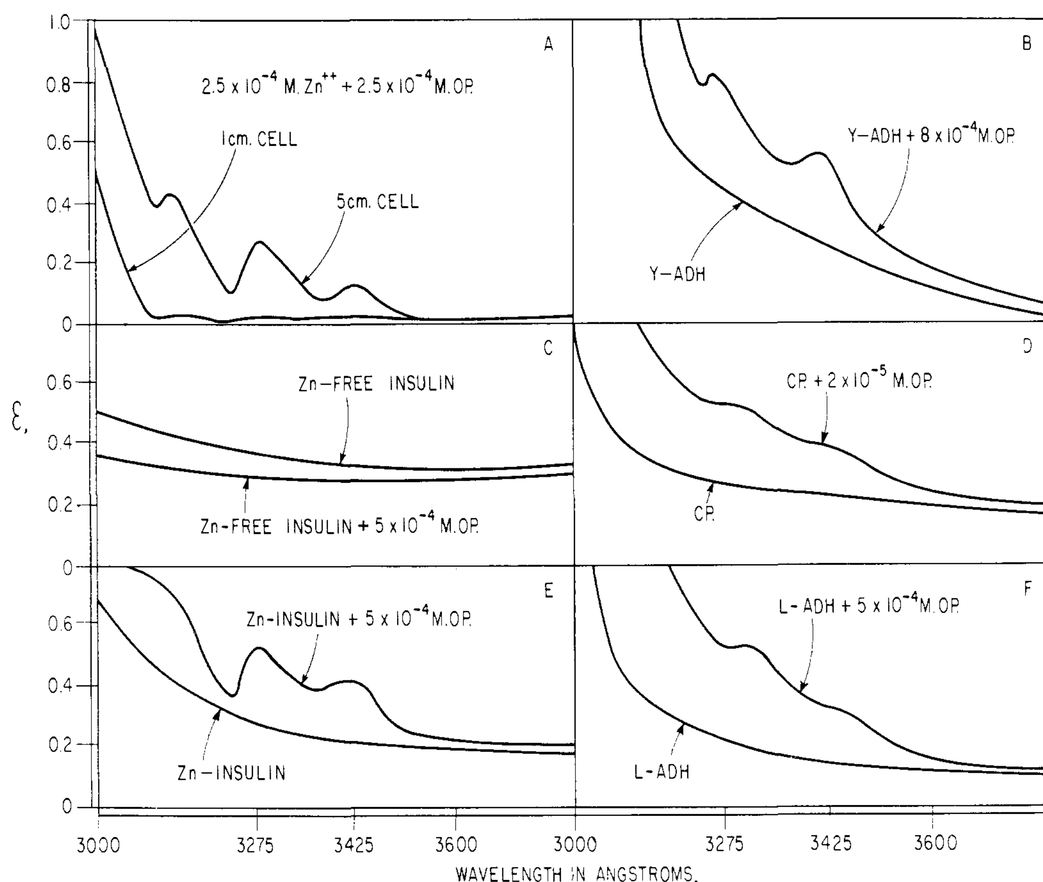


Fig. 1.—Difference spectra of Zn^{++} and Zn-proteins in presence and absence of OP: (A) Zn^{++} using a 1 cm. and a 5 cm. cell; (B) Y-ADH; (C) Zn-free insulin; (D) carboxypeptidase; (E) Zn-insulin; (F) L-ADH (5 cm. cells only). Measured against a reference solution containing buffer or buffer and OP at the same concentration as in the sample solutions. Buffer: 0.1 *M* Tris *p*H 7.5 except for carboxypeptidase where 0.1 *M* Tris + 1 *M* NaCl, *p*H 7.5 was used. [OP] as shown.

method for zinc analysis with 1,10-phenanthroline has been proposed.[6]

The 8-hydroxyquinoline-5-sulfonic acid complexes with Zn^{++} ions are stable over the *p*H range 5.0–8.5 and obey the Beer–Lambert law. The

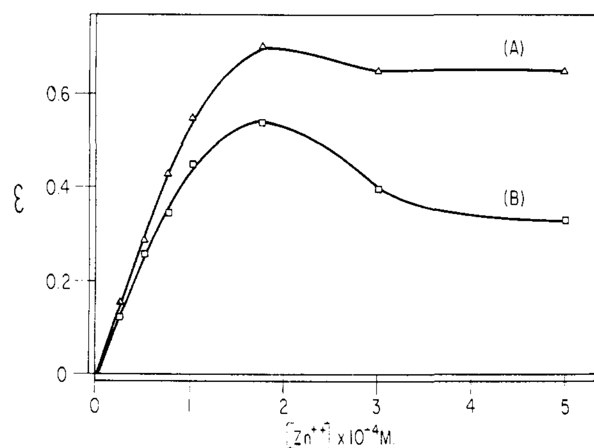


Fig. 2.—Absorption of $[Zn(OP)]^{++}$ *versus* Zn^{++} concentration (A) at 3275 Å.; (B) at 3425 Å. when Zn^{++} is added to a constant amount of OP. [OP] = 5×10^{-4} *M*. Buffer: 0.1 *M* Tris, *p*H 7.5.

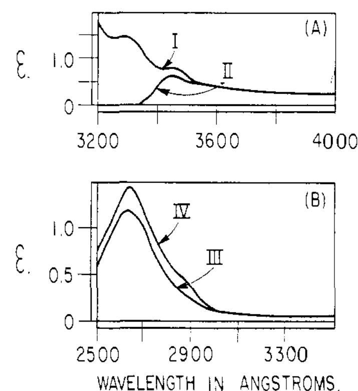


Fig. 3.—(A) Effect of dialysis on the absorption of LADH-Zn-OP complex. Difference spectrum of LADH–Zn–OP: (I) before dialysis; (II) after dialysis. Measured against a reference solution containing the same concentration of OP as was added to the LADH. [OP] = 7.7×10^{-4} *M*. Buffer: 0.1 *M* Tris, *p*H 7.5. (B) Comparison of the absorption spectrum of (III) the dialysate *after* dialysis to (IV) that of OP, 8.3×10^{-5} *M* in 0.1 *M* Tris *p*H 7.5. Dialyzing conditions: 6.5 ml. LADH–Zn-OP in 0.1 *M* Tris *p*H 7.5 dialyzed for 18 hr. at 4° against 600 ml. 0.1 *M* Tris, *p*H 7.5.

conversion of a linear rise to a constant absorption at 3700 Å. (Fig. 5) indicates a change in extinction coefficient when $Zn[8OHQ5SA]_2$ changes to Zn-[8OHQ5SA]. Thus these extinction coefficients can be used to differentiate between 1:2 and 1:1 complexes with this agent. The rising total absorption with rising *p*H due to the dissociated free 8-hydroxyquinoline-5-sulfonic acid limits the usefulness of this system for analytical purposes to *p*H's less than 8.0.

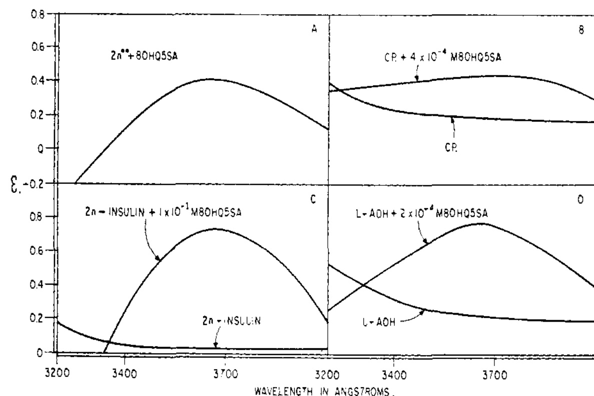


Fig. 4.—Difference spectra of Zn^{++} and Zn-proteins in the presence and absence of 8OHQ5SA: (A) Zn^{++}; (B) carboxypeptidase; (C) Zn-insulin; (D) L-ADH. Measurements were performed in 5 cm. cells against a reference solution containing buffer or buffer + 8OHQ5SA at the same concentration as in the sample solutions. Buffer 0.1 *M* Tris, *p*H 7.5, except for carboxypeptidase where 0.1 *M* Tris + 1 *M* NaCl, *p*H 7.5, was used.

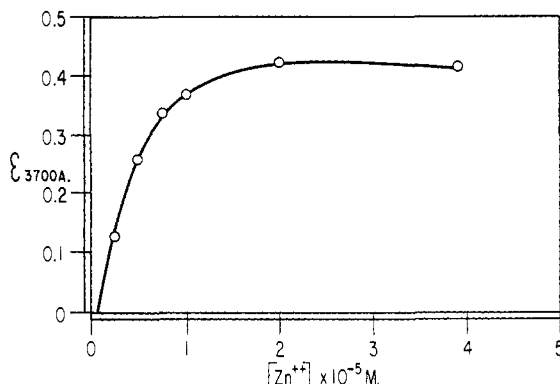


Fig. 5.—Absorption of [Zn-(8OHQ-5-SA)] at 3700 Å. *versus* Zn^{++} concentration when Zn^{++} was added to a constant amount of 8OHQ5SA. Buffer: 0.1 *M* Tris, *p*H 7.5. [8OHQ5SA] = 4.0×10^{-5} *M* and using as reference solution the same concentrations of 8OHQ5SA and buffer.

The method of molar proportions, applied to the interaction of zinc and both 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid (Figs. 2, 5 and 6) yield results in accord with those of previous investigators. In the presence of excess zinc a 1:3 complex forms with OP and a 1:2 complex forms with 8OHQ5SA resulting in maximal absorption. The extinction decreases when the molar ratio to zinc of either agent exceeds this optimal proportion due to the formation of 1:1 and 1:2 complexes, the existence of which is a function of the concentration of free chelating agent.

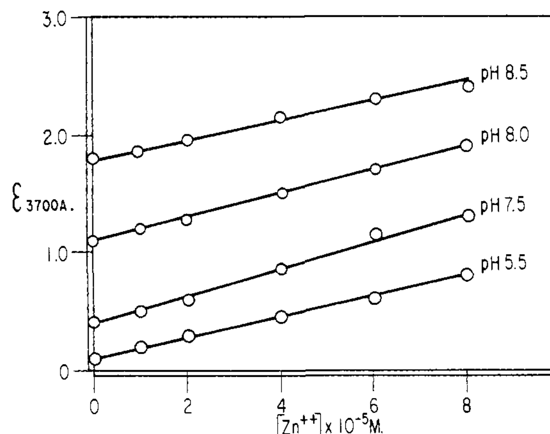
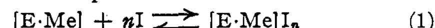


Fig. 6.—Effect of *p*H on the changes in absorption of [Zn(8OHQ-5-SA)] at 3700 Å. when Zn^{++} is added to a constant amount of 8OHQ5SA. Buffers: *p*H 5.5, 0.1 *M* acetate, *p*H's 7.5, 8.0 and 8.5, 0.1 *M* Tris. [8OHQ5SA] = 1.0×10^{-3} *M*.

The interaction of ionic zinc with these chelating agents constitutes models similar to the interaction of zinc containing proteins with and without enzymatic activity. The inhibition of liver alcohol dehydrogenase, yeast alcohol dehydrogenase, glutamic dehydrogenase, lactic dehydrogenase and carboxypeptidase by chelating agents such as 1,10-phenanthroline, 4,7-dihydroxy-1,10-phenanthroline, 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, sodium diethyldithiocarbamate, thiourea and diphenyldithiocarbazone[4b] suggested that the observed inhibition could be attributed to the interaction of these chelating agents with the zinc of the enzyme in a manner similar to or identical with that of the ionic systems. This interference with the functional catalytic action of zinc by the formation of a chelate with the inhibitor can be postulated to act in one of two ways.

An enzymatically inactive, mixed complex between the metalloenzyme and the chelating agent may form in a manner similar to that shown by Klotz and Loh-Ming[15] and by Watters[16] for non-enzymatic systems. The apoenzyme (E) constitutes one ligand to which the metal (Me) is firmly bound. A second ligand, the chelating agent acting as an inhibitor (I) then interacts to form the complex $E\text{–}Me\text{–}(I)_n$ where n is an integral number and a function of the available coördination sites of the metal as in equation 1



$$[E{\cdot}Me] + nI \rightleftharpoons [E{\cdot}Me]I_n \qquad (1)$$

This equation indicates that the active metalloenzyme [E·Me] reversibly combines with n moles of inhibitor (I) to form the enzymatically inactive, mixed complex $[E{\cdot}Me]\ I_n$.

Alternatively, inactivation can be postulated to occur through the removal by the chelating agent of the metal bound to the apoenzyme to form an ionic metal chelate as in equation 2

$$[E{\cdot}Me] + nI \rightleftharpoons E + MeI_n \qquad (2)$$

indicating that this reaction may be reversible.

(15) I. M. Klotz and W. C. Loh-Ming, THIS JOURNAL, **76**, 805 (1954).

(16) J. I., Watters, *et al.*, (a) *ibid.*, **75**, 4819 (1953); (b) **75**, 5212 (1953); (c) **76**, 3810 (1954).

Now n need not be the same in equation 1 and equation 2 so that if criteria for distinguishing different degrees of coördination of Me by I can be found, we may be able to differentiate between equations 1 and 2.

Both these schemes demand that the inhibitory effects of chelating agents be brought about solely by interaction with the metal moiety of the enzyme and that the apoenzyme is otherwise unaffected. If these premises be correct, the system should be susceptible to physical-chemical treatment akin to that possible for simple chelate systems, provided that variables, such as time, temperature, concentration and ionic strength, etc., do not affect the protein moiety adversely. Previous studies have demonstrated that the removal of zinc ions by lowering pH values simultaneously brings about irreversible changes in the protein and in enzymatic activity. Such changes are not observed under the conditions here employed for obtaining these spectra, making the removal of the metal unlikely as a mechanism, at least for LADH[2]; the other enzymes are under study.

Previous conclusions concerning the interaction of metalloenzymes and chelating agents were inferred solely from enzymatic activities. The validation of such conclusions by more direct methods of observation seemed necessary. The changes in spectra seemed to provide a simple and direct experimental approach to this problem.

For this purpose, the typical absorption spectrum of the metal chelate should ideally be at wave lengths free from interference by other components of the system to be studied. Thus the absorption of the aromatic amino acids of the protein may interfere with the absorption maxima of any reagent. The physical and chemical parameters of the system should closely resemble those optimal for the enzymatic assay to allow for comparison of the enzymatic and spectrophotometric data. The reagent should not combine with other groups of the protein or of the enzyme assay system, *e.g.*, the reaction between CN^- and DPN in the alcohol dehydrogenase systems. The agent should form stable metal chelates, absorption of which obeys the Beer–Lambert law. Non-specific adsorption of the agent to the protein is an undesirable complication to be borne in mind.

While it would be most desirable if an agent were specific for a single metal ion, few exhibit this capacity. Alternatively, the specific metal of the protein should be identified by analysis and shown to be unique to the system. Though these requirements limit the selection of possible reagents, 1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid satisfy these conditions. While these agents do not interact with zinc exclusively, here this circumstance is of little import since the systems under study contain no metal other than zinc.

The present data demonstrate the appearance of the characteristic zinc–1,10 phenanthroline and zinc–8-hydroxyquinoline-5-sulfonic acid absorption maxima when liver alcohol dehydrogenase, yeast alcohol dehydrogenase, carboxypeptidase and insulin are exposed to these reagents, respectively. The maxima occur at the wave lengths identical to those observed in the inorganic system save for the liver alcohol dehydrogenase–1,10-phenanthroline complex which exhibits small bathochromic shifts of 3275 → 3287 Å. and 3425 → 3450 Å. The insulin preparation, shown by analysis to contain trace quantities of zinc only, did not exhibit the spectral changes with 1,10-phenanthroline (Fig. 1C) shown by zinc insulin. Thus the absorption spectra cannot be due to an interaction of 1,10-phenanthroline with other, non-specific groupings of the insulin molecule or, by analogy, with those of the other zinc enzymes. In all the zinc proteins studied, the protein and its ligand groups to which zinc is bound do not appear to induce any major shift in the absorption of the zinc–1,10 phenanthroline and zinc–8-hydroxyquinoline-5-sulfonic acid chelates (small changes in the LADH·OP complexes will be discussed), in contrast to shifts in the mixed complexes of oxalate–Cu^{++}–ethylenediamine, pyrophosphate–Cu^{++}-ethylenediamine and pyrophosphate–Cu^{++}–ammonia, and of iminodiacetic acid–Cu^{++}–ethylenediamine, where the absorption band is due to the cation, and of protein–Zn^{++}–pyridine–2–azo-p-dimethylaniline observed by Watters, *et al.*,[16] Bennet,[17] and Klotz and Loh-Ming.[15] Thus the protein ligand groups are not able to modify any electronic transitions in the OP or 8OHQ5SA molecules through the electron bridge formed by the Zn atom, such as can be produced by auxochromic, bathochromic or hypsochromic groups substituted directly into these chelate molecules. The above authors also noted changes in extinction coefficients on mixed complex formation. Such changes will be discussed subsequently in our cases.

The reversibility of the protein–zinc–1,10 phenanthroline was tested by dialysis of the liver alcohol dehydrogenase–1,10-phenanthroline complex against Tris buffer. The disappearance, after dialysis, of the zinc–1,10-phenanthroline spectrum and the appearance of only the 1,10-phenanthroline spectrum in the dialysate demonstrate that the reaction is reversible and that the Zn is not removed from the protein in accord with equation 1 and is in full accord with enzymatic evidence previously described.[4h]

These studies demonstrate that zinc containing proteins with and without enzymatic activity form zinc complexes with chelating agents in a manner entirely analogous to ionic zinc. The mechanism for the inactivation of zinc enzymes by chelating agents previously proposed[4b] seems to be substantiated, therefore, by the spectrophotometric demonstration of an enzymatically inactive protein–metal–chelating agent complex, the behavior of which closely resembles that of the model system. Dissociability of this inactive enzyme–inhibitor complex, previously shown enzymatically, has now also been demonstrated spectrophotometrically. The significance of the metal atom as an active enzymatic site has thus been shown in terms of its specific chemical reactivity retained while being a part of a much larger protein molecule and the configurational properties of the locus of action of these enzymes further delineated.

BOSTON, MASS.

(17) W. E. Bennett, THIS JOURNAL, 79, 1290 (1957).