Inhibition of Acetylcholinesterase by Chelates I

Inhibition by Copper Chelates

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Enzyme kinetic studies were used in an effort to determine the nature of the inhibition of acetylcholinesterase by cupric chelates of glycine and ethylenediamine. Since the chelate solutions contain mixtures of species in equilibrium, it was necessary to calculate the concentrations of pertinent species under experimental conditions. Correlation of the observed inhibition with species concentration under various conditions indicate that (*a*) 2–1 ligand–copper chelates show negligible inhibition, (*b*) 1–1 chelate species exert significant inhibition, and (*c*) free copper may be responsible for inhibition if the affinity for copper in the chelate system is small. Conditions conducive to copper mediated inhibition include low pH, small chelate stability constants, and low ratio of ligand–copper in chelate solutions.

DURING A recent investigation of chelates as potential reactivators of poisoned acetylcholinesterase, it was noted that cupric and nickelous chelates of pyridine-2-aldoxime acted as enzyme inhibitors (1). Since the composition of the pyridine-2-aldoxime chelates under the experimental conditions in this study was in doubt (2), a further investigation of enzyme inhibition by chelate mixtures of known composition seemed worth while. The present paper is concerned with an attempt to elucidate the nature of the inhibition of acetylcholinesterase by cupric chelates of glycine and ethylenediamine (en).

The initial problems in such a study are formidable, since the chelate solutions contain an qeuilibrium mixture of several species. The questions to be resolved are which species are inhibitors and how do they exert their inhibition. In the present case (*a*) free ligand, (*b*) free metal ion, (*c*) 1–1 chelate, and (*d*) 2–1 chelate may possibly act as enzyme inhibitors. It was possible to measure the inhibition caused by free cupric ion and free ligand with use of the pure components. However, it is impossible to prepare solutions containing either of the two chelate species in the absence of the other. It was necessary, therefore, to design an experiment which would identify the species in the chelate mixture responsible for inhibition. Equations were derived which could be used to calculate the concentrations of individual species in an equilibrium mixture of ligand and metal under fixed conditions of concentration and pH as follows:

$$M + L \rightleftharpoons ML \qquad K_1 = (ML)/(M)(L) \qquad \text{(Eq. 1)}$$

$$ML + L \rightleftharpoons ML_2 \qquad K_2 = (ML_2)/(ML)(L) \qquad \text{(Eq. 2)}$$

where L is the ligand, and M is the metal ion.

$$H_2L \rightleftharpoons HL + H^+ \qquad K_{a_1} = (HL)(H^+)/(H_2L) \qquad \text{(Eq. 3)}$$

$$HL \rightleftharpoons L + H^+ \qquad K_{a_2} = (L)(H^+)/(HL) \qquad \text{(Eq. 4)}$$

$$(L_t) = (H_2L) + (HL) + (L) + (ML) + 2(ML_2) \qquad \text{(Eq. 5)}$$

where (L_t) is total ligand in the system. Combining Eq. 5 with Eqs. 1–4,

$$(L_t) = (L)\,[1 + (H^+)/K_{a_2} + (H^+)^2/K_{a_1}K_{a_2} + K_1(M) + 2K_1K_2(L)(M)] \qquad \text{(Eq. 6)}$$

$$(M_t) = (M) + (ML) + (ML_2) \qquad \text{(Eq. 7)}$$

where (M_t) is total metal in the system. Then,

$$(M) = (M_t) - (ML) - (ML_2) \qquad \text{(Eq. 8)}$$

Combining Eq. (8) with Eqs. 1 and 2

$$(M) = (M_t)/[1 + K_1(L) + K_1K_2(L)^2] \qquad \text{(Eq. 9)}$$

With a knowledge of (M_t), (L_t), K_1, K_2, K_{a_1}, K_{a_2}, and (H^+), Eqs. 6 and 9 can be used to solve for (L) and (M) by a method of approximations.[1] The values of (L) and (M) may be used then to calculate the concentrations of all species. Inhibition by chelate mixtures of various ratios[2] at pH's 7.0 and 8.0 was correlated with the calculated equilibrium concentrations of species in solution. This approach appeared to differentiate the inhibiting species: (*a*) 2–1 species inhibit the enzyme to a negligible extent; (*b*) 1–1 species are inhibitors; (*c*) cupric ion alone is an inhibitor above a critical concentration; (*d*) chelates may act as a reservoir of copper, which is available for interaction with the enzyme.

Direct evidence of interaction of individual

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

[2] In the remainder of this paper, chelate mixture ratios refer to $(L_t)/(M_t)$, whereas 1–1 and 2–1 helates refer to the individual chelate species in equilibrium.

TABLE I.—ACID DISSOCIATION CONSTANTS AND CHELATE STABILITY CONSTANTS FOR GLYCINE AND ETHYLENEDIAMINE AND THEIR CUPRIC CHELATES

Ligand	pKa_1	pKa_2	Ionic Strength	Log K_1	Log K_2
Ethylenediamine	7.08	9.86	0.162	10.34	8.89
Glycine	2.35	9.445	0.162	8.175	6.68
		Lit. Values (4)			
Ethylenediamine	7.31	10.05	1.0	10.75	9.30
Glycine	...	9.78	0	8.62	6.97

species in the chelate mixtures with the enzyme did not seem possible. For example, the amount of cupric ion bound to the enzyme was so small that no suitable analytical techniques were available. Also, the spectra of the chelate mixtures did not noticeably change after equilibration with the enzyme.

EXPERIMENTAL

Reagents.—Glycine, acetylcholine iodide, and acetylcholinesterase (Nutritional Biochemicals Corp.) were used. At pH 7.0, 1 mg. of the enzyme preparation decomposed approximately 0.8 mg. of acetylcholine iodide per minute at 3×10^{-3} M substrate concentration. Ethylenediamine dihydrochloride (Eastman Kodak), sodium chloride, magnesium chloride, EDTA, and cupric nitrate, all analytical reagent grade, were also employed. The purity of the cupric nitrate was checked, as previously described (1). The diluent in all studies consisted of an unbuffered solution of 0.9% NaCl and 0.266% $MgCl_2$ in double distilled water.

Procedure.—All experiments were run at 30° ± 0.1°. Double distilled water was used to make all solutions, and the ionic strength of all solutions was adjusted to 0.162 with NaCl. Acid dissociation constants and chelate stability constants were determined potentiometrically according to previously described methods (3). Enzyme activity was determined by titrating the acid formed during acetylcholine hydrolysis with approximately 0.005 N NaOH so that the pH was kept constant within 0.02 pH units. This was accomplished by an adaptation of a Beckman automatic titrator in conjunction with a Leeds and Northrup model 7401 pH meter. The effects of the chelate mixtures on enzyme activity were determined by a method, previously described (1), with slight modification. In general, 1 ml. of enzyme solution was added to a solution of the chelate, preset to the pH of the experiment, in a jacketed container connected to a circulating water bath. A stream of nitrogen was passed over the solution to exclude carbon dioxide. The enzyme and chelate were allowed to remain in contact for approximately 1 hr. (except during time dependence experiments) before acetylcholine was added. The final concentration of acetylcholine was 1.07×10^{-3} M for all experiments. The final volume of the reaction mixture was 10 ml. In experiments where cupric ion alone was used as an inhibitor, equilibration time was faster if cupric nitrate was added gradually to the enzyme solution. The values of V_0/V, (velocity with no inhibitor)/(velocity with inhibitor), were taken as the relative times necessary to consume 0.30 ml. of base during which the hydrolysis was linear as a function of time. The value of V_0 was checked at the beginning and end of each series of experiments to ensure that enzyme activity remained constant.

RESULTS

Acid Dissociation and Chelate Stability Constants.—The values of the constants needed to calculate the concentrations of the species in chelate mixtures from Eqs. 6 and 9 are shown in Table I. Previously reported values are included for comparison.

Inhibition by Ligand and Cupric Ion.—Neither glycine nor en alone in concentrations much beyond those used in this study, caused inhibition of the enzyme. Cupric ion alone in concentrations above approximately 10^{-5} M inhibited the activity of the enzyme. This inhibition was time dependent, approaching a steady value in about 1 hr. As expected, the degree of inhibition was dependent on enzyme concentration, and lower inhibition was observed at higher enzyme concentrations. Table II shows some representative results at pH's 7.0 and 8.0. The inhibition at these pH values is reversible. Addition of an equivalent amount of EDTA to the reaction mixture, after 1 hr. of equilibration with cupric nitrate, resulted in no noticeable inhibition of the enzyme.

Time Dependence of Inhibition by Chelate Mixtures.—Chelate mixtures were tested for time dependence of inhibition; some pertinent results are shown in Table III. It is apparent that solutions containing relatively large amounts of free cupric ion show a time dependence, whereas little or no time dependence is exhibited by solutions containing small concentrations of free cupric ion. Although none of the solutions contain enough free cupric ion as such to cause inhibition (see Table II), it

TABLE II.—INHIBITION OF ACETYLCHOLINESTERASE BY CUPRIC ION

M Concn. $Cu^{++} \times 10^5$	pH	Concn. Enzyme/ml.	V_0/V
0.1	7.0	0.0625	0.96
0.5	...	...	1.00
1.0	...	...	1.01
2.0	...	...	1.10
3.0	...	...	1.33
4.0	...	...	3.75
2.0		0.0500	1.44
1.0	8.0	0.0625	1.00
2.0	...	...	1.03
3.0	...	...	1.22
4.0	...	...	1.44
5.0	...	...	1.54
1.0	...	0.0450	1.08
2.0	...	...	1.38
3.0	...	...	1.51

TABLE III.—TIME DEPENDENCE OF SOME CHELATE–ENZYME INTERACTIONS

Chelate	(Ligand)/(Cu) Ratio	*M* Concn., Total Cu	pH	*M* Concn., Free Cu	Equil. Time, min.	V_0/V
en	1.95–1	4×10^{-3}	7.0	4.8×10^{-7}	15	1.22
					60	1.22
	2.00–1	8×10^{-3}	7.0	8.2×10^{-8}	5	1.12
					60	1.12
Glycine	2.00–1	2×10^{-3}	7.0	1.8×10^{-6}	3	1.28
					10	1.37
					30	1.40
					50	1.43
					60	1.43
	2.20–1	2×10^{-3}	7.0	5.8×10^{-7}	3	1.18
					30	1.24
					60	1.27
		8×10^{-3}	7.0	2.7×10^{-7}	15	1.24
					30	1.24
					60	1.26
en	1.95–1	4×10^{-3}	8.0	3.5×10^{-7}	15	1.62
					60	1.89
		8×10^{-3}	8.0	6.9×10^{-7}	15	2.28
					30	2.90
					60	3.39
		2×10^{-3}	8.0	1.8×10^{-7}	15	1.39
					60	1.40
	2.00–1	1.2×10^{-2}	8.0	7.3×10^{-9}	10	1.19
					60	1.195
Glycine	2.00–1	1×10^{-3}	8.0	1.9×10^{-7}	30	1.29
					60	1.32
	2.20–1	1×10^{-3}	8.0	2.2×10^{-8}	20	1.12
					40	1.13
		4×10^{-3}	8.0	6.8×10^{-9}	20	1.10
					40	1.10

TABLE IV.—INHIBITION BY CHELATES AT pH 7.0

Chelate	(Ligand)/(Cu) Ratio	*M* Concn. Chelate $\times 10^3$	*M* Concn. 1–1 Species $\times 10^4$	*M* Concn. Free Cu $\times 10^8$	V_0/V
en	1.95–1	1	0.74	20.7	1.065
		2	1.27	29.9	1.115
		4	2.26	47.7	1.20
		8	4.2	81.9	1.34
		10	5.17	98.6	1.40
en	2.00–1	1	0.45	7.6	1.04
		4	0.94	7.9	1.075
		6	1.16	8.0	1.10
		8	1.36	8.2	1.12
		10	1.52	8.3	1.13
en	2.10–1	4	0.20	0.35	1.03
		10	0.21	0.15	1.03
Glycine	2.00–1	0.25	0.94	183	1.26
		0.50	1.42	183	1.32
		1	2.12	183	1.38
		2	3.10	183	1.43
		4	4.50	183	1.50
Glycine	2.20–1	0.50	1.12	105	1.25
		1	1.43	76.9	1.26
		2	1.81	57.6	1.27
		4	2.15	39.1	1.25
		8	2.40	24.3	1.26
Glycine	3.00–1	4	0.56	2.6	1.07
		8	0.57	1.3	1.08

will be shown that some of the mixtures probably release bound copper to the enzyme, and that time dependence of inhibition is an indication that free cupric ion is contributing to the inhibition.

Inhibition by Chelate Mixtures.—Ligand–metal mixtures of different ratios were used to inhibit the enzyme at pH's 7.0 and 8.0. One of the problems in these studies was to select mixtures which contained one species in predominance, so that its inhibition could be observed to the exclusion of the other species with which it is in equilibrium. Systems containing large ligand–metal ratios result in

TABLE V.—INHIBITION BY CHELATES AT pH 8.0

Chelate	(Ligand)/(Cu) Ratio	*M* Concn. Chelate × 10^3	*M* Concn. 1–1 Species × 10^4	*M* Concn. Free Cu × 10^8	V_0/V
en	1.95–1	1	0.52	10.0	1.215
		2	0.98	17.6	1.40
		4	1.94	34.7	1.89
		8	3.85	69.0	3.39
en	2.00–1	4	0.25	0.55	1.09
		8	0.38	0.64	1.145
		12	0.49	0.74	1.195
		16	0.60	0.81	1.25
en	2.10–1	4	0.011	0.001	1.02
		8	0.011	0.0005	1.03
		12	0.011	0.0003	1.04
Glycine	2.00–1	0.5	0.52	18.8	1.24
		1	0.74	18.7	1.32
		2	1.05	18.5	1.43
		4	1.48	18.2	1.59
		4.84	1.63	18.1	1.64
Glycine	2.20–1	1	0.26	2.21	1.13
		2	0.28	1.26	1.10
		4	0.29	0.68	1.10
		10	0.30	0.28	1.13
Glycine	3.00–1	2	0.06	0.057	1.02
		4	0.06	0.029	1.02
		8	0.06	0.014	1.03

solutions containing 2–1 chelate in equilibrium with negligible amounts of free cupric ion and 1–1 chelate. These solutions showed negligible inhibition (see Tables IV and V); therefore, it appeared that in other mixtures inhibition was caused by free cupric ion, 1–1 chelate, or both. Since it is impossible in these systems to prepare a chelate solution containing one of these species without a significant amount of the other, the only practical approach seemed to be an attempt to correlate inhibition caused by the mixtures with the calculated concentrations of free cupric ion and/or 1–1 chelate species. Solutions could not be prepared in which the ratio of ligand to metal ion was less than 1.95–1 for en and 2–1 for glycine due to solubility considerations.

Some representative results are shown in Tables IV and V. At pH 7.0, 2–1 en–copper solutions are in equilibrium with small concentrations of free cupric ion. Although 1.95–1 en–copper mixtures are in equilibrium with larger amounts of free cupric ion, the inhibition in both 2–1 and 1.95–1 systems at pH 7.0 was attributed to the 1–1 species because (*a*) no time dependence was observed; (*b*) if inhibition is attributed only to 1–1 chelate species, there is a close correlation of inhibition caused by 1.95–1 and 2–1 mixtures (see Fig. 1); (*c*) there is no correlation between concentration of free cupric ion and inhibition caused by these mixtures.

At pH 7.0 in the glycine systems, the results indicate that free cupric ion and 1–1 chelate are inhibitors. There is no linear correlation between inhibition and concentration of 1–1 chelate (see Fig. 1). Free cupric ion is present in relatively high amounts, and there is no direct relationship between the concentration of free cupric ion and the degree of inhibition (see Table IV). The free cupric ion concentration stays approximately constant in a wide concentration range of 2–1 glycine–copper mixtures; yet the inhibition in these systems increases with concentration of the 2–1 mixtures. If the inhibition caused by these mixtures is due to a constant amount of free cupric ion plus increasing amounts of 1–1 chelate, the type of inhibition curve observed in Fig. 1 would be expected. That the inhibition is probably due to both of these species is more dramatically illustrated in 2.2–1 mixtures. Here the concentration of free cupric ion decreases, and 1–1 chelate concentration increases as the concentration of the 2.2–1 mixture increases. Table IV shows that the inhibition is relatively constant throughout a wide concentration range of 2.2–1 mixtures, which is strongly suggestive of the simultaneous effect of the two inhibiting species. It is of interest that both 2–1 and 2.2–1 glycine–copper mixtures showed time dependence (except for high concentrations of 2.2–1, where cupric ion concentration becomes small) in their inhibition of the enzyme at pH 7.0 (see Table III).

Table V shows the results of chelate inhibition at pH 8.0. Again 2–1 chelate species exert negligible inhibition, indicated by results in solutions in which ligand is in excess. In other solutions, the amount of free cupric ion is still well below that which would

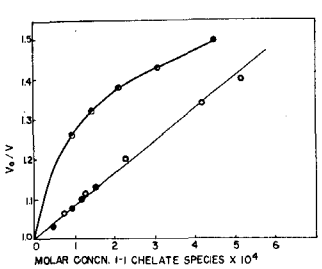

Fig. 1.—Correlation of inhibition with concentration of 1–1 chelate species at pH 7.0. Ligand – copper ratios refer to (total ligand)/(total copper). Key: ○, en–Cu, 1.95–1; ●, en–Cu, 2.00–1; ◐ 2.00–1 glycine–Cu.

cause inhibition if the free metal were alone, as is the case in the pH 7.0 studies. That free cupric ion is not contributing to inhibition in the glycine mixtures is apparent from the fact that although free cupric ion decreases as the concentration of the glycine–copper mixtures increases, inhibition increases with the concentration of the mixtures. This increase can be related directly to the concentration of 1–1 chelate in these mixtures, as illustrated in Fig. 2. Neither 2–1 or 2.2–1 mixtures show a time dependence in their inhibition. In 2–1 en–copper mixtures, the 1–1 chelate also seems to be solely responsible for inhibition. However, at higher concentrations of 1.95–1 mixtures, a time dependence was observed, and the inhibition could not be accounted for by the 1–1 species alone. Relatively large concentrations of free cupric ion are in equilibrium with these solutions; it appears that this species is contributing to the inhibition. At lower concentrations of 1.95–1 en–copper mixtures, where the concentration of free cupric ion is lower, inhibition is probably due only to 1–1 chelate. Inhibition in these solutions shows excellent agreement with inhibition observed in 2–1 mixtures if inhibition is attributed to the 1–1 chelate only (see Fig. 2).

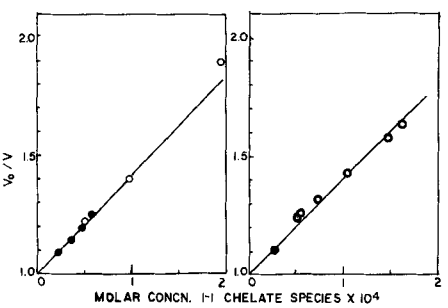

Fig. 2.—Correlation of inhibition with concentration of 1–1 chelate species at pH 8.0. Ligand–copper ratios refer to (total ligand)/(total copper). Key: O, 1.95–1 en–Cu; ●, 2.00–1 en–Cu (left). O, 2.00–1 glycine–Cu; ●, 2.20–1 glycine–Cu (right).

DISCUSSION

It has been demonstrated that in glycine and en–copper chelate systems both 1–1 chelate and free cupric ion may act as inhibitors. Binding of the four coordination sites of copper is probably responsible for the ineffectiveness of the 2–1 chelate species as enzyme inhibitors. The following schemes indicate possible equilibria in the enzyme–chelate mixture interactions:

1 ligand–Cu + enzyme $\rightleftharpoons$ ligand–Cu–enzyme

2 Cu + enzyme $\rightleftharpoons$ Cu–enzyme $\quad Ke = (\text{Cu–enzyme})/(\text{Cu})(\text{enzyme})$

3 ligand–Cu + enzyme $\rightleftharpoons$ Cu–enzyme + ligand $\quad K'e = (\text{Cu–enzyme})(\text{ligand})/(\text{ligand–Cu})(\text{enzyme})$

$$K'e = Ke/K_1$$

where K_1 = first chelate stability constant.

In the mixtures used in this study, the concentration of free cupric ion was well below that which would cause inhibition were the cupric ion alone. However, there is strong evidence that free cupric ion may be responsible for inhibition in certain cases. This effect would correspond to scheme *3* above. If K_1 is sufficiently small compared with Ke, the enzyme can compete for the copper bound to the ligand. This appears to be the case, for example, in the 2–1 and 2.2–1 glycine systems at pH 7.0. Another way of looking at scheme *3* is

$$(\text{Cu-enzyme})/(\text{enzyme}) = K'e\,(\text{ligand-Cu})/(\text{ligand}) = Ke(\text{Cu})$$

(Cu-enzyme)/(enzyme) represents, in a sense, inhibited enzyme; this ratio is directly proportional to the concentration of free cupric ion. The concentration of free cupric ion which, of course, is an indication of its affinity to the ligand in a chelate system, will depend on pH, ratio of ligand to metal, stability constants, and acid dissociation constants of the ligand. When experimental conditions result in a relatively high concentration of free cupric ion, the above analysis would account for inhibition by free cupric ion.

In the cases where inhibition was attributed to 1–1 chelate species, scheme *1*, the possibility exists that scheme *3* may describe the interaction better since both equilibria are dependent on the concentration of 1–1 species. If this were the case, however, the 1–1 glycine chelate should be a significantly stronger inhibitor than the 1–1 en species. (See values of K_1 in Table I.) Since the 1–1 chelates of these ligands are approximately equally effective (see Fig. 2), it seems that scheme *2* is the best description for interactions where cupric ion is strongly bound in the chelate system.

Although the exact site of inhibition cannot be determined from the present data, the anionic site of the enzyme or some other negatively charged site is probably involved. This hypothesis is supported by the fact that the 1–1 chelate species is a better inhibitor at pH 8.0 than pH 7.0. If the chelate is interacting with an ionizing group(s) in the enzyme, the positively charged chelate might be expected to exert a stronger effect at higher pH. It seems that the affinity of the enzyme for chelate bound copper is also greater at pH 8.0 than pH 7.0. For example, at pH 8.0, in the 0.004 *M* 1.95–1 en–copper mixture, free cupric ion probably contributes to enzyme inhibition. At pH 7.0, where cupric ion is less strongly bound by the chelate, the same mixture shows no evidence of inhibition by free cupric ion. The fact that cupric ion alone does not seem to exhibit this effect can be explained on the basis of greater competition for cupric ion by hydroxyl ions at higher pH.

At present, further efforts are being made to elucidate the mechanism of acetylcholinesterase inhibition by chelates. This work includes studies with chelate compounds which may better fit the enzyme surface.

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