mass spectral molecular ion peak, 606. The NMR spectrum (CDCl₃) showed $\tau = 6.08, 6.15, 6.25$ (9H, O-methyl), 8.01 (3H, N-methyl). The infrared spectrum showed a band at 2.88 μ, indicative of the presence of a phenolic hydroxyl group, and a band at 6.22 μ, indicative of the presence of a double bond.

Anal.—Calcd. for C₃₇H₃₈N₂O₆: C, 73.24; H, 6.31; N, 4.62; 3(OCH₃), 15.33; 1(NCH₃), 4.77. Found: C, 72.92; H, 6.26; N, 4.56; (OCH₃), 16.15; (NCH₃), 4.07.

O-Methylcissampareine.—Cissampareine (1.093 Gm.) in methanol was treated with an excess of an ethereal solution of diazomethane, and the solution was allowed to stand at room temperature for 48 hr. The solution was concentrated partially, then treated with a second charge of excess diazomethane in ether for 24 hr. The solvent was evaporated to yield a syrupy residue, shown to be homogeneous upon paper chromatography. The residue was dissolved in 2% sulfuric acid, washed with ether, made alkaline with 5% sodium hydroxide solution, and extracted with ether. The ethereal solution was dried over anhydrous potassium carbonate and evaporated to give a yellowish residue (1.02 Gm.), which was crystallized easily from acetone to yield colorless rods (0.62 Gm.), m.p. 192–194°; $[\alpha]_D^{26}$ −121° (c 1.36, chloroform); λ_{max}. 279 mμ (ϵ 13,500). The NMR spectrum (CDCl₃) showed $\tau = 6.00, 6.14, 6.24$ (12 H, O-methyl) and 7.64 (3H, N-methyl). The infrared spectrum showed no absorption in the 2.7–3.0-μ region.

Anal.—Calcd. for C₃₈H₄₀N₂O₆: C, 73.52; H, 6.50; N, 4.51; 4(OCH₃), 19.97. Found: C, 73.29; H, 6.52; N, 4.50; (OCH₃), 18.97.

Dihydrocissampareine.—A solution of cissampareine (35 mg.) in methanol (5 ml.) was treated portionwise with sodium borohydride (40 mg.), and the mixture was allowed to stand at room temperature for 3 hrs. Evaporation yielded a colorless residue, which was suspended in water and extracted with ether. The ethereal solution was dried over anhydrous sodium sulfate and evaporated to yield a colorless residue. Crystallization from methanol afforded colorless prisms (17 mg.), m.p. 208–212°; $[\alpha]_D^{27}$ −157° (c 0.7, chloroform); λ_{max}. 274 mμ (ϵ 3,800), 284 mμ (ϵ 3,400).

Anal.—Calcd. for C₃₇H₄₀N₂O₆: C, 73.00; H, 6.62; N, 4.60. Found: C, 72.62; H, 6.94; N, 4.83.

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Inhibition of Acetylcholinesterase by Chelates II

By SANFORD BOLTON

Inhibition of acetylcholinesterase by 1–1 cupric chelates of ethylenediamine and glycine has been analyzed and shown to be essentially of a noncompetitive type. Inhibition by the 1–1 nickel chelates is weaker than the corresponding cupric chelates; but in contrast to the lack of activity of 2–1 cupric chelates, 2–1 nickel chelates exert significant inhibition. This suggests that in these chelates the metal is binding to the enzyme and that the availability of coordination sites in the metal is more important than chelate charge. This fact plus the noncompetitive nature of the inhibition suggests that the binding does not occur at the active site of the enzyme. Increased inhibition at higher pH in the nickel systems is further evidence that the chelate is interacting with an ionizing group, as previously reported.

IN THE FIRST paper of this series it was shown that inhibition of acetylcholinesterase by cupric chelates of ethylenediamine (en) and glycine could be attributed to the 1–1¹ chelate species and, under certain conditions, free cupric ion (1). The 2–1 chelate species did not inhibit the enzyme noticeably. This investigation has been extended to include nickel chelates of these compounds and to elucidate further the nature of the cupric chelate inhibition.

Results of this study show that acetylcholinesterase inhibition by nickel chelates of ethylenediamine and glycine at pH's 8.0 and 9.0 can be described by simultaneous interaction of both 1–1 and 2–1 chelate species. A more detailed analysis of the 1–1 cupric chelate inhibition indicates that the inhibition is essentially noncompetitive.
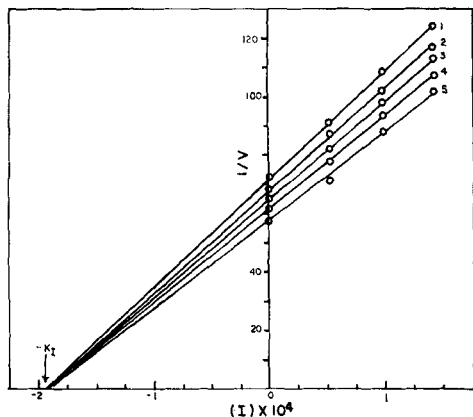
Fig. 1.—Analysis of inhibition by 1–1 en–Cu^{2+} chelate (*I*) at pH = 8.0. Key: *1*, (*S*) = 1.17 × 10^{-3} *M*; *2*, (*S*) = 7.8 × 10^{-3} *M*; *3*, (*S*) = 5.85 × 10^{-4} *M*; *4*, (*S*) = 4.68 × 10^{-4} *M*; *5*, (*S*) = 3.9 × 10^{-4} *M*.
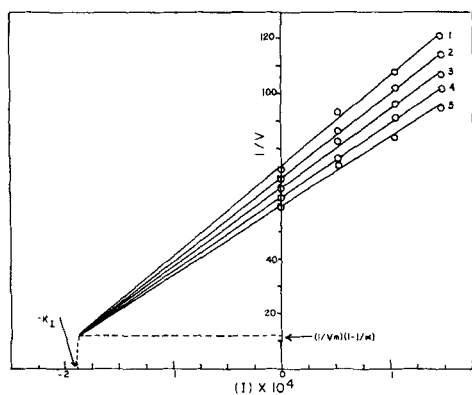

Fig. 2.—Analysis of inhibition by 1–1 glycine–Cu^{2+} chelate (*I*) at pH = 8.0. Curves *1–5* have the same meanings as in Fig. 1.

THEORY AND CALCULATIONS

Theory.—The Michaelis-Menten theory of enzyme kinetics, extended to the case of a dissociable inhibitor, has been discussed by Friedenwald and Maengwyn-Davies (2). They derived an equation which can be used, with certain transformations, to determine graphically the unknown constants describing the inhibition

$$Vm/V = 1 + Ks/(S) + (I)Ks/[(S)K_I] + (I)/[\alpha K_I] \quad (\text{Eq. 1})$$

where

Vm = maximum velocity, a constant
Ks = Michaelis-Menten constant
(S) = substrate concentration
(I) = inhibitor concentration
(K_I) = (inhibitor-enzyme) dissociation constant
α = a constant describing the nature of the inhibition, *i.e.*, when α = 1, the reaction is noncompetitive; when α = ∞, the inhibition is competitive. (Intermediate values of α represent varying degrees of "competitiveness.")

Usually, $1/V$ is plotted *versus* $1/(S)$ at constant values of (*I*), yielding a family of straight lines

intersecting in a point. Also, a plot of $1/V$ *versus* (*I*) at constant values of (*S*) yields a series of lines intersecting in a point. The points of intersection may be used to determine the unknown constants.

Because of the dependency of chelate composition on pH and because of the lack of solubility of the 1–1 chelates, it was necessary in part of this study to observe the simultaneous effect of two inhibitors, *i.e.*, a mixture of 1–1 and 2–1 chelate species. The following is the derivation of the equation used to solve this problem.

Friedenwald and Maengwyn-Davies have derived an equation describing the effect of a dissociable inhibitor and activator on an enzyme reaction (2). Using their model, but substituting a second inhibitor for the activator, the following equation may be derived

$$Vm/V = 1 + Ks/(S) + Ks(I)/K_I(S) + Ks(I')/K_{I'}(S) + (I)/\alpha K_I + [Ks(I)(I')]/[K_I \times K_{I'}u(S)] + (I')/xK_{I'} + [Ks(I)(I')]/[\alpha \, xpKs \times K_I K_{I'} u] \quad (\text{Eq. 2})$$

where (*I'*) = concentration of second inhibitor, $K_{I'}$ = dissociation constant for (enzyme–*I'*) interaction, and *x* is similar to α, but describes nature of *I'* inhibition. *u* and *p* are related to the dissociation constant of (enzyme–*I*–*I'*) interaction and dissociation constant of the (enzyme–*S*–*I*–*I'*) interaction, respectively. The remaining terms are the same as in Eq. 1.

The equation is greatly simplified if the interactions involving (enzyme–*I*–*I'*) and (enzyme–*S*–*I*–*I'*) are considered to occur to a negligible extent. This assumption seems reasonable because (*I*) and (*I'*) probably bind at the same sites; it is doubtful that both can bind simultaneously. The data obtained in this study substantiate the validity of this assumption. Thus, the following equation results:

$$Vm/V = 1 + Ks/(S) + Ks(I)/K_I(S) + Ks(I')/K_{I'}(S) + (I)/\alpha K_I + (I')/x \, K_{I'} \quad (\text{Eq. 3})$$

For this study, a more useful form of Eq. 3 is

$$1/V = 1/Vm[1 + K_s/(S)] + (I)(1/Vm) \times [Ks/K_I(S) + 1/\alpha K_I] + (I')(1/Vm) [Ks/K'_I(S) + 1/xK'_I] \quad (\text{Eq. 4})$$

Since the first term on the right-hand side of the equation and the coefficients of (*I*) and (*I'*) are constant (under experimental conditions in this study), the equation may be represented as

$$1/V = K_m + (I) K_n + (I')Kp \quad (\text{Eq. 5})$$

where the K's are appropriate constants.

Equation 5 was used to analyze the inhibition by nickel chelates.

Calculations.—The calculation of individual species in cupric chelate mixtures has been described (1). In the nickel system, the computations are more complex because of the presence of a 3–1 chelate species. A derivation by the author similar to the case where only 1–1 and 2–1 species are

TABLE I.—K_I VALUES FOR 1–1 CUPRIC CHELATES

Chelate	pH	K_I
En–copper	7.0	8.6 × 10^{-4}
En–copper	8.0	1.9 × 10^{-4}
Glycine–copper	8.0	1.9 × 10^{-4}

TABLE II.—CONSTANTS FOR NICKEL CHELATES

Ligand	pKa₁	pKa₂	Ionic Strength	Log K_1	Log K_2	Log K_3
Glycine	2.35	9.445	0.162	5.71	4.60	3.40
En	7.08	9.86	0.162	7.26	5.885	4.115
Lit. Values (4)						
Glycine	...	9.68	0.5	5.77	4.80	3.61
En	7.31	10.05	1.0	7.66	6.40	4.55

TABLE III.—INHIBITION BY EN-NICKEL MIXTURES AT pH 9.0

En/Ni²⁺ Ratio and M Concn. Total (Ni²⁺)		M Concn. Free Ni²⁺ × 10⁶	M Concn. 1–1 Chelate × 10⁴	M Concn. 2–1 Chelate × 10⁴	$1/V^a$ Observed	$1/V^a$ Calcd.
0	...	...	...	...	76.	...
1.75–1	1.135 × 10⁻³	6.8	3.88	9.2	88.	87.
2.00–1	3.30 × 10⁻³	2.3	3.88	26.	94.	92.
2.125–1	6.25 × 10⁻³	1.3	3.88	48.	101.	99.5
2.25–1	1.2 × 10⁻²	0.75	3.88	84.6	112.	111.5
1.75–1	1. × 10⁻³	5.3	2.92	6.8	83.	84.
	2. × 10⁻³	9.8	5.64	13.7	94.	92.
	4. × 10⁻³	19.	11.1	27.6	108.	108.
	8. × 10⁻³	37.	21.9	55.3	137.	139.
2.00–1	1. × 10⁻³	1.0	1.37	7.9	82.	81.5
	2. × 10⁻³	1.6	2.43	15.8	86.5	86.
	4. × 10⁻³	2.7	4.51	31.7	97.5	95.5
	8. × 10⁻³	5.0	8.65	63.4	111.	114.
	1.2 × 10⁻²	7.2	12.8	95.0	130.	133.
2.125–1	4. × 10⁻³	0.92	2.61	31.1	92.5	91.5
2.25 –1	4. × 10⁻³	0.34	1.52	29.0	89.	88.5
2.50 –1	4. × 10⁻³	0.06	0.57	23.3	86.	85.

^{a} $1/V$ was the time in seconds to consume 0.30 ml. of approximately 0.005 N base.

formed yields the following equation of the fourth degree:

$$A4 + [(C + 3K_3M_t - K_3A_t)/CK_3]A^3 +$$
$$[(C + 2K_2M_t - K_2A_t)/CK_2K_3]A^2 +$$
$$[(C + K_1M_t - K_1A_t)/CK_1K_2K_3]A -$$
$$A_t/CK_1K_2K_3 = 0 \quad (Eq. 6)$$

where

C	$= 1 + (H^+)/Ka_2 + (H^+)^2/Ka_1Ka_2$
A	= free (en) or (glycine)
K_1, K_2, K_3	= chelate stability constants
M_t	= total metal ion in system
A_t	= total ligand in system
Ka_1, Ka_2	= acid dissociation constants

A was evaluated with a 7094 IBM computer; all other species could then be calculated as previously described (1).

EXPERIMENTAL

The reagents were the same as those used in the first paper (1). Nickelous nitrate, analytical reagent, was standardized according to a previously described method (3).

The experimental conditions and procedures were also essentially the same as that described in the first paper (1). In the nickel studies, acetylcholine concentration was 1.17×10^{-3} M. In the analysis of the cupric chelate inhibitions, substrate concentration and inhibitor concentration were varied. The values of $(1/V)$ were taken as the time necessary to consume 0.30 ml. of approximately 0.005 N base if the hydrolysis was linear as a function of time. Otherwise, the initial straight portion of the "base consumed *versus* time" curve was extrapolated to 0.30 ml. of base to obtain the value $(1/V)$.

RESULTS

The inhibition of acetylcholinestrase by the 1–1 cupric chelates of en at pH 7.0 and 8.0 and glycine at pH 8.0 was analyzed according to methods suggested by Friedenwald and Maengwyn-Davies (2). Typical results of these studies at pH 8.0 are depicted in Figs. 1 and 2, and K_I values obtained from these studies are listed in Table I.

Mixtures of 1.95–1 en-Cu⁺⁺ and 2–1 glycine–Cu⁺⁺ were used as inhibitors. $1/V$ was plotted against the calculated concentration of 1–1 species in these mixtures. Since it has been shown that free cupric ion contributes to the inhibition in glycine–Cu⁺⁺ mixtures at pH 7.0 (1), this system was not examined. The inhibition by the en chelate appeared to be noncompetitive at both pH 7.0 and 8.0. Analysis of the glycine chelate interaction at pH 8.0 revealed a small competitive component with an α value of 1.3 (see under *Theory*). That these analyses are dependent on the accuracy of the calculations of equilibrium concentrations of the 1–1 species (which depends on experimentally determined constants) and the assumption that these concentrations are not significantly changed in the presence of enzyme is indicative of the limitations of the analytical method. In view of the results in the en systems, the representation of the glycine inhibition as being partially competitive may be due to experimental error.

The constants necessary to calculate the species concentrations in nickel chelate mixtures according to Eq. 6 were determined from potentiometric data and are listed with previously reported values in Table II.

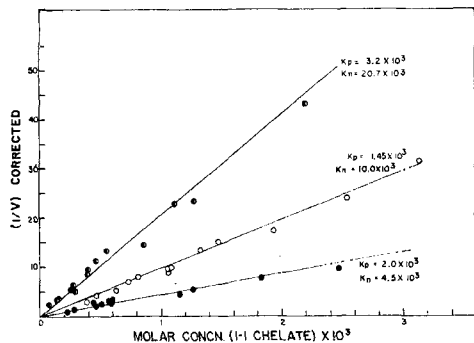Nickelous ion alone is a relatively weak enzyme

Fig. 3.—Plot used to calculate value of Kn for 1–1 Ni chelate. (See text.) $(1/V)$ corrected = $1/V - 1/Vo - Kp(2-1$ chelate). Key: $\bullet\!\!\circ$ = en–Ni²⁺ at pH 9.0; $\bigcirc$ = glycine–Ni²⁺ at pH 9.0; $\bullet$ = en–Ni²⁺ at pH 8.0.

TABLE IV.—K_I VALUES FOR NICKEL CHELATES

Chelate	pH	K_I
En 1–1	8.0	1.6×10^{-2}
2–1		3.7×10^{-2}
En 1–1	9.0	3.6×10^{-3}
2–1		2.3×10^{-3}
Glycine 1–1	9.0	7.3×10^{-3}
2–1		5.1×10^{-2}

inhibitor. For example, at pH 8.0, $(Vo/V)^*$ for 2×10^{-4} M Ni²⁺ is 1.10; at pH 9.0, (Vo/V) for 10^{-4} M Ni²⁺ is 1.20. At approximately 4×10^{-4} M Ni²⁺, enzyme activity rapidly decreased with time, the rate showing no linearity. This effect which appears at high concentrations of Ni²⁺ may be due to complexing of acetylcholine with Ni²⁺.

Results obtained for the inhibition of enzyme by en–Ni²⁺ mixtures at pH 9.0 are analyzed in Table III. Results of other inhibition studies by en and glycine–Ni²⁺ chelates at pH 8.0 and 9.0 are not listed here but were analyzed similarly. It was not possible to observe the inhibition caused by 3–1 chelate species since solutions containing this species in significant amounts are highly buffered. However, the assumption that these species contribute negligibly to enzyme inhibition seems valid in view of the following analysis where inhibition can be fully attributed to 1–1 and 2–1 species. That the 2–1 chelate exerts an inhibiting effect was demonstrated by observing the inhibition caused by a series of solutions in which the 1–1 chelate concentration was constant, and the 2–1 concentration varied. (See Table III.) From these data, an approximate value of Kp for the 2–1 species was calculated from Eq. 5. K_n for the 1–1 species then can be evaluated (rearranging Eq. 5) as

$$K_n(1\text{–}1 \text{ chelate}) = 1/V - 1/Vo - \\ K_p(2\text{–}1 \text{ chelate}) \quad (\text{Eq. 7})$$

A plot of (1–1 chelate) *versus* the right-hand side of Eq. 7 should yield a straight line with slope K_n. These plots are shown in Fig. 3.

Calculated values of $1/V$, using the experimentally determined values of K_a and K_p are listed in Table III and compare favorably with experimental

results. Equally close agreement was observed in the other systems studied. The data for glycine–Ni²⁺ inhibition at pH 8.0 have been omitted because of evidence that free Ni²⁺ was contributing to the inhibition; these solutions generally contained relatively large amounts of free Ni²⁺. However, results of inhibition by a few of these mixtures, where Ni²⁺ appeared to have little influence, indicated that glycine–Ni²⁺ chelates were as effective as the en chelates at this pH.

Approximate values of K_I may be evaluated for the Ni²⁺ chelate interactions if the inhibition is assumed to be noncompetitive, *i.e.*, $\alpha = 1$:

$$K_{n,p} = (1/V_m) \lfloor K_s/(S)K_I + 1/K_I \rfloor \quad (\text{Eq. 8})$$

$$K_I = (1/V_m) [K_s/(S) + 1]/K_{n,p} \quad (\text{Eq. 9})$$

V_m and K_s may be determined experimentally. Table IV lists the values of K_I thus calculated.

DISCUSSION

That 2–1 nickel chelates of en and glycine act as enzyme inhibitors, and that the 2–1 cupric chelates do not, suggest that the chelate–enzyme interaction occurs *via* the metal; *i.e.*, inhibition can only occur if coordination sites in the metal are available. The positive charge on the chelate apparently is not, in itself, sufficient to cause enzyme inhibition. For example, the neutral 2–1 glycine–Ni²⁺ species exerts inhibition, whereas the doubly charged 2–1 en–Cu²⁺ chelate does not. This effect is in contrast to the inhibition by 4° ammonium compounds which are competitive inhibitors (5). This result is not surprising since the inhibition by the 1–1 Cu²⁺ chelate species have been shown to be essentially noncompetitive; the chelates are not binding at the active sites of the enzyme. Nickel chelates are stronger inhibitors at pH 9.0 than pH 8.0; this is further evidence that the chelates are binding to an ionizing group. (See *Reference 1.*)

It is of interest that the en–Ni²⁺ chelates are stronger inhibitors than glycine–Ni²⁺ chelates at pH 9.0; whereas at pH 8.0, the Cu²⁺ and Ni²⁺ chelates of these compounds exert approximately equal inhibition, respectively. Perhaps different sites, which have a greater affinity for charged species, are available to the chelates at higher pH.

As expected, the 1–1 Ni²⁺ chelates are significantly weaker inhibitors than the cupric chelates; Cu²⁺ is a stronger coordinating ion than Ni²⁺. Also, the fact that the 1–1 Ni²⁺ chelate is a better inhibitor than the 2–1 species was anticipated on the basis of less steric hindrance and more available coordination sites.

Although this study has shed some light on the mechanism of chelate mediated enzyme inhibition, there are still many questions to be answered. At present, efforts are being made to elucidate the nature of these interactions.

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* (V_0/V) is the ratio of velocities of uninhibited and inhibited enzymatic decomposition of acetylcholine.