the sulfur–phosphorus bonds for form (2-mercaptoethyl) ammonium iodide. The rate of this reaction was found to be first order with respect to hydroxyl ion. The second-order rate constant at 25° was calculated to be 59.7 M^{-1} min.$^{-1}$. The apparent activation energy was determined to be 22 Kcal./mole with a standard error of 1 Kcal./mole. Since this energy of activation includes the heat of ionization of water, the energy of activation for the hydroxyl reaction would be 10 Kcal./mole.

3. Below pH 5 the main degradative route was the hydrolytic cleavage at the carbon oxygen bonds to form ethanol and trimethyl (2-hydroxylethyl) ammonium iodide, *S*-ester with phosphorothionic and *O*-ethyl ester. The rate of this reaction was not influenced by changes in concentration of hydroxyl ion but was increased slightly by increased buffer concentration. The attacking species were postulated to be a water molecule and the activation energy was determined to be 23 Kcal./mole.

4. The overall first-order rate constant for echothiophate iodide in aqueous solution was presented at 25° as a function of pH.

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Keyphrases

Echothiophate iodide degradation
pH effect—echothiophate iodide degradation
Hydrolysis, echothiophate iodide—temperature effect
Degradation products—acidic, alkaline media
GLC—analysis
TLC—analysis
UV spectrophotometry—analysis
Flame photometry—analysis
Iodometric analysis

Inhibition of Acetylcholinesterase by Chelates III

By ERNEST MARIO* and SANFORD BOLTON†

Inhibition of acetylcholinesterase by 1–1 cupric chelates of 1,3-diaminopropanol-2 and 1,3-diaminopropane is analyzed in the pH range of 8.0–9.0. The terdentate chelate of 1,3-diaminopropanol-2 exhibits weak inhibitory activity at pH values below 8.5 and increased activity at pH values above 8.5. The bidentate chelate of 1,3-diaminopropane exerts significant inhibition at pH 8.0. The bidentate system exhibits essentially noncompetitive inhibition while the terdentate system appears to be essentially competitive. Increased inhibition at elevated pH in the terdentate system is further evidence that the chelate is interacting with an ionizing group(s) on the enzyme surface, as previously reported.

As PART of a continuing study to investigate metal chelate inhibition of the acetylcholinesterase–acetylcholine enzyme reaction system, copper chelates of 1,3-diaminopropanol-2 (AOH) and 1,3-diaminopropane (AH) were prepared. These two structurally similar ligands were chosen because (*a*) the cupric chelates of AOH and AH had been clearly elucidated in an earlier investigation (1) and (*b*) it was of interest to examine the comparative effects of the two ligands, one of which is capable of forming both a 1–1 and 2–1 and the other only a 1–1 chelate.

In the first papers of this series, Bolton (2, 3) described some of the problems which are incurred in a study of this type. Since the chelate solutions contain an equilibrium mixture of several species, it is necessary to monitor closely the absolute concentrations of free ligand, free metal, 1–1 chelate, and 2–1 chelate under specific experimental conditions to determine which specie(s) is responsible for enzyme inhibition. The information obtained from the first two papers in this series may be briefly summarized as follows: (*a*) metal chelates inhibit the acetylcholinesterase–acetylcholine interaction; (*b*) in general, free ligand does not inhibit the interaction; (*c*) cupric

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ion alone is an inhibitor above concentrations of approximately $3 \times 10^{-5} M$ (the inhibitory effects of free metal ion may be eliminated if the chelate stability constant is large, or high pH values are used); (*d*) available coordination sites on the metal ion appear to be necessary prerequisites for inhibition; (*e*) overall chelate charge has a significant increasing effect on enzyme inhibition at elevated pH values; and (*f*) the 1–1[1] chelate species of Cu^{2+} appears to inhibit the enzyme system in a primarily "noncompetitive" manner.

The concentrations of individual species in an equilibrium mixture of ligand and metal under fixed conditions of concentration and pH may be calculated with a knowledge of the total metal, total ligand, chelate formation constants, ligand dissociation constants, and (H^+). The equations used to calculate the individual species in cupric chelate mixtures have been described (2). The values for free ligand (L) and free metal (M) can be solved from these equations by a method of successive approximations.[2] The values of (L) and (M) may then be used to calculate the concentrations of the chelate species present. In the case of the AOH-cupric interaction, a very strong 1–1 terdentate chelate is formed (1) with no discernible amount of 2–1 chelate species present. In the AH-cupric interaction, both a 1–1 bidentate chelate and a 2–1 tetradentate chelate are formed. The 1-1 AOH-Cu chelate carries a net (+1) charge while the 1–1 AH-Cu chelate carries a net (+2) charge. Thus, the two systems present an opportunity to compare the effects of metal coordination vacancies and overall chelate charge on enzyme inhibition.

EXPERIMENTAL

Reagents—AOH and AH (Aldrich Chemical Corp.) were obtained in the impure, free amine form. The di-HCl salts of these two ligands were prepared by dissolving the appropriate diamine species in excess concentrated hydrochloric acid (analytical reagent grade), flash evaporating to dryness and recrystallizing several times from methanol–ethanol mixtures (for AOH) and methanol–ethanol–water mixtures (for AH). Salt purity was checked by titration with standard base and the pKa values were compared to literature values, where available. Acetylcholine iodide and acetylcholinesterase (Nutritional Biochemicals Corp.) were used. Sodium chloride, magnesium chloride, and cupric nitrate, all analytical reagent grade, were also employed. The cupric nitrate solutions prepared were standardized according to the chelatometric titration method described by Wilson and Wilson (4) using murexide as an indicator. The diluent in all studies was prepared as previously reported (2).

[1] The 1–1 and 2–1 chelate species refer to (ligand–metal) and (ligand$_2$–metal) complexes, respectively.

[2] An IBM 7094 computer was used to solve the equations. The solution yielded values so that (MT) and (M) + (ML) + (ML_2) agreed to 10 decimal places.

Procedure—The experimental conditions and procedures were essentially the same as previously described (2). The values of V_0/V, (velocity with no inhibitor/velocity with inhibitor), and the values of $1/V$ were taken as the relative time necessary to consume 0.30 ml. of approximately 0.005 *N* base if the hydrolysis was linear as a function of time. The data were analyzed using the Michaelis-Menten theory of enzyme kinetics as extended by Friedenwald and Maengwyn-Davies to the case of a dissociable inhibitor (3, 8).

RESULTS AND DISCUSSION

Acid Dissociation and Chelate Stability Constants—The values of the constants required to determine the concentrations of species present in the chelate equilibrium mixtures are shown in Table I. Literature values are included where available.

Inhibition by Chelate Mixtures—Ligand-metal mixtures of different ratios[3] were used to inhibit the enzyme-substrate reaction at various pH values in the range of 8.0–9.0. The selection of pH values and chelate mixture ratios was governed by the desire to obtain one chelate species in predominance to all other species and the necessity of keeping the free metal concentration below approximately 3×10^{-5} *M* to prevent free metal initiated enzyme inhibition. Typical results of enzyme inhibition by solutions containing a 2.1–1 ligand–metal ratio of AOH-Cu at several pH values are shown in Table II. The concentration of 1–1 chelate is directly related to V_0/V. The choice of chelate mixture ratios with this system was not critical; the formation constant of the terdentate chelate attachment being very large, results in constant molar values for the 1–1 chelate species and extremely minute quantities of free metal. The values for V_0/V shown in Table II indicate a decrease in inhibition from pH 8.0 to pH 8.5 and then an increase in inhibition in the pH range of 8.5 to 9.0. Since the 1–1 chelate species formed has been shown to be stable up to pH 10, the increased inhibition cannot be due to chelate dissociation and may therefore be attributed to charge changes on the enzyme surface. The strong possibility exists that the pKa of the group(s) on the enzyme surface directly responsible for chelate attachment or proximal to the point of attachment has been reached in the pH range of 8.5–9.0. That the anionic site of the enzyme or some other negatively charged site is probably involved, was the hypothesis Bolton offered in the first paper of this series (2), and the data shown in Table II appears to support this proposed mechanism of inhibition.

The results of enzyme inhibition by several ligand-metal ratio solutions of AH–Cu^{2+} at pH 8.0 are shown in Fig. 1. The concentration of 1–1 chelate species is seen to be directly related to V_0/V. It was not possible to obtain meaningful data at pH values above 8.0 using the above-mentioned chelate mixture ratios. There was no apparent linear relationship between any of the species present and V_0/V. Some possible explanations for this behavior are: (*a*) the double plus charge in Cu^{2+} chelates of AH changes either the site or mechanism of binding on the enzyme surface and/or (*b*) the concentration of 1–1 chelate species was so small compared to 2–1 chelate species (at pH values above 8.0) that a

[3] Chelate mixture ratios refer to (LT)/(MT)

TABLE I—ACID DISSOCIATION CONSTANTS AND CHELATE STABILITY CONSTANTS FOR AOH AND AH AND THEIR CUPRIC CHELATES

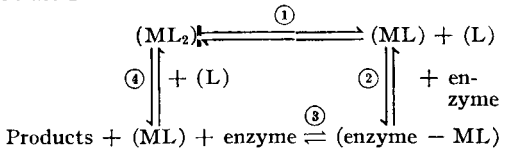
Ligand	pKa_1	pKa_2	pKa_3	Ionic Strength	Temp., ° C.	Ref.
AOH	8.01	9.58	14.20	0.162	30	...
AH	8.50	10.24	...	0.162	30	...
			Literature Values			
AOH	8.23	9.68	...	0.100	20	(5)
AH	8.88	10.64	...	0.100	20	(6)

Ligand	log K_1	log K_2	Ionic Strength	Temp., ° C.	Ref.
AOH	18.40	...	0.162	30	...
AH	9.29	6.96	0.162	30	...
		Literature Values			
AOH	...	...	...	..	...
AH	9.62	7.00	1.000	20	(7)

TABLE II—INHIBITION BY 1-1 AO-Cu^+ CHELATE IN A 2.1-1 CHELATE MIXTURE RATIO SOLUTION

pH	Molar Concn. Chelate × 10^{-3}	Molar Concn. 1-1 Chelate × 10^{-3}	Molar Concn. Free Cu^{2+} × 10^{-10}	V_0/V
8.0	1	0.9	0.45	1.021
	2	1.9	0.45	1.048
	3	2.9	0.45	1.072
	4	3.9	0.45	1.096
8.25	1	0.9	0.11	1.020
	2	1.9	0.11	1.037
	3	2.9	0.11	1.058
	4	3.9	0.11	1.080
8.50	1	0.9	0.031	1.016
	2	1.9	0.031	1.034
	3	2.9	0.031	1.057
	4	3.9	0.031	1.070
8.75	1	0.9	0.028	1.019
	2	1.9	0.028	1.039
	3	2.9	0.028	1.060
	4	3.9	0.028	1.080
9.00	1	0.9	0.003	1.042
	2	1.9	0.003	1.089
	3	2.9	0.003	1.134
	4	3.9	0.003	1.184

rate-determining equilibrium was achieved between the enzyme and the chelate solution according to Scheme I.



Scheme I

Analysis of Inhibition—The inhibition of acetylcholinesterase by the 1–1 cupric chelate of AOH at pH 8.5 was analyzed according to the methods suggested by Friedenwald and Maengwyn-Davies (8). The results of this study are tabulated in Table III and shown graphically in Fig. 2. The inhibition by the AOH chelate appeared to be essentially competitive at pH 8.5 with a small, noncompetitive component. This small noncompetitive component may in fact be the result of experimental error since the inhibitory properties of the 1–1 AO-Cu^+ chelate were relatively weak. The inhibition of acetylcholinesterase by the 1–1 cupric chelate of AH at pH 8.0 was analyzed by the same method, and the results of this study are tabulated in Table IV and shown graphically in Fig. 3. The inhibition by the 1-1 AH-Cu^{2+} chelate appeared to be essentially

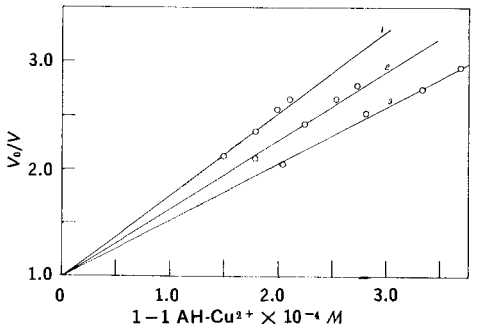


Fig. 1—Inhibition by 1–1 AH–Cu^{2+} chelate in several ligand–metal ratios at pH 8.0. Key: 1, ligand-metal ratio = 2.3–1; 2, ligand–metal ratio = 2.2–1; 3, ligand–metal ratio = 2.1–1.

TABLE III—INHIBITION BY 1–1 AO–Cu^+ CHELATE IN A 2.2–1 CHELATE MIXTURE RATIO SOLUTION AT pH 8.5 WITH VARYING SUBSTRATE CONCENTRATIONS

Molar Concn. of Substrate × 10^{-3}	Molar Concn. of Chelate Mixture × 10^{-3}	Molar Concn. of 1-1 Species × 10^{-3}	Reaction Velocity $(1/V)$
1.22	0	0.0	49.0
	1	0.9	49.5
	2	1.9	51.0
	3	2.9	52.0
	4	3.9	53.5
0.99	0	0.0	50.0
	1	0.9	52.0
	2	1.9	53.0
	3	2.9	54.5
	4	3.9	57.0
0.73	0	0.0	53.5
	1	0.9	55.0
	2	1.9	57.0
	3	2.9	58.5
	4	3.9	62.0
0.49	0	0.0	58.0
	1	0.9	61.0
	2	1.9	63.5
	3	2.9	66.0
	4	3.9	69.0

noncompetitive at pH 8.0 in agreement with earlier results reported by Bolton (3).

Relation of Inhibition to Net Chelate Charge and Coordination Vacancies—While the concentration of the 1–1 AO–Cu^+ chelate did not change between

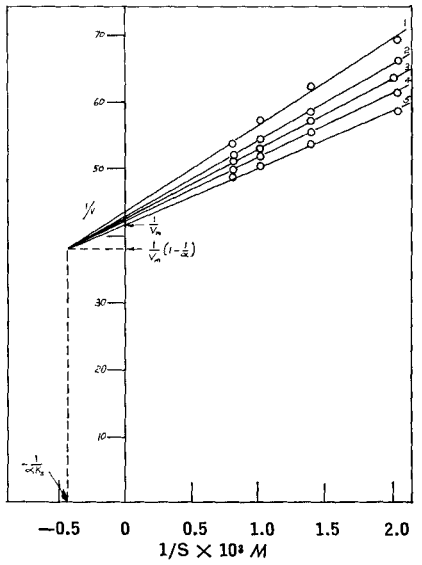


Fig. 2—Analysis of inhibition by 1–1 AO–Cu⁺ chelate at pH 8.5 using 1/S values. Key: 1, (I) = 3.9 × 10⁻³ M; 2, (I) = 2.9 × 10⁻³ M; 3, (I) = 1.9 × 10⁻³ M; 4, (I) = 0.9 × 10⁻³ M; 5, (I) = 0.

TABLE IV—INHIBITION BY 1-1 AH-Cu²⁺ CHELATE IN A 2.1-1 CHELATE MIXTURE RATIO SOLUTION AT pH 8.0 WITH VARYING SUBSTRATE CONCENTRATION

Molar Concn. of Substrate × 10⁻³	Molar Concn. of Chelate Mixture × 10⁻³	Molar Concn. of Free Cupric Ion × 10⁻⁴	Molar Concn. of 1-1 Species × 10⁻⁴	Molar Concn. of 2-1 Species × 10⁻³	Reaction Velocity 1/V
1.22	0	0.00	0.00	0.00	50.5
	1	2.50	2.06	0.79	101.0
	2	2.17	2.82	1.72	124.5
	3	1.96	3.34	2.67	138.0
	4	1.79	3.73	3.63	149.0
0.99	0	0.00	0.00	0.00	53.0
	1	2.50	2.06	0.79	111.0
	2	2.17	2.82	1.72	133.0
	3	1.96	3.34	2.67	148.0
	4	1.79	3.73	3.63	161.0
0.73	0	0.00	0.00	0.00	57.5
	1	2.50	2.06	0.79	125.0
	2	2.17	2.82	1.72	148.5
	3	1.96	3.34	2.67	162.0
	4	1.79	3.73	3.63	170.5
0.49	0	0.00	0.00	0.00	65.6
	1	2.50	2.06	0.79	142.0
	2	2.17	2.82	1.72	169.0
	3	1.96	3.34	2.67	192.0
	4	1.79	3.73	3.63	211.0

pH 8.0 and 9.0, the inhibition of the enzyme system increased when the pH exceeded 8.5 (see Table II). Since there is only one available coordination vacancy in the terdentate 1–1 AOH chelate, the increased inhibition may be attributed to the net +1 charge on the chelate being attracted to some negatively charged site on the enzyme surface with a pKa value between 8.5 and 9.0. At pH values below 8.5 the inhibition was slight. This may be expected considering the assumption that metal coordination vacancies are necessary at low pH values for inhibition to occur (the 1–1 AOH chelate having only one available coordination site, access to which is spacially hindered by the strong terdentate attachment). The results of studies with the AH system lend support to this hypothesis. The 1–1 AH chelate has two coordination vacancies and

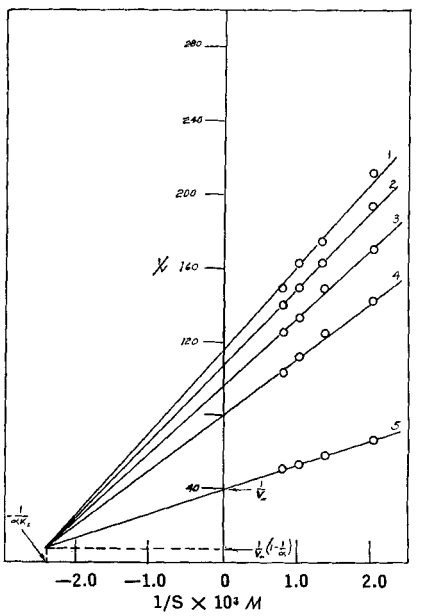


Fig. 3—Analysis of inhibition by 1–1 AH–Cu²⁺ chelate at pH 8.0 using 1/S values. Key: 1, (I) = 3.73 × 10⁻⁴ M; 2, (I) = 3.34 × 10⁻⁴ M; 3, (I) = 2.82 × 10⁻⁴ M; 4, (I) = 2.06 × 10⁻⁴ M; 5, (I) = 0.

is a stronger inhibitor than the 1–1 AOH chelate at pH 8.0 (see Fig. 1). At elevated pH values it was not possible to correlate inhibition with any individual species concentration present. This was of particular interest since the 1–1 AH chelate carries a net +2 charge and would probably be attracted to a negatively charged site on the enzyme surface more strongly than the single +1 charge of the AO–Cu⁺ chelate.

SUMMARY

Previous analyses of enzyme-chelate interactions have shown that 1–1 chelate species of ethylenediamine and glycine yield noncompetitive enzyme inhibition at pH 8.0 (see *Reference 3*). This study has shown that the 1–1 chelate species of AH also inhibits acetylcholinesterase in an essentially noncompetitive manner at pH 8.0. This was not unexpected since all of the above-mentioned chelate species are similar in that they have two metal coordination vacancies. The 1–1 chelate species of AOH was found to inhibit the enzyme weakly in an essentially competitive manner that may be attributed to the single, hindered, metal coordination vacancy present in the terdentate chelate attachment. At pH values above 8.5, the 1–1 chelate species of AOH exhibited stronger inhibitory properties. This can be explained on the basis of the predominance of a charge attraction between the chelate and the enzyme surface compared to a purely metal–enzyme attachment *via* the coordination vacancy. At pH values above 8.0, the 1-1 chelate species of AH was no longer linearly related to inhibition. Since this chelate carries a doubly positive net charge, the magnitude of any charge affinity between the chelate and a negatively charged

site on the enzyme surface would be large. If in fact, the mechanism of chelate–enzyme interaction changes from metal coordination attachment to charge attraction at elevated pH values, as the data would appear to indicate, it may be possible to identify the specific amino acid(s) responsible for the charge attraction from the estimated pKa. Further investigations are being contemplated to pursue these avenues.

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Keyphrases

Acetylcholinesterase inhibitors
Cupric chelate synthesis—1,3-diaminopropanol and 1,3-diaminopropane
Dissociation constants—acid
Chelate stability constants
Inhibition, acetylcholinesterase—analysis
pH Effect—acetylcholinesterase inhibition

Comparison of the Absorption and Excretion of Calcium from Precipitated Calcium Carbonate USP XVII and Egg Shells

By ARMEN H. MIRZAIAN and E. BLANCHE SOMMERS

Experiments were conducted to evaluate relative absorption and excretion of calcium from precipitated calcium carbonate USP and powdered egg shells (93.7 percent). Samples from 24-hr. specimens of urine and feces were collected after the fourth day of a repeated daily diet of the same food containing approximately 1 Gm. of calcium. The subjects were continued on the same regimen and samples were collected for 10 consecutive days from each person tested. The calcium content of the samples was determined by means of a flame photometer and a spectrophotometer. This procedure was repeated with addition of 2.5 Gm. of the official precipitated $CaCO_3$ and again with 2.6 Gm. of powdered egg shells to each daily diet. The calcium balance, that is, the difference between the quantity of calcium ingested and the total amount excreted, was positive for each type of experiment. When the diet was supplemented with the precipitated $CaCO_3$ and egg shells, calcium retention was increased 86.3 and 97.9 percent, respectively. Increased calcium absorption was reflected in increased urinary calcium. Egg shells compared favorably with the precipitated calcium carbonate as a source of absorbed calcium.

EXTENSIVE STUDIES have been concerned with nutritional standards for calcium. The National Research Council recommends a daily dietary allowance of 0.8 Gm. of calcium for an adult (1). Goodman and Gilman state that the average adult's daily requirement is about 10 mg./Kg. of body weight (2).

Calcium is the fifth most abundant element in the body, and the major fraction is in the bony structure (99%). It is present in small quantities in the extracellular fluid and to a minor extent in the structure and cytoplasm of cells of soft tissue. In the instance of hypocalcia, daily intake is frequently supplemented with a calcium salt. Wohl (3) prescribed the clinical use of calcium carbonate in preference to other calcium salts since it has a higher percentage of available calcium (40%).

The high calcium content of egg shells—*Gallus domesticus* Temminck (family, *Phasianidae*): calcium carbonate, 93.7%; magnesium carbonate, 1.3%; phosphorus pentoxide, 0.76%; and organic matter, 4.15% (4)—prompted investigation in regard to their value as a source of calcium. Limited studies of this nature have been done. However, they were primarily concerned with the palatability of foods containing powdered egg shells.

The studies presented here are concerned with comparison of calcium balances when the diet is

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