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INHIBITION OF ACETYLCHOLINESTERASE BY COPPER CHELATES

Ernest Mario
University of Rhode Island

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BY

ERNEST MARIO

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ERNEST MARIO

Approved:

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Chairman_____

_____

_____

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ABSTRACT

As part of a study to investigate metal chelates as possible inhibitors of acetylcholinesterase, copper chelates of 1,3 diaminopropanol-2, 1,3 diaminopropane, and hydroxy-L-proline are examined. Since the chelate solutions contain mixtures of species in equilibrium, it is necessary to calculate the concentrations of pertinent species under experimental conditions. Equations are derived which can be used to calculate the concentrations of individual species in an equilibrium mixture of ligand and copper under fixed conditions of concentration and pH with the aid of an IBM 1410 computer. Initial measurements of reaction velocity of enzyme-substrate breakdown to enzyme plus acid products, are made by observing the speed of pH drop with the aid of a Beckman Automatic Titrator. Selection of equilibrium pH values and ligand-metal ratios is dependent on the formation constants calculated for each system. Enzyme kinetic studies are used in an effort to determine the nature of the inhibition of acetylcholinesterase.

It is found that (a) 1-1 ligand-copper chelate inhibit the acetylcholinesterase system, (b) free cupric ion may inhibit the enzyme system if concentrations greater than 1×10^{-6} M are present in the equilibrium mixture, (c) coordination vacancies on the metal ion are necessary prerequisites for inhibition, (d) 1-1 ligand-copper chelates of 1,3 diaminopropanol inhibit the enzyme in an essentially "competitive" manner, (e) 1-1 ligand-copper chelates of 1,3 diaminopropane inhibit the enzyme in an essentially "non-competitive" manner, (f) 1-1 ligand-copper chelates of hydroxy-

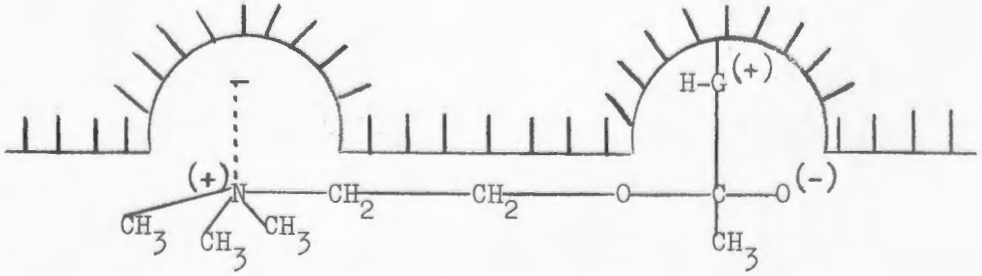
L-proline exhibit a "yield value" (or critical concentration requirement) before any inhibition occurs, and (g) the equilibrium pH of the experiments affects both the extent and nature of the inhibition.

I. INTRODUCTION

In recent years, the study of enzyme inhibition has gained widespread attention. Enzyme activity may be affected by a variety of factors such as pH changes (1) and chemical or physical interference (2,3). For example, recent findings concerning the use of ultraviolet light to impair the succinate oxidase enzyme system have been reported by several investigators (4-7). Unfortunately, in most instances, the exact mechanism(s) behind the inhibition of enzymes remains obscure. This is not unexpected since enzymes are, by nature, extremely complex molecules (8).

Acetylcholinesterase is a typically complex enzyme. It has a protein-like structure with a molecular weight of approximately three million, and is found in erythrocytes, nervous and electrical tissue (9). Acetylcholinesterase, in vivo; hydrolyzes the ester, acetylcholine, into choline andacetic acid and the following mechanism has been suggested (8):

ACETYLCHOLINESTERASE



ACETYLCHOLINESTERASE-ACETYLCHOLINE COMPLEX

The complex formed dissociates to yield enzyme plus products. The model has two active sites to which the substrate may attach, an anionic site that attracts the quarternary nitrogen group of acetylcholine and an esteratic site which reacts with the carbonyl moiety of the ester. Wilson and Bergmann (10) have found that acetylcholinesterase exhibits optimum activity at pH 8.3. Above or below this pH, the activity of the enzyme decreases. They also found that there are dissociable groups near the active sites which have pK values of 7.2 and 9.3. These dissociable groups can interact or bind with various substrates resulting in enzyme activity at pH values above and below the optimum value of 8.3.

Prostigmine and eserine are two classical examples of acetylcholinesterase inhibitors. Prostigmine contains a quarternary ammonium group which is charged throughout the pH range of enzyme activity. Eserine, on the other hand, contains a basic group with a pK value near 8.2. When fixed concentrations of these inhibitors were studied at different pH values, the inhibitory effect of prostigmine was constant; whereas the inhibitory effect of eserine was higher on the acid side of pH 8.2 and lower on the alkaline side (10). It was concluded from this information that the inhibitors attach to the anionic site and a cationic group in the inhibitor is essential for inhibition to take place (11). This conclusion was further substantiated by observing the effects of simple ammonium compounds on the

activity of acetylcholinesterase. Wilson (12) showed that the inhibition of acetylcholinesterase by simple ammonium compounds is dependent on the charge of the ammonium moiety. At high pH values, where there is less of the cationic form, the inhibitory effects of these ammonium compounds are less pronounced. This reduction of inhibitory effect, as pH is increased, illustrated a significant point. Since there is a dissociable group on the enzyme surface, near the active site, with a pK value of 9.3, the influence of the positively charged ammonium group might be expected to be more apparent when this pH is approached. The fact that the inhibition falls off as pH is raised, indicates that the ammonium moiety is binding not to an adjacent dissociable group, but, rather, directly to the active anionic site on the enzyme surface. Furthermore, if the binding occurs only at the active site, the inhibition will appear as "competitive", (i.e., the inhibitor and the substrate will vie for the same site) when the data are analyzed using conventional theoretical enzyme kinetic theory methods (13-17). If the binding occurs at adjacent sites, the inhibition is often found to be "non-competitive". Unfortunately, most inhibitors do not show a sharp delineation between "competitive" and "non-competitive" inhibition, but exhibit instead, a combination of both types (18).

The present investigation was undertaken in an attempt to elucidate the nature of enzyme inhibition by metallo-organic

compounds. It was felt that the chelates might act as inhibitors due to the presence of the positively charged metallic ion. Since the interaction of chelates with the enzyme may be related to chelate properties, it would be of value to review some of the pertinent chemistry of chelate compounds.

The term "chelate", first introduced by Morgan and Drew (19), is taken from the Greek term chele, meaning crab's claw. The analogy is obvious, since both a claw and a chelating agent or ligand may hold an object (i.e., the metal ion) through two or more points of attachment. These attachments are the result of ligand molecules successfully competing with the water molecules normally associated with metal ions. The number of water molecules associated with a given metal ion under known conditions is referred to as the coordination number of the metal. The binding forces between ligand molecules and metal ions may be electrostatic in nature (i.e., the attraction of a positive metal ion for a negative ion or the attraction of a positive metal ion for the negative portion of the dipole of a neutral molecule such as water). On the other hand, the binding may be primarily covalent in nature with electron-pair bonds accounting for the major portion of the affinity. Whether the binding is primarily electrostatic, primarily covalent, or something intermediate, the function of the ligand is always that of donating electrons to the metal (20). The overall net charge of the chelate molecule will depend on the nature of the ligand

molecule and the type of binding which occurs. If the ligand forms a stable, water soluble metal chelate, it is called a sequestering agent. This sequestering property can be shown by elevating the pH of the ligand-metal mixture and observing the presence or absence of metal hydroxide precipitation. If no precipitate appears at elevated pH values, the metal is said to be "sequestered."

When one ligand molecule contains sufficient electron donor groups to saturate all the coordinating positions on the metal ion, a single, stable 1-1 ligand-metal chelate is usually formed. The interaction of ethylenediaminetetraacetic acid with several metal ions as described by Schwarzenbach and co-workers (21-23) is a good example of a metal chelate species containing a ligand-metal ratio of unity. If, however, the introduction of one ligand molecule does not saturate all the coordinating positions on the metal ion, it is possible in certain cases to bind one or more additional ligand molecules to the same metal ion and still retain solubility of the chelate. The formation of higher ligand-metal ratios occurs in a stepwise manner not dissimilar to the dissociation of protons from a polyprotic acid. A typical example of stepwise chelate formation has been reported by Chaberek and Martell (24) and concerns the interaction of imino-diacetic acid with Cu^{++}, Ni^{++}, and Co^{++} ions. The nature of the interaction of this acid with

metal ions was interpreted from the curves obtained by titration of various ligand-metal mixtures with standard base. The formation or stability constants of the 1-1 and 2-1 ligand-metal species can be calculated using a direct algebraic method outlined originally by Schwarzenbach and co-workers (21,25). It should be noted that this algebraic treatment of the data is only approximate. Certain assumptions are made which are not strictly accurate and the true values can be obtained only through the use of a series of approximations (26). Employing this algebraic technique, Schwarzenbach and co-workers (23,27, 28) have determined the chelating tendencies of several alkaline earth metals.

Bjerrum (29) introduced a method of calculating formation constants which relies on the experimental determination of free ligand concentrations at various points during the titration of a ligand-metal mixture. He also introduced the function $\bar{n}$ which is defined as the average number of ligand molecules bound per mole of metal. This $\bar{n}$ function may be expressed mathematically as follows:

$$\bar{n} = \frac{(ML+2(ML_2)+.....+n(ML_n)}{(M)+(ML)+(ML_2)+.....+(ML_n)} \qquad (1)$$

With a plot of $\bar{n}$ versus the negative log of free ligand concentration, the formation constants may be obtained directly (see Experimental). However, this method is also approximate unless the numerical difference between the successive constants

is large. If the numerical difference is small, the true values for the successive formation constants may be determined by an iteration procedure (see Appendix A).

As previously mentioned, the initial purpose of this series of investigations was to observe the effects of metallo-organic chelate compounds on the acetylcholinesterase-acetylcholine system. To this end, Bolton and Beckett (30) prepared Cu^{++} chelates of pyridine-2-aldoxime. In this preliminary study, it was felt that metal chelates, having a positive charge, would bind directly to the active anionic sites on the enzyme surface and inhibit enzyme activity in a "competitive" manner. Further, if the enzyme was poisoned with organic phosphates such as DFP, the chelated species might act as an enzyme reactivator by displacing the poison through a competitive process. Also, certain chelates have been reported which, in fact, increase the rate of decomposition of organic phosphate poisons (31,32) and pyridine-2-aldoxime methiodide has been shown by Wilson (33) to reactivate the phosphate poisoned acetylcholinesterase enzyme. Thus the choice of pyridine-2-aldoxime chelates as potential enzyme reactivators seemed reasonable.

The results of this preliminary study showed that metal chelates of pyridine-2-aldoxime were poor reactivators of phosphate poisoned acetylcholinesterase. The absence of good reactivation properties was attributed to steric factors. In other words, the configuration of the aldoxime chelates appar-

ently was not as stereospecific for the active sites on the enzyme surface as the aldoxime-methiodide structure. Nevertheless, an interesting observation recorded was that the aldoxime-copper chelate species, in some way, exerted an inhibitory effect on the activity of acetylcholinesterase. Unfortunately, the exact composition of the pyridine-2-aldoxime-Cu^{++} chelate is in doubt (34). Repeated efforts to elucidate the exact ratio of ligand to metal in the chelated species were unsuccessful.

Since one of the main purposes of this investigation was to correlate chelate charge and configuration with inhibitory activity, it was essential that the nature of the ligand-metal interactions be known. Because of this requirement regarding the nature of the ligand-metal interaction, simple, well defined chelate systems were sought. In the first of a series of papers concerning inhibition of acetylcholinesterase by metal chelates, Bolton (35) used the Cu^{++} chelates of glycine and ethylenediamine. The choice of these two ligands was prompted by the fact that the chelation properties of these ligands have been extensively studied (36). Using a set of derived equations (34), Bolton was able to determine the precise concentrations of all species present in the ligand-metal mixture at specific pH values. On the basis of his observations, Bolton concluded that: (1) Both 1-1 ligand-metal species and free cupric ion may act as inhibitors; (2) 2-1 ligand-metal species did not

inhibit significantly, possibly due to steric factors; (3) the exact site of binding could not be determined on the basis of data obtained, but the anionic site or some other negatively charged site is probably involved; (4) the inhibitory influence of the chelate mixture is greater at pH 8.0 than pH 7.0, indicating a possible approach to the pK value of the dissociable group at the binding site (10).

In the second paper of this series, Bolton (37) extended his study to include Ni^{++} chelates of glycine and ethylenediamine. Further, the data concerned with the inhibition of the enzyme by the 1-1 cupric chelates were analyzed according to the method described by Friedenwald and Maengwyn-Davies (18). The conclusions reached in this second paper were as follows: (1) Both 1-1 and 2-1 ligand-metal species of Ni^{++} chelates exhibit inhibitory activity and the 1-1 species is a stronger inhibitor than the 2-1 species; (2) the 3-1 Ni^{++} chelate species does not inhibit the enzyme significantly, probably because all the coordination sites of the metal ion are saturated; (3) the inhibitory mechanism of the 1-1 Cu^{++} chelates of both glycine and ethylenediamine appears to be primarily "non-competitive" in nature, indicating that binding is not occurring at the active anionic sites; (4) the inhibitory influence of free metal ion can be eliminated by working at higher pH values provided the chelate remains stable; and (5) the inhibition is more pronounced at elevated pH values with those chelate

species having an overall positive charge, indicating a greater affinity for charged sites available to the chelates at high pH values.

The information available at the beginning of this study could be summarized as follows: (1) Metal chelates inhibit the acetylcholinesterase-acetylcholine interaction; (2) available coordination sites on the metal ion are necessary prerequisites for inhibition; (3) overall chelate charge has a significant effect at elevated pH values; (4) the 1-1 chelate species of Cu^{++} appears to inhibit the enzyme in a primarily "non-competitive" manner; and (5) the inhibitory effects of free metal ion may be eliminated if the chelate stability constant is large or high pH values are used.

As a continuation of the above study, we were interested in finding additional chelate systems with well-defined properties and examining their effects on the acetylcholinesterase-acetylcholine system. In view of previous results and our objectives, the choice of suitable ligand species was restricted as follows: (a) The chelate formation constants of the ligand must be of sufficient magnitude to preclude any interference by free metal ion; (b) a large pH range of stability is necessary to aid in observing the relationship between pH and inhibition; and (c) the chelate should be completely soluble throughout the pH range employed. Approximately ten potentially useful ligand molecules were examined prior to the inhibition

studies. In all but three cases, the compounds studied were found to be unsuitable. For example, kojic acid chelates proved to be too insoluble in aqueous systems; tetramethylethylenediamine formed very weak 1-1 and 2-1 chelate species; and several other ethylenediamine derivatives proved to be sterically hindered when interacted with cupric ion, resulting in weak ligand-metal interactions. The three ligands which seemed suitable for this study were 1,3 diaminopropanol-2-(AOH), 1,3 diaminopropane(AH), and hydroxy-L-proline(HP). All three compounds formed water soluble chelate species with substantially large pH ranges of stability and formation constants of sufficient magnitude to preclude free metal ion interference. The nature of the interaction of AOH with Cu^{++} was in doubt (38,39), and the interaction was studied in some detail. Attempts to duplicate previously published results were unsuccessful. It was found, from our studies, that the interaction of AOH and Cu^{++} resulted in a stable 1-1 ligand-metal species with a terdentate attachment (40).

The stability constants of the interaction of AH with Cu^{++} as reported by Schwarzenbach and co-workers (41), were reproduced, within experimental error, in our laboratory. The inhibition caused by the two homologues (AOH and AH) could be compared at identical pH values because they were both stable and soluble within the limits of our studies and the contribution of charge and coordination site vacancies to inhibitory activity could be observed.

The interactions of the (HP)-Cu^{++} chelate were determined and compared favorably with published results (42,43). This ligand was chosen because of the presence of a free hydroxyl group in the chelated molecule. A three dimensional scale model showing the approximate positions of the active sites on acetylcholinesterase was prepared using available information (8), and the model was compared with the possible configurations of the 1-1 (HP)-Cu^{++} chelate species to observe the spacial allignment and direction of the free hydroxy group. It appeared that the hydroxy group was directed towards the esteratic site on the enzyme and it was felt that this potentially significant stereospecificity might result in increased inhibition.

II. EXPERIMENTAL

Reagents

Analytical reagent grade $Cu(NO_3)_2$ was used as the source of metal ion and the solutions prepared were standardized according to the chelatometric titration method described by Wilson and Wilson (44) using murexide as an indicator. The substrate, acetylcholine iodide, was obtained in pure form from Nutritional Biochemicals Corporation.[1] The unbuffered physiological solution used as the diluent in all experiments, was prepared by dissolving 0.9 per cent analytical reagent grade sodium chloride and 0.0567 per cent magnesium nitrate in double distilled water. Acetylcholinesterase was obtained from Nutritional Biochemicals Corporation and a stock solution of 60 mg of the enzyme in 100 mls of diluent was used for the experimental runs. It was not essential to know the precise purity of the enzyme material since comparative velocity studies were made at the beginning and end of each series of runs. The enzyme solution, stored in a refrigerator, was allowed to stand for three days, after which time its activity remained relatively constant for at least three weeks.

The 1,3 diaminopropanol-2(AOH) and 1,3 diaminopropane(AH) ligands were obtained in the free amine state from Aldrich Chemical Corporation[2]. Since the di-HCl salts of these two

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[1] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Aldrich Chemical Corporation, 2371 North 30th Street, Milwaukee, Wisconsin.

ligands were used in the chelation studies, methods were sought to obtain these salts in pure form. The di-HCl salt of AOH was prepared by dissolving the impure diamine species in excess concentrated hydrochloric acid, reagent grade, flash evaporating to dryness, and recrystallizing several times from methanol-ethanol mixtures. The di-HCl salt of AH was prepared in a similar manner, but the recrystallization step required a methanol-ethanol-water mixture for best results. The hydroxy-L-proline(HP) ligand was obtained in pure form from Nutritional Biochemical Corporation and the mono-hydrochloride salt of this ligand was prepared in a manner identical to that for the AH ligand. The melting points of these salts is accompanied by decomposition and, therefore, their purity was checked by titrations with standard base and subsequent comparisons of literature pK_a vlaues, when available (see Results).

Description of pH STAT Instrumentation

Because one of the primary purposes of this study was the effect of pH on inhibition, an accurate means of measuring pH throughout the reaction process was sought. Furthermore, it was essential to the success of the experiments that a predetermined pH value be held relatively constant when measurements were taken. The importance of this requirement will become more apparent when the general procedure is described. Suffice it to say, at this point, that an extremely sensitive pH-STAT

type of instrumentation was essential.

A Beckman Automatic Titrator[1] was selected because this instrument anticipates a preset titration end-point. Unfortunately, the titrator alone was not sufficiently sensitive to pH changes and modifications were necessary. A Leeds-Northrup pH Meter[2] was incorporated into the titrator circuit to increase the sensitivity or response of the titrator to changes in hydrogen ion concentration. The titrator alone responded to 0.84 volts for full scale deflection from pH one to pH fourteen. By tying in the Leeds-Northrup amplifier circuit, the input voltage from the electrodes to the titrator was magnified approximately twelve times. In other words, the titrator response to hydrogen ion concentration changes was increased by a factor of twelve. This modification of the Beckman titrator resulted in a highly accurate means of controlling and following the pH of the experiments.

Free Cupric Ion Determination

Since free cupric ion has been shown to inhibit the enzyme (34), a method was sought to determine the effects of this metal ion on the rate of hydrolysis of acetylcholine in the enzyme system.

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[1] Beckman Instruments Inc., 2500 Harbor Boulevard, Fullerton, California.

[2] Leeds-Northrup Company, 4901 Stenton Avenue, Philadelphia, Pennsylvania.

Initially, an attempt was made to determine the precise amount of cupric ion that would bind to a fixed concentration of enzyme. A Sargent Polarograph, model XV[1], was used to prepare a calibration curve for various free cupric ion concentrations (i.e., a plot of diffusion current versus Cu^{++}). A ten per cent solution of enzyme stock solution (identical with the stock solution used in the inhibition studies) was treated with known increments of cupric ion. Polarograms were taken after each addition and the i_D values calculated. The total (Cu^{++}) added was calculated after each addition taking into consideration any volume changes. The concentrations of Cu^{++} added and the amounts obtained from the calibration curve were then compared. It was anticipated that any reduction in free Cu^{++} in the enzyme mixture could then be attributed to enzyme attachment.

Another analytical approach to the determination of Cu^{++}, described by Jenkins (45), entails the use of sodium diethyldithiocarbamate as a complexing agent specific for Cu^{++}. The complex formed in this method is colored and is found to absorb in the ultraviolet range of the spectrum. A calibration curve was prepared by plotting absorption versus concentration of Cu^{++}. Then, solutions of known concentration of enzyme were dialyzed for various lengths of time at constant temperature

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[1]E.H. Sargent & Company, Chicago, Illinois.

and the dialysate analyzed spectrophotometrically for free Cu^{++}. To detect possible absorption of the metal ion on the dialysing membrane, dialysis studies were made using cupric ion solutions of known concentration in the absence of enzyme.

Chelation Studies

The acid dissociation constants for all three ligands were determined potentiometrically according to the method of Chaberek and Martell (23). To be consistent with subsequent inhibition studies, these constants were determined at 30°C. $\pm$ 0.1°C. in solutions of 0.162 ionic strength adjusted with sodium chloride, with a stream of nitrogen passing over the solution to exclude carbon dioxide. A Beckman Zeromatic pH Meter[1] was used to follow the titrations. The dissociation constants were determined directly from the titration curves taking mid-point values in the usual manner.

The extent of chelation of AOH, AH, and HP with Cu^{++} was followed potentiometrically by recording equilibrium pH values after addition of increments of standard base to the ligand-metal mixtures. The conditions for these determinations were the same as those described for the determination of dissociation constants. The ratio of ligand to metal in the chelate was analyzed by titration of ligand-metal solutions of various ratios (i.e., 1-1, 2-1, 3-1, etc.)--see Results.

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[1]Beckman Instruments Inc., 2500 Harbor Boulevard, Fullerton, California.

Enzyme Inhibition Studies

In all inhibition studies, one ml of the enzyme stock solution was added to a solution of the chelate preset to the pH of the experiment. The adjustment of pH before the addition of the enzyme was essential since the composition of the chelate mixture is pH dependent. If, for example, the initial pH of the chelate solution were low, excessive amounts of free cupric ion might be available for attachment to the enzyme surface.

A glass, jacketed vessel of approximately 20 ml volume was used to contain the reaction mixture and the temperature of the system was controlled with a Labline circulating water bath[1]. The reaction mixture was constantly stirred using a Magnestir. A plexiglass cover, fitted to the top of the reaction vessel, contained openings for the Beckman combination electrode, the nitrogen gas inlet, and the plastic extension from the titrator burette. The final volume of the reaction mixtures was adjusted to nine ml with diluent.

The enzyme and chelate were allowed to remain in contact for fifteen minutes at the preset pH of the experiment (except during time dependence studies) before the substrate (acetylcholine) was added. The final concentration of acetylcholine was 1.22×10^{-3}M for all experiments except those concerned with the determination of the "competitiveness" of the inhibition (see Results).

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[1]Labline Instruments Inc., Chicago, Illinois.

The values of Vo/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 velocity of the hydrolysis reaction corresponded to the time required to use 0.30 ml of base, provided the relationship between base used and time remained linear. In certain cases, where the velocity was very slow, it was necessary to extrapolate the line to 0.30 ml of base. The value of Vo was checked at the beginning and end of each series of experiments to ensure that enzyme activity remained constant.

Derivations and Calculations

The dissociation constants for all three ligands, with the exception of the dissociation of the ethanolic hydrogen in AOH, were obtained directly from the titration data. (The special situation in the case of the OH group in AOH will be discussed in detail in the Results.)

By titrating solutions containing various ligand-metal ratios with standard base, it was possible to calculate the chelate stability constants for all three ligands according to the method described by Bjerrum (29). A brief description of this method for a diprotic ligand-metal interaction, with successive formation constants corresponding to 1-1 and 2-1 chelate species, is as follows (disregarding the charge of the species). The acid dissociation constants and the chelate

stability constants are defined as follows:

$$H_2L \rightleftharpoons HL + H \quad , \quad ka_1 = \frac{(HL)(H)}{(H_2L)} \tag{1}$$

$$HL \rightleftharpoons L + H \quad , \quad ka_2 = \frac{(H)(L)}{(HL)} \tag{2}$$

$$M + L \rightleftharpoons ML \quad , \quad K_1 = \frac{(ML)}{(M)(L)} \tag{3}$$

$$ML + L \rightleftharpoons ML_2 \quad , \quad K_2 = \frac{(ML_2)}{(ML)(L)} \tag{4}$$

where H_2L represents undissociated ligand and M represents metal ion.

Also,

$$L_T = (L)+(HL)+(H_2L)+(ML)+2(ML_2) \tag{5}$$

and $$M_T = (M)+(ML)+(ML_2) \tag{6}$$

where L_T represents total ligand in the system and

M_T represents total metal in the system.

Let a represent moles of OH added/mole of ligand. Then $a(L_T)$ will equal the number of moles of base added and $(2-a)(L_T)$ will equal the number of moles of titratable H^+ remaining in solution.

Thus, $$(2-a)(L_T)=(H^+)+(HL)+2(H_2L)-OH \tag{7}$$

Substituting Equations 1 and 2 into Equation 7, yields:

$$(2-a)(L_T)=(H^+)-(OH)+\frac{(H)(L)}{ka_2}+\frac{2(H)^2(L)}{ka_1ka_2} \tag{8}$$

Solving Equation 8 for (L) yields:

$$(L) = \frac{(2-a)(L_T)-(H)+(OH)}{\frac{2(H)^2}{ka_1ka_2} + \frac{(H)}{ka_2}} \tag{9}$$

The use of Equation 9 allows the calculation of free ligand species present in solution (at a known pH after a known addition of base) during the titration.

Now, let $\bar{n}$ represent the average number of ligand molecules bound per mole of metal.

Then,

$$\bar{n} = \frac{ML+2(ML_2)}{M_T} \tag{10}$$

Rearranging Equation 5 yields:

$$(ML)+2(ML_2)=L_T-(L)+(HL)+(H_2L) \tag{11}$$

Substituting Equations 1 and 2 into Equation 11 and dividing both sides by M_T yields:

$$\frac{(ML)+2(ML_2)}{M_T} = \frac{L_T-\left[1 + \frac{(H)}{ka_2} + \frac{(H)^2}{ka_1ka_2}\right](L)}{M_T} \tag{12}$$

The left side of Equation 12 is equal to $\bar{n}$.

Therefore,

$$\bar{n} = \frac{L_T-\left[1 + \frac{(H)}{ka_2} + \frac{(H)^2}{ka_1ka_2}\right](L)}{M_T} \tag{13}$$

It is possible to obtain the values of the consecutive formation constants by plotting $\bar{n}$ versus -log (L). K_1 is approximately equal to the -log (L) value at $\bar{n} = 0.5$ and K_2 approximately equal to the -log (L) value at $\bar{n} = 1.5$. It should be noted that this method is only valid if the numerical difference between K_1 and K_2 is large. In the case of a small difference, an iteration procedure is required (see Appendix A).

The problems involved in a study of this type are formidable since the chelation solutions contain an equilibrium mixture of several species, i.e., free ligand, free metal, 1-1 and 2-1 chelate species. It was possible to measure the inhibitory effects caused by free ligand and free cupric ion by using the pure components. However, it was not possible to prepare chelate solutions containing either of the two chelate species in the absence of the other (except in the case of AOH where only a 1-1 species formed). It was necessary, therefore, to design an experiment which would identify the species present in the equilibrium chelate mixtures which was responsible for inhibition. The first step in this approach was to calculate the concentration of individual species present in the equilibrium mixture of ligand and metal under fixed conditions of concentration and pH. The following is a description of these calculations.

In the AOH system, where only a 1-1 chelate species formed, the following expression was derived. The symbols have the same

meaning as those described in the explanation of Bjerrum's $\bar{n}$ function.

$$M + L \rightleftharpoons ML \quad , \quad K_1 = \frac{(ML)}{(M)(L)} \tag{1}$$

$$H_3L \rightleftharpoons H_2L + H \quad , \quad ka_1 = \frac{(H_2L)(H)}{(H_3L)} \tag{2}$$

$$H\,L \rightleftharpoons HL + H \quad , \quad ka_2 = \frac{(HL)(H)}{(H_2L)} \tag{3}$$

$$HL \rightleftharpoons L + H \quad , \quad ka_3 = \frac{(L)(H)}{(HL)} \tag{4}$$

$$L_T = H_3L + H_2L + HL + L + ML \tag{5}$$

Combining Equation 5 with Equations 1 to 4 yields:

$$L_T = (L)\left[1 + \frac{H}{ka_3} + \frac{H^2}{ka_2ka_3} + \frac{H^3}{ka_1ka_2ka_3}\right] + K_1(M)(L) \tag{6}$$

And,

$$M_T = M + ML \tag{7}$$

Setting $\left[1 + \frac{H}{ka_3} + \frac{H^2}{ka_2ka_3} + \frac{H^3}{ka_1ka_2ka_3}\right]$ equal to α

And combining Equation 6 with Equations 7 and 1 yields:

$$L_T = L\alpha + \frac{K_1LM_T}{1+K_1L} \tag{8}$$

Rearranging terms to obtain the quadratic form yields:

$$L^2+L\left[\frac{K_1M_T-K_1L_T+\alpha}{\alpha K_1}\right]-\frac{L_T}{K_1\alpha}=0 \qquad (9)$$

With a knowledge of (M_T), (L_T), K_1, ka_1, ka_2, ka_3, and (H), Equation 6 and 9 could be solved for (L) and (M) with the aid of a computer[1]. The concentration of (ML) could then be obtained from Equation 7 and K_1 solved from Equation 1.

In the AH and HP systems, the calculations were further complicated because of the presence of a 2-1 chelate. The following expression was derived following the original method outlined by Bolton (34).

$$M + L \rightleftharpoons ML \quad , \quad K_1 = \frac{(ML)}{(M)(L)} \qquad (1)$$

$$ML + L \rightleftharpoons ML_2 \quad , \quad K_2 = \frac{(ML_2)}{(ML)(L)} \qquad (2)$$

$$H_2L \rightleftharpoons HL + H \quad , \quad ka_1 = \frac{(HL)(H)}{(H_2L)} \qquad (3)$$

$$HL \rightleftharpoons H + L \quad , \quad ka_2 = \frac{(H)(L)}{(HL)} \qquad (4)$$

$$L_T = H_2L + HL + L + ML + 2ML_2 \qquad (5)$$

---

[1] IBM 1410; Fortran IV Language.

Combining Equation 5 with Equations 1 to 4 yields:

$$L_T = (L)\left[1 + \frac{H}{ka_2} + \frac{H^2}{ka_1 ka_2}\right] + K_1(M)(L) + 2K_1K_2(M)(L)^2 \qquad (6)$$

And,

$$M_T = M + ML + ML_2 \qquad (7)$$

Setting $\left[1 + \frac{H}{ka_2} + \frac{H^2}{ka_1 ka_2}\right]$ equal to α

And combining Equation 6 with Equations 1, 2, and 7 yields:

$$L_T = L\alpha + \frac{K_1 L M_T}{1 + K_1 L + K_1 K_2 L^2} + \frac{2K_1 K_2 L^2 M_T}{1 + K_1 L + K_1 K_2 L^2} \qquad (8)$$

Rearranging to obtain the cubic form yields:

$$L^3 + L^2\left[\frac{\alpha + 2K_2 M_T - K_2 L_T}{K_2 \alpha}\right] + L\left[\frac{\alpha + K_1 M_T - K_1 L_T}{K_1 K_2 \alpha}\right] - \frac{L_T}{K_1 K_2 \alpha} = 0 \qquad (9)$$

With a knowledge of (M_T), (L_T), K_1, K_2, ka_1, ka_2, and (H), Equations 6 and 9 could be solved for (L) and (M) by a method of approximations. The computer programs used for the solution of these expressions are shown in the Appendix, (see Appendix B and C).

III. RESULTS AND DISCUSSION

Free Cupric Ion Determinations

In the polarographic studies, a calibration curve of diffusion current (i_D) versus cupric ion was prepared (Figure 1). It was anticipated that very small concentrations of Cu^{++} could be detected by extrapolating the calibration curve to very low Cu^{++} values. Unfortunately, the total concentration of Cu^{++} involved in the enzyme-Cu^{++} interaction was too small for accurate measurement by this method. Repeated efforts to detect these small Cu^{++} concentrations were unsuccessful.

The colorimetric method for cupric ion determinations (see Experimental) also presented problems. It was found that although a suitable calibration curve could be made by plotting absorption versus concentration at relatively high concentrations of metal (Figure 2), attempts to analytically reproduce the minute quantities of Cu^{++} apparently associated with enzyme binding were unsuccessful. Several modifications of the literature procedure were tried including dialysis of large volumes and subsequent evaporation techniques to increase the per cent concentration of Cu^{++}. The major difficulty encountered was color fading. The colored complex formed between the complexing agent and Cu^{++} was found to be relatively unstable. Attempts were made to carry out the complexation directly in the spectrophotometer cuvette, but the results obtained using this technique proved to be unreproducible.

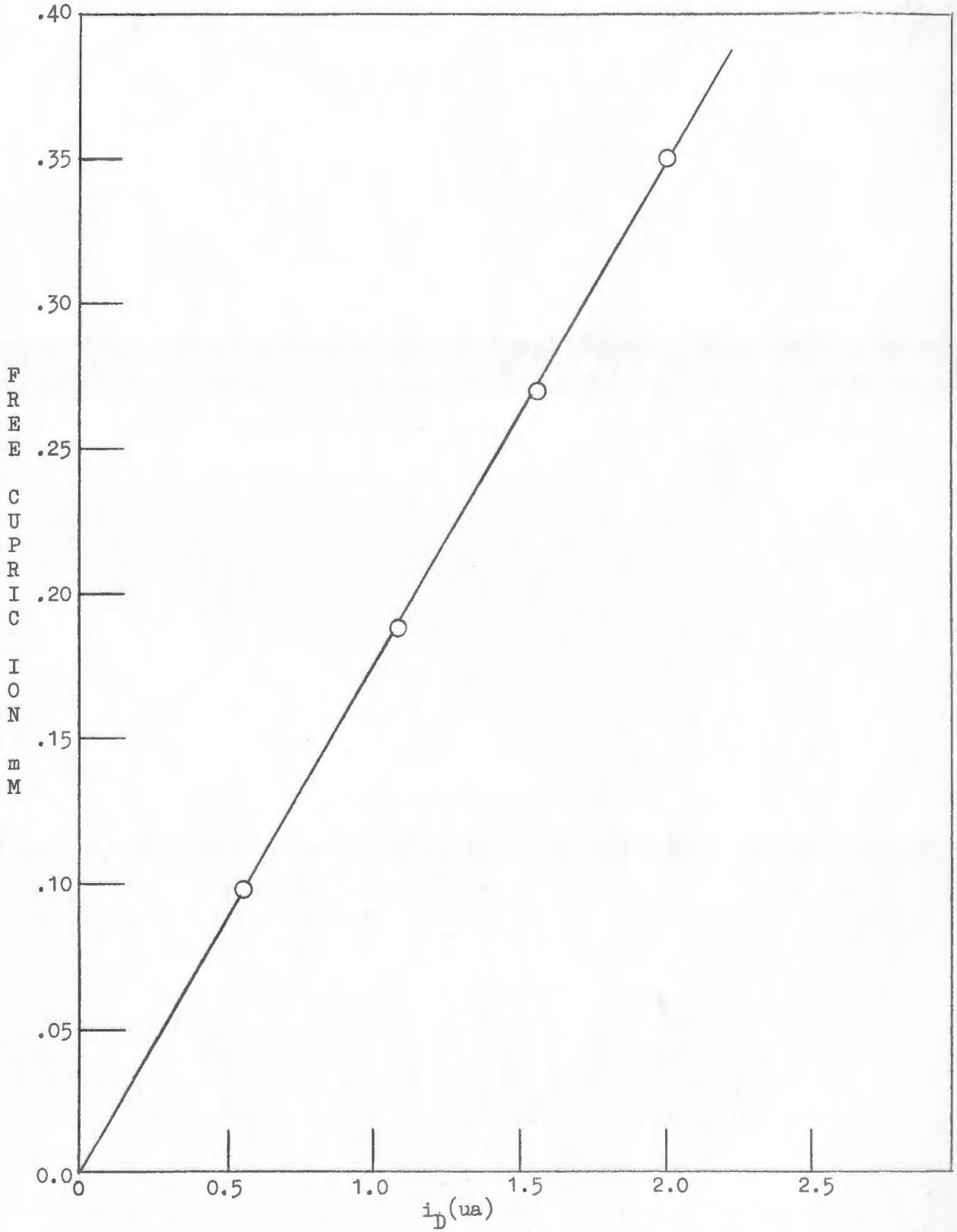


Figure 1. Calibration curve for free cupric ion versus diffusion current (i_D)

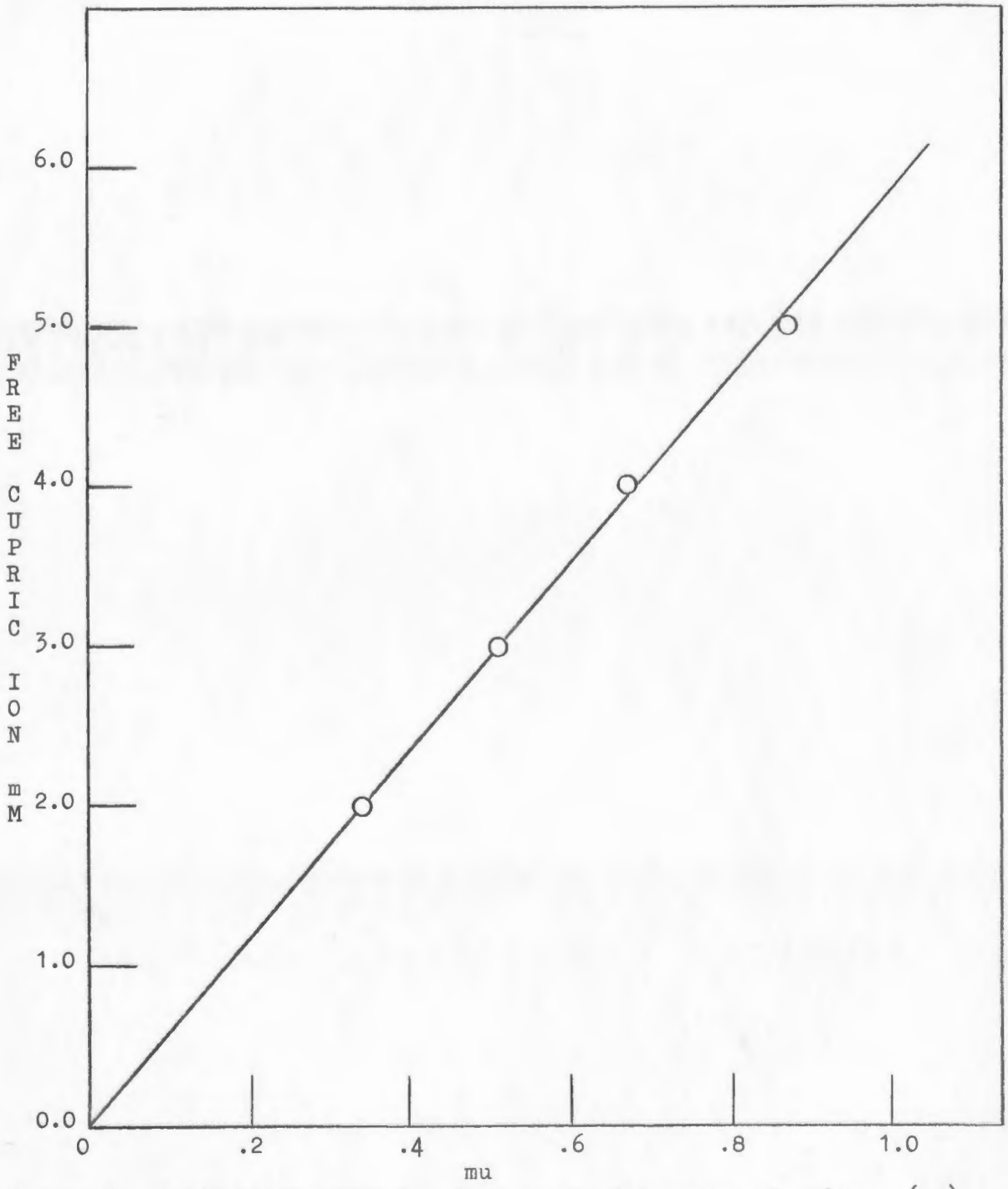


Figure 2. Calibration curve for free cupric ion versus absorbance (mu).

Although the results of these studies were inconclusive, certain observations of interest were noted. It appeared that the amount of cupric ion bound to the enzyme or capable of binding was extremely small. Although the actual quantities of metal ion involved were not determined, they were found to be less than 1×10^{-6}M. Further, when relatively large amounts of cupric ion were added to the enzyme and the mixture dialysed, analyses for Cu^{++} were not reproducible. This lack of reproducibility could have been due to metal adsorbance on the dialysing membrane, but attempts to determine the actual concentration of Cu^{++} capable of adsorbing on the membrane were unsuccessful.

Ligand Dissociation Constants

The dissociation constants for all three ligands determined by potentiometric titrations with standard base are shown in Table I. Literature values are indicated when available. Differences between the literature values and experimental values may be attributed to differences in ionic strength and the temperature at which the determinations were made.

With the exception of AOH (see below), the acid dissociations were determined from the pH at the mid-points in the titration curves for each ligand.

Stability Constants

A summary of the chelate stability constants for all three ligands appears in Table II. Literature values are in-

TABLE I

LIGAND DISSOCIATION CONSTANTS

Ligand	pKa_1	pKa_2	pKa_3	Ionic Strength	Temp. °C	Reference
AOH	8.01	9.58	14.20	0.162	30	....
AH	8.50	10.24	....	0.162	30	....
HP	2.20	9.36	....	0.162	30	....
			Literature Values			
AOH	8.23	9.68	....	0.100	20	39
AH	8.88	10.64	....	0.100	20	46
HP	1.92	9.73	....	0.100	20	47

TABLE II

STABILITY CONSTANTS FOR LIGAND INTERACTIONS WITH Cu^{++}

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	...
HP	8.88	7.21	0.162	30	...
		Literature Values			
AOH	....	....	....	...	...
AH	9.62	7.00	1.000	30	53
HP	....	....	....	...	...

dicated when available. In general, the formation constants for all three ligand-metal interactions were determined by titrating several ligand-metal ratios with standard base. Because each ligand exhibited different chelating properties, their interactions with Cu^{++} will be discussed separately.

AOH. Titrations of Cu^{++}-AOH$(HCl)_2$ mixtures as well as free amine di-HCl are shown in Figure 3. AOH-Cu^{++} ratios of 1-1 and greater, all yielded exactly three equivalents of H^+ per metal ion (Figure 4). This could only be explained on the basis of formation of a 1-1 terdentate chelate with simultaneous release of the ethanolic proton. That a 2-1 chelate did not form was evident from a comparison of curves A, B and C in Figure 3; curve C was found to be a summation of curves A and B. Since a 2-1 chelate did not form, the possibility that the third proton came from Cu^{++}-bound water was excluded.

The system was also analysed according to the method of Job (48), utilizing the peak at 623 mu exhibited by the Cu^{++} chelate. Unfortunately, mixtures containing a mole fraction of amine less than 0.5 precipitated at the pH of the experiment. Therefore, no maximum could be observed in a 1-1 ratio system, but solutions containing larger ratios of amine (at constant total concentration) showed decreasing absorption which is corroborative evidence of only 1-1 chelate formation.

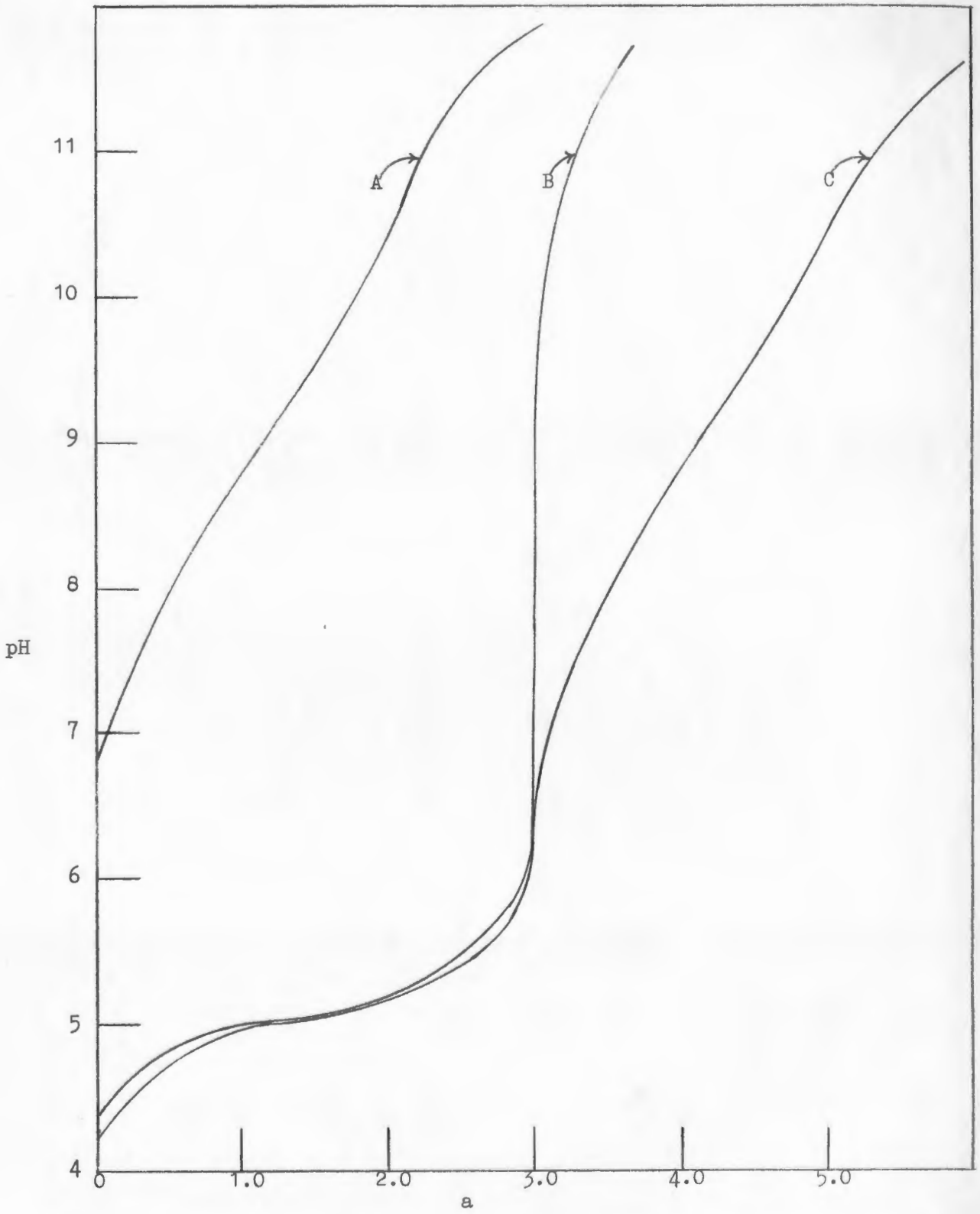


Figure 3. Titration of 1,3 diaminopropanol-2(diHCl) in the absence of, and in the presence of, Cu^{++}. A, titration of 1 x 10^{-2}M AOH with NaOH; B, equimolar amounts of Cu^{++} and AOH; C, 100 per cent excess of AOH; a denotes moles of base added per mole of AOH.

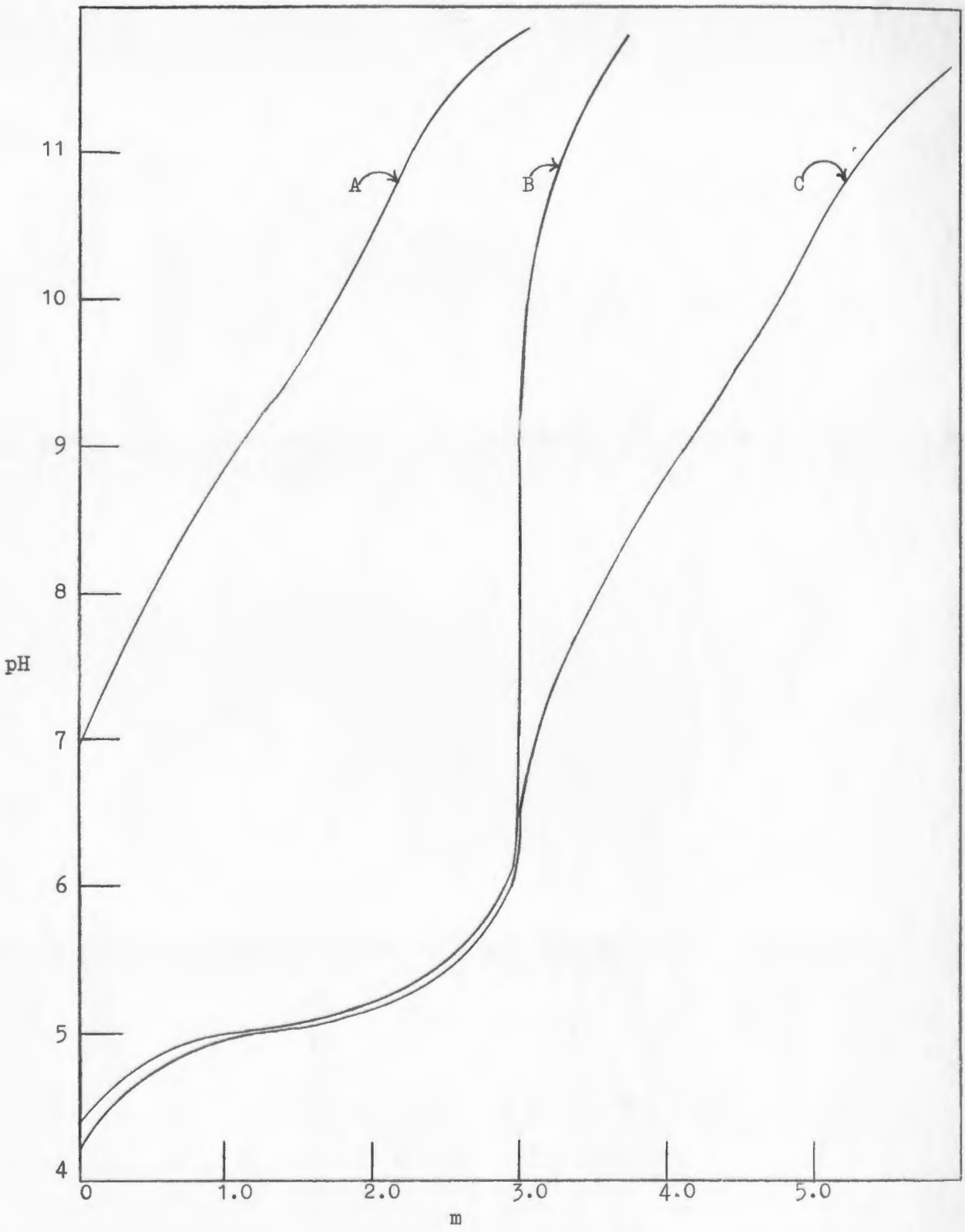


Figure 4. Titration of 1,3 diaminopropanol-2(diHCl) in the absence of, and in the presence of, Cu^{++}. A, titration of 1×10^{-2}M AOH with NaOH; B, equimolar amounts of Cu^{++} and AOH; C, 100 per cent excess of AOH; m denotes moles of base added per mole of metal.

Since the ethanolic hydrogen was released during the chelation of AOH and Cu^{++}, evaluation of the stability constant for this 1-1 species required the evaluation of the ka of this ethanolic group. It seemed reasonable to believe that this ka value could be determined spectrophotometrically. Ideally, two distinct peaks corresponding to the dissociated and undissociated species would appear.

Unfortunately, the necessity of using systems of very high pH introduced solvent effects which prevented clear-cut resolution of the peaks. Instead one peak appeared which continually shifted toward higher wavelengths with a corresponding lowering of absorption as the pH was increased. Then at pH 14.06 the absorption began to increase while the peak continued to shift as before. This increase in absorption was attributed to $(-O)^-$ formation and the pH of the solution when this was first observed was considered to be the pK_a of the ethanolic hydrogen.

Admittedly, the method was only an approximation and some corroborative evidence was needed. A theoretical calculation of the pK_a of the ethanolic hydrogen was made using the method of inductive effects outlined by Hine (49).

For a series of substituted acetic acids (50), γ, the measure of electron donation or acceptance, was +3.651. When this value was used in the expression derived for substituted alcohols and corrections were made for carbon chain lengths

and the presence of two positively charged nitrogens (51), the pK_a was calculated to be 14.35. The estimated pK_a value of 14.06 found in the spectrophotometric method appears, therefore, to be of the correct order of magnitude.

Our calculation of the stability constant for the $(CuAO)^+$ chelate (K_1) agreed well with the log K_s value of 3.57 ± 0.17 obtained by Bertsch, Fernelius, and Block (52,53), if the following relationships are considered:

$$Cu^{++} + AOH \rightleftharpoons CuAO^+ + H^+ \qquad (1)$$

Then,

$$K_s = \frac{(CuAO+)(H^+)}{(Cu^{++})(AOH)} \qquad (2)$$

but

$$K_1 = \frac{(CuAO^+)}{(Cu^{++})(AO^-)} \qquad (3)$$

And, because

$$ka_3 = \frac{(H^+)(AO^-)}{(AOH)} \qquad (4)$$

then,

$$K_1 = \frac{K_s}{ka_3} \qquad (5)$$

the value of K_1 is depencent on the ka. Using our estimated ka we found an average value of 2.5×10^{18} for K_1.

AH. Preliminary examination of the titration curves of several ligand-metal ratios of AH and M^{++} indicated the for-

mation of both a stable 1-1 and a stable 2-1 chelate (Figure 5).

As can be seen, in Figure 6, four protons were titrated per mole of metal corresponding to 2-1 chelate formation. The numerical values for the two successive stability constants (K_1 and K_2) were calculated using the $\bar{n}$ relationship derived earlier in this thesis. Table III contains the results of calculated free ligand species at various $\bar{n}$ values. Since the K_1 and K_2 constants exhibited a large numerical difference, the log values of free ligand species at $\bar{n} = 0.5$ and 1.5 were taken as the actual values of K_1 and K_2 respectively.

HP. Potentiometric titrations of the ligand salt and 2-1 HP-M^{++} mixtures with standard base are shown in Figure 7. Titration curves of ratios greater than 2-1, indicated no higher chelates and preliminary examination of these curves indicated the formation of a 1-1 chelate and a relatively stable 2-1 chelate. Further, the absence of a strong inflection corresponding to 1-1 formation appeared to indicate a close proximity between the stability constants for the 1-1 and 2-1 chelate species. Further evidence supporting 2-1 formation was noted when pH was plotted versus moles of base per mole of metal in Figure 8. The end-point occurred when two protons were titrated per mole of metal ion indicating the attachment of two ligand molecules per metal ion. Calculations of free ligand species at various $\bar{n}$ values is shown in Table IV and preliminary eval-

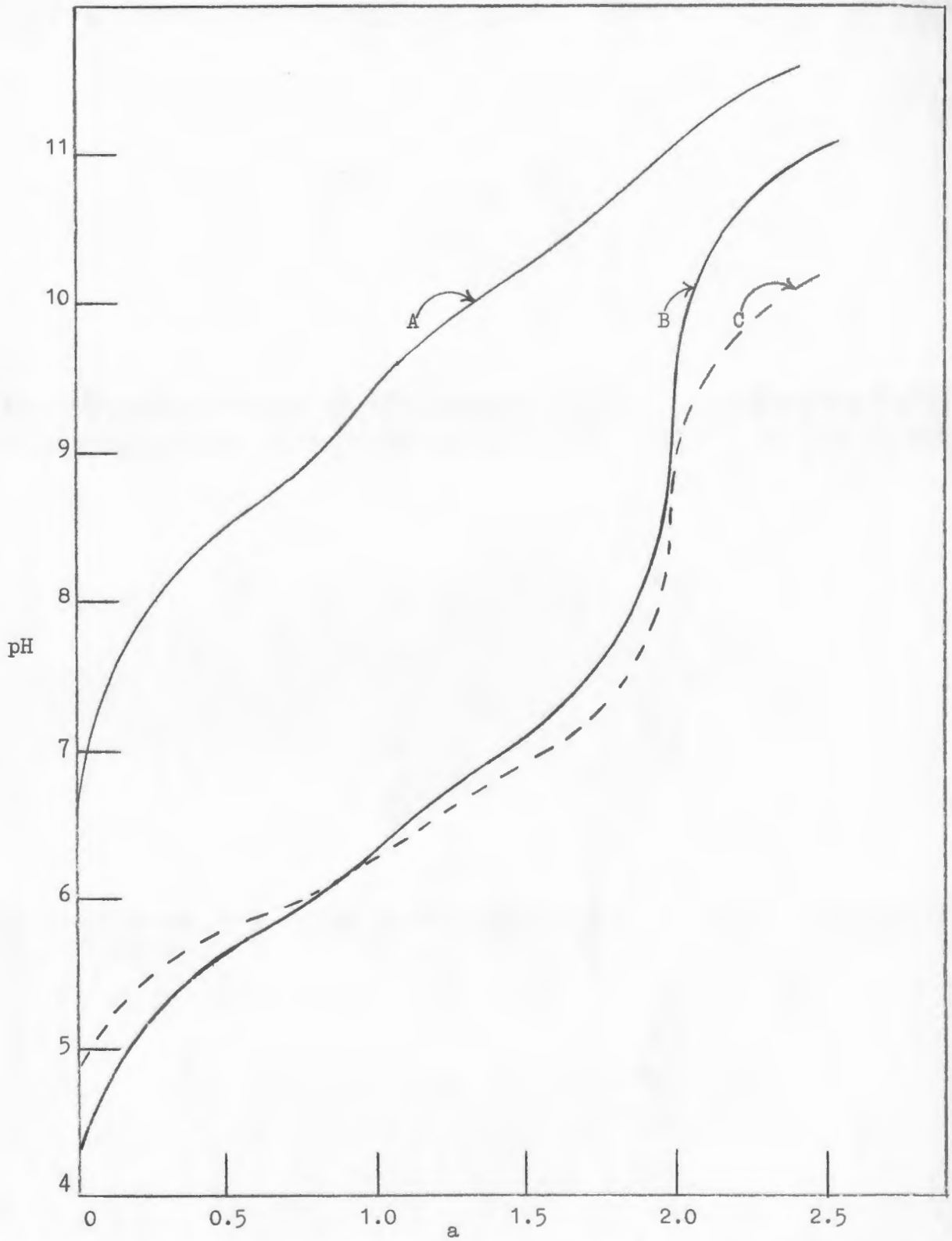


Figure 5. Titration of 1,3 diaminopropane(diHCl) in the absence of, and in the presence of, Cu^{++} ion. A, titration of 1 x 10^{-2}M AH with NaOH; B, ligand-metal ratio of 2-1; C, ligand-metal ratio of 3-1; a denotes moles of base added per mole of AH.

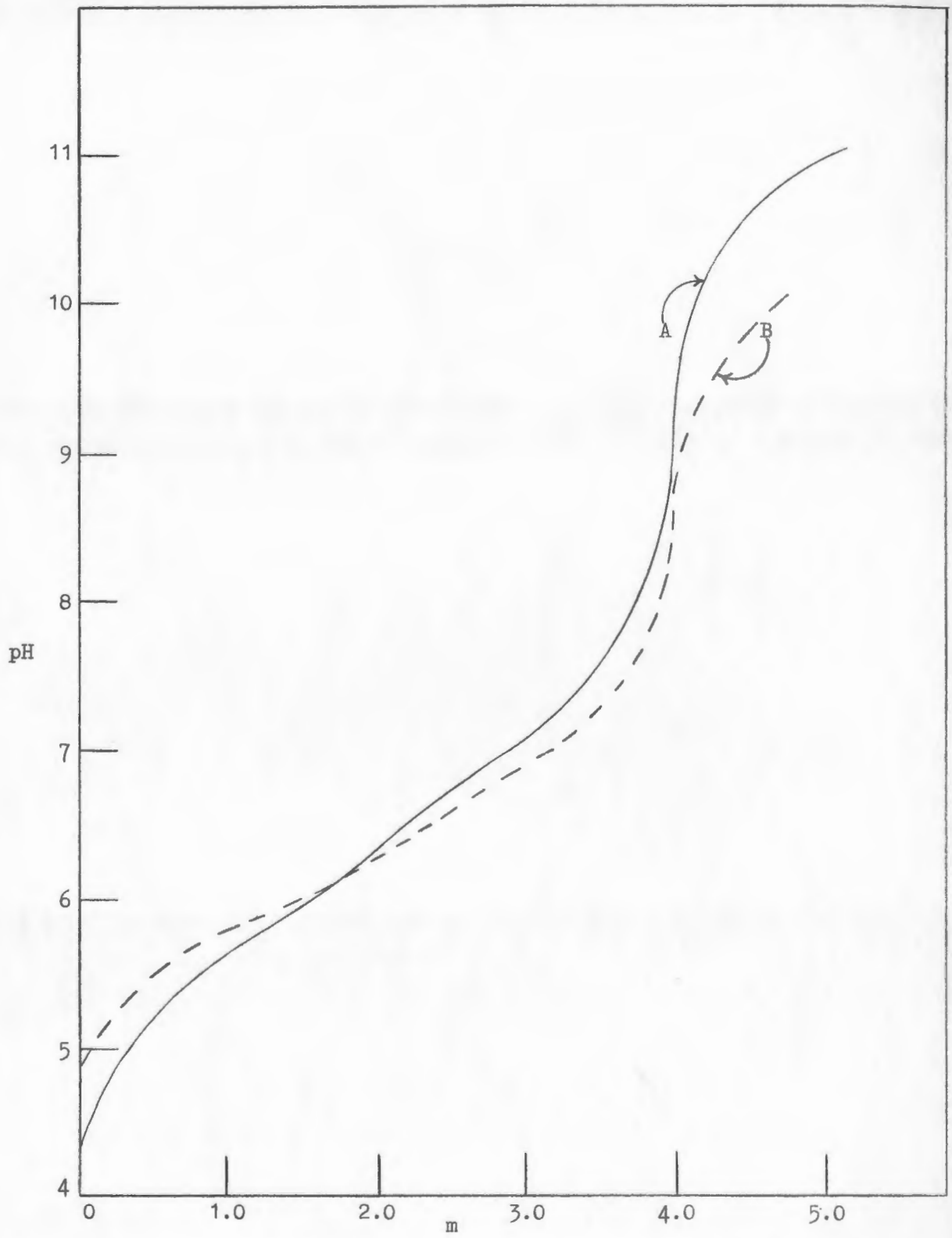


Figure 6. Titration of 1,3 diaminopropane(diHCl) in the presence of Cu^{++}ion. A, ligand-metal ratio of 2-1; B, ligand-metal ratio of 3-1; m denotes moles of base added per mole of metal.

TABLE III

CALCULATED FREE LIGAND SPECIES AT VARIOUS $\bar{n}$ VALUES FOR THE AH-Cu^{++} INTERACTION

pH	(L^-)	$\bar{n}$	-log (L)
5.39	1.85×10^{-10}	0.350	9.73
5.55	3.70×10^{-10}	0.402	9.43
5.66	5.18×10^{-10}	0.500	9.23
5.81	9.17×10^{-10}	0.650	9.04
6.75	3.62×10^{-8}	1.250	7.44
6.88	5.54×10^{-8}	1.360	7.26
7.08	1.08×10^{-7}	1.472	6.97
7.33	2.28×10^{-7}	1.640	6.62

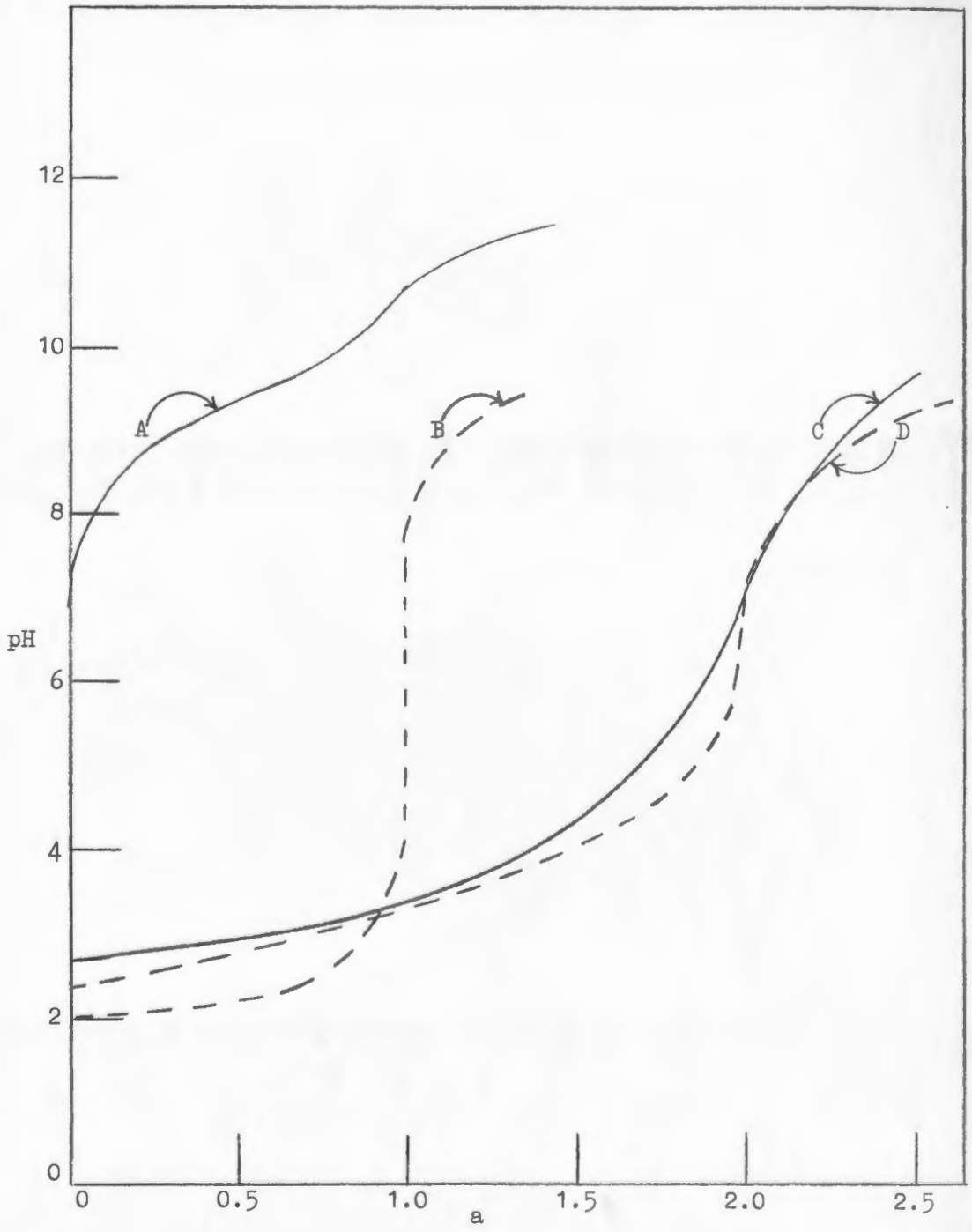


Figure 7. Titration of hydroxy-L-proline(HCl) in the absence of, and in the presence of Cu^{++} ion. A, titration of 1×10^{-2}M free amine with NaOH; B, titration of 1×10^{-2}M mono-HCl salt with NaOH; C, ligand-metal ratio of 2-1; D, ligand-metal ratio 3-1; a denotes moles of base added per mole of HP.

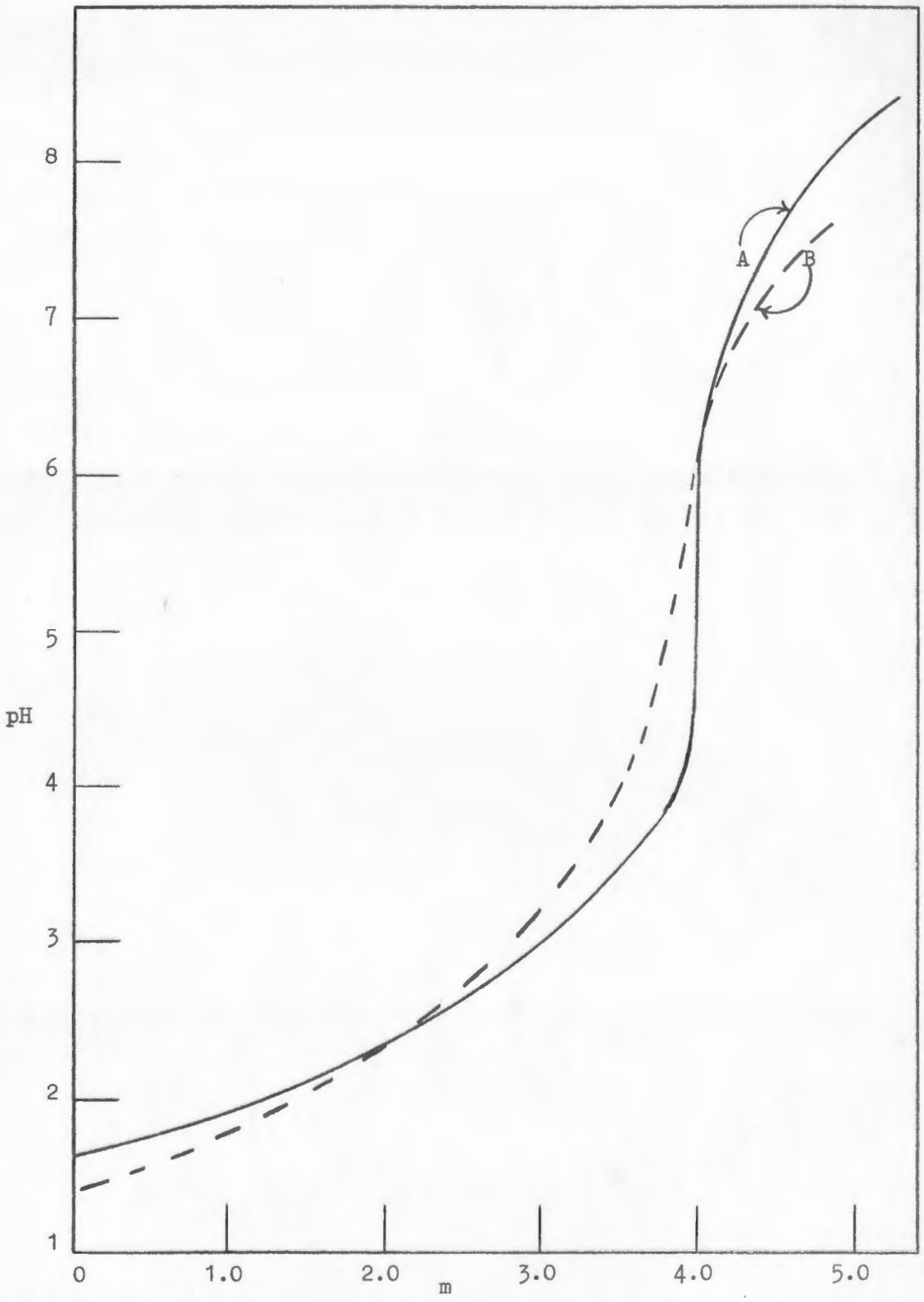


Figure 8. Titration of hydroxy-L-proline(HCl) in the presence of Cu^{++} ion. A, ligand-metal ratio of 2-1; B, ligand-metal ratio of 3-1; m denotes moles of base added per mole of metal.

TABLE IV

CALCULATED FREE LIGAND SPECIES AT VARIOUS $\bar{n}$ VALUES FOR THE HP-Cu^{++} INTERACTION

pH	(L^-)	$\bar{n}$	-log(L)
2.04	6.67×10^{-10}	0.303	9.18
2.12	8.31×10^{-10}	0.383	9.08
2.21	1.07×10^{-9}	0.461	8.97
2.31	1.39×10^{-9}	0.539	8.86
3.36	1.51×10^{-8}	1.061	7.82
3.88	3.72×10^{-8}	1.314	7.43
4.28	6.75×10^{-8}	1.504	7.17
4.52	9.37×10^{-8}	1.602	7.03

uation of K_1 and K_2 at $\bar{n}$ = 0.5 and 1.5, respectively, indicated a small numerical difference between the two successive formation constants. In order to obtain more accurate values for K_1 and K_2, it was necessary to use the iteration procedure referred to in the introduction of this thesis. A brief description of this procedure, using actual values necessary for the evaluation of K_1 and K_2 for the interaction of HP-Cu^{++}, is shown in the Appendix. Figure 9 shows the results of plotting $\bar{n}$ versus the negative log of free ligand concentration (pL) for the AH and HP systems.

Inhibition Studies

As was mentioned earlier, the chelate solutions contained an equilibrium mixture of several species. The questions to be resolved were which species acted as inhibitors and how did they exert their inhibition. In the case of the AOH ligand, free ligand, free metal and/or 1-1 chelate could be responsible for inhibition. With the AH and HP compounds, the additional possibility of inhibition by the 2-1 chelate species was also considered.

Inhibition by Free Cupric Ion. The influence of free cupric ion on the hydrolysis of acetylcholine by acetylcholinesterase, has been reported by Bolton (34). The results of this study indicated that concentrations of free cupric ion below 2 x 10^{-5}M did not exert a significant inhibitory action

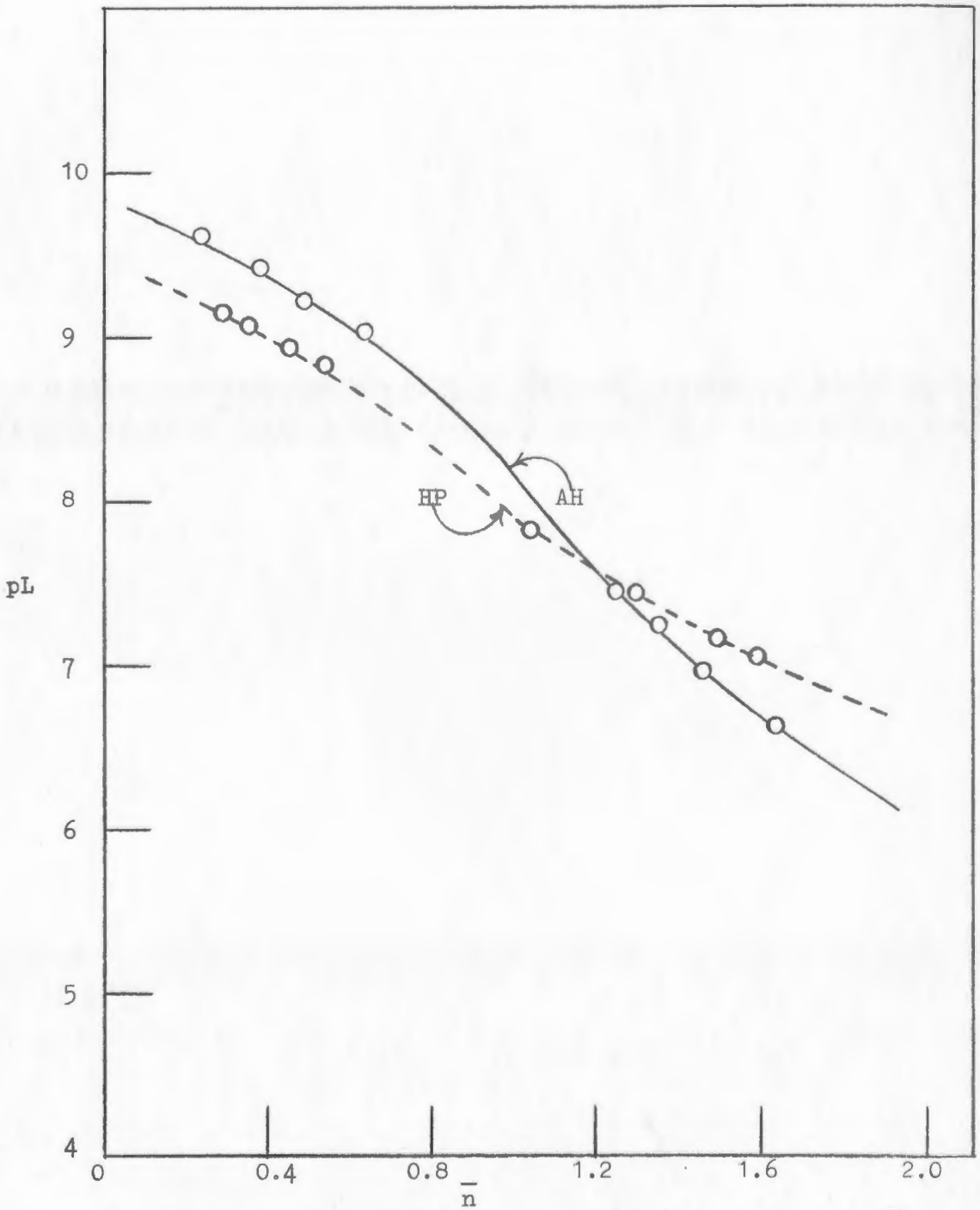


Figure 9. Formation functions of copper chelates of AH and HP; $\bar{n}$ = average number of ligands bound per mole of metal ion; pL = negative logarithm of L.

on the acetylcholinesterase system, although this value was somewhat dependent on the concentration of enzyme. Further, it was found that inhibition by free cupric ion was usually accompanied by a time dependence. When the concentration of free cupric ion was relatively large, the measured velocity of the inhibited enzyme-substrate reaction changed as a function of time, whereas, when the concentration of free cupric ion was relatively small, the observed velocity remained constant for periods of time up to one hour. Because of this possible interference from cupric ion, all chelate solutions used contained less than 1×10^{-6} M free cupric ion at the experimental pH values used in this thesis. The concentration of cupric ion was controlled by a careful choice of the pH, the concentration of chelate solution, and the ratio of ligand to metal. The individual effects of these three experimental conditions will be discussed separately for each ligand. As a further check for possible Cu^{++} interference, time dependence studies were performed initially on all systems. Whenever cupric ion interference was suspected, the data could not be analysed and has been omitted.

Inhibition by Free Ligand. The contribution of free ligand to the inhibition of the acetylcholinesterase system was examined by Bolton (34,36). Results showed that within the concentrations used in these experiments, free ligand exhibited no inhibitory

effects. Studies run with AOH, AH, and HP also showed that these ligand molecules exhibited no inhibitory properties even when concentrations of total ligand used were far in excess of the concentrations normally encountered in our inhibition studies.

Inhibition by the AOH System. The results of enzyme inhibition by a 2.1-1 ligand-metal ratio solution of AOH-Cu^{++} at several pH values, are shown in Table V. The choice of ligand-metal ratio was not critical with this system because the formation constant resulting from the terdentate attachment was very large. Because of the very large 1-1 formation constant, the concentration of free cupric ion was very low over a pH range from 7.5 to 9.0. The kinetic data obtained for this system was found to be independent of time. Examination of Figure 4 shows a large pH change at the end-point of the titration, corresponding to 1-1 chelate formation. Analysis of the equilibrium chelate solution at pH values from 7.5 to 9.0 using the computer approximation method described in the Appendix, revealed no change in the concentration of 1-1 chelate species throughout the pH range used. Also, when the ligand-metal ratio was changed to 2.2-1 and the equilibrium chelate solution analysed, it was found that the concentration of cupric ion was lowered, but the concentration of 1-1 species did not change.

TABLE V

INHIBITION BY 1-1 AOH CHELATES IN A 2.1-1 LIGAND-METAL RATIO SOLUTION

pH	Molar Conc. Chelate x 10^{-3}	Molar Conc. 1-1 Chelate x 10^{-3}	Molar Conc. Free Cu^{++} x 10^{-10}	Vo/V
7.5	1	0.9	9.3	1.074
	2	1.9	9.3	1.091
	3	2.9	9.3	1.104
	4	3.9	9.3	1.111
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
9.0	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

Since the concentration of free cupric ion throughout the pH range used, apparently, was too low to cause inhibition and since the concentration of 1-1 chelate species did not change when the pH of the equilibrium mixture was changed, any variation of inhibition could reasonably be attributed to charge changes on the enzyme surface. It was anticipated that if inhibition changed as a function of pH, an optimum pH value would appear corresponding to the pK_a of the group on the enzyme surface involved in the binding.

With the 2.1-1 ligand-metal ratio solution, the inhibition of the enzyme system at all pH values except pH 7.5 followed a straight line relationship, passing through zero, when Vo/V was plotted against 1-1 chelate species concentration (Figure 10). At pH 7.5, the resultant straight line did not pass through the origin but yielded, instead, a positive y-intercept. This odd behavior at pH 7.5 could possibly be attributed to a different mechanism inhibition or the presence of significant amounts of cupric ion in equilibrium with the enzyme. (It should be noted, however, that no time dependence was observed). pH values above and below the range of 7.5 to 9.0 could not be used, since the system was too highly buffered at low pH values and the chelate dissociated at high pH values. As can be seen in Figure 10, the inhibition appears to decrease as the pH is increased above 7.5. In the pH range of 8.5 to 8.75, the inhibition increases, possibly due to the fact that the pK_a value

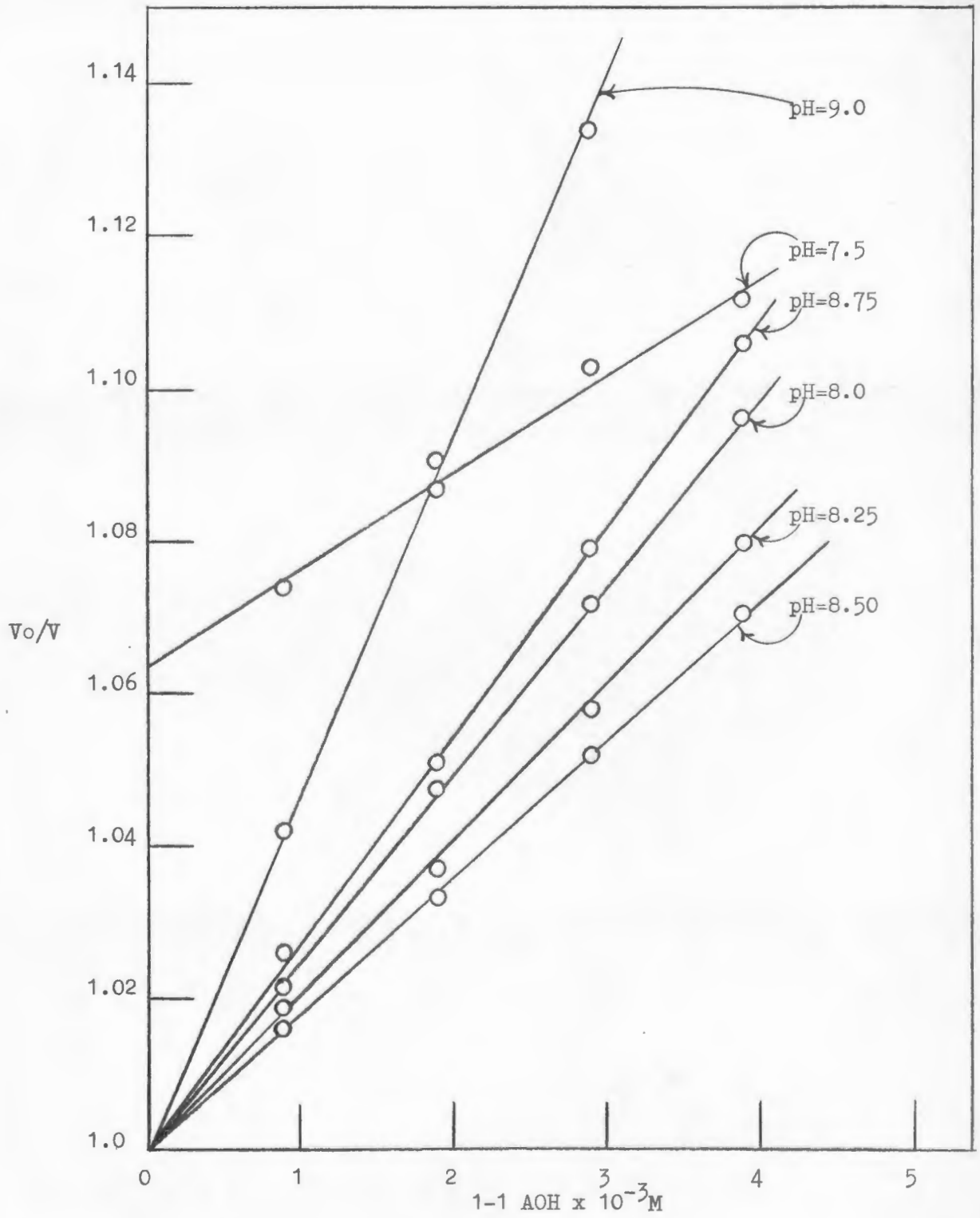


Figure 10. Comparison of inhibition by 1-1 AOH chelate at various pH values. The ratio of ligand-metal is 2.1-1.

of the ionizing group on the enzyme surface involved in inhibitor binding, had been reached.

In order to substantiate these results, a 2.2-1 ligand-metal ratio solution was used as the inhibitor. This solution has less free cupric ion but the concentration of 1-1 chelate species was the same as that present in the 2.1-1 mixture. It seemed reasonable to expect less inhibition with this ratio compared to the 2.1-1 ratio, if free cupric ion was contributing to the inhibition. The results of the inhibition studies at pH 8 and 8.5 are shown in Figure 11. The inhibition, expressed as Vo/V, was found to be the same, within experimental error, as the data obtained using the 2.1-1 ratio, under the assumption that the 1-1 species is the inhibitor. Thus, the possibility that free copper ion is contributing to the inhibition, seems unlikely.

In an attempt to elucidate the nature of the inhibition by the 1-1 chelate species, the 2.2-1 ratio solution (at pH 8.5) was used to observe the inhibition of the enzyme at various substrate and inhibitor concentrations. (The choice of both the ratio of ligand to metal and the pH were arbitrary in this system, since the concentration of 1-1 species did not change throughout the pH range under consideration, and free cupric ion concentration was negligible.) This procedure followed the general method of Michaelis and Menten (13) and the numerical results appear in Table VI. Graphical analysis of this data

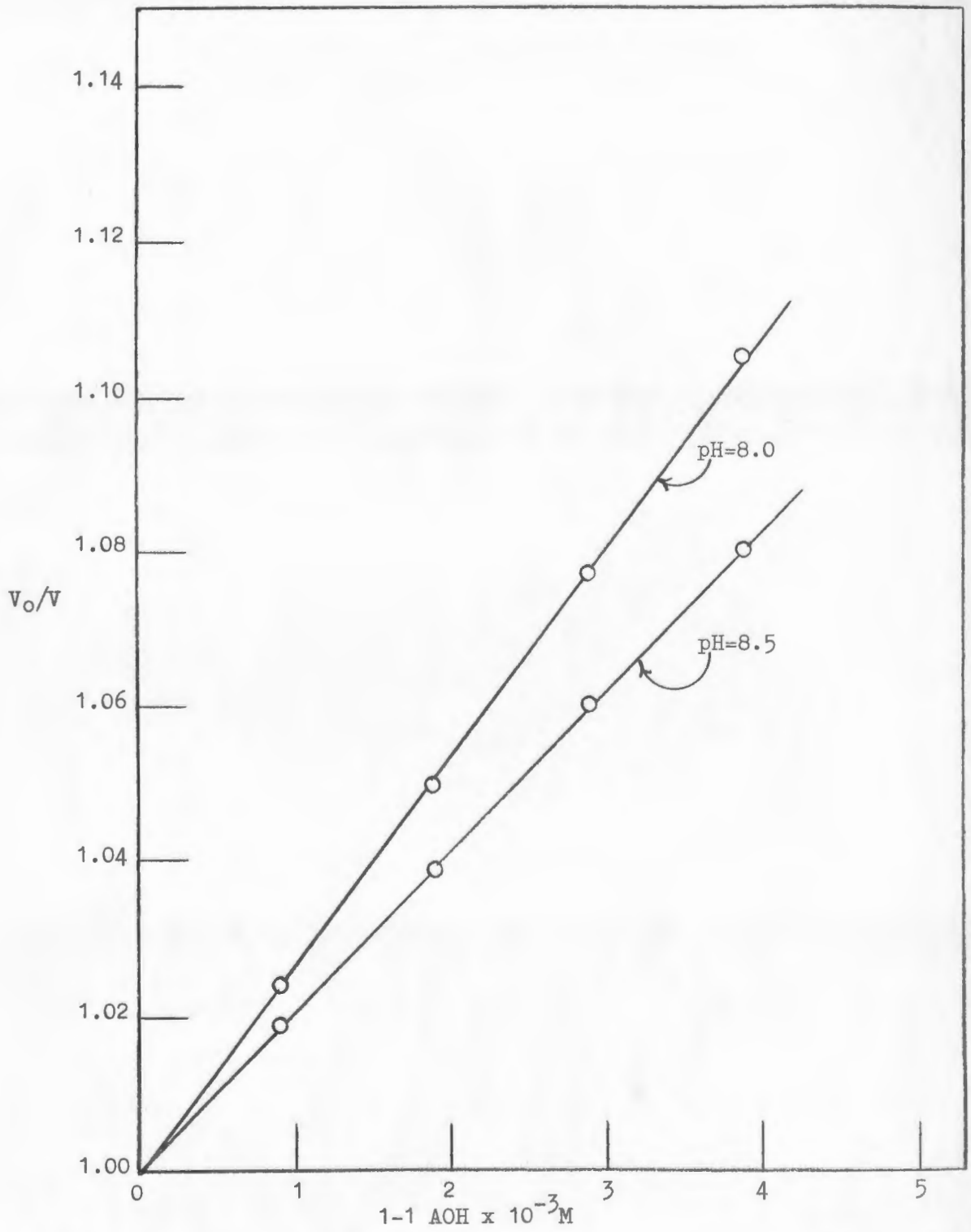


Figure 11. Comparison of inhibition by 1-1 AOH chelates at pH 8.0 and 8.5. The ratio of ligand-metal is 2.2-1.

TABLE VI

INHIBITION BY 1-1 AOH CHELATE IN A 2.2-1 LIGAND-METAL RATIO SOLUTION AT pH 8.5 WITH VARYING SUBSTRATE CONCENTRATIONS

Molar Conc. of Substrate x 10^{-3}	Molar Conc. of Chelate Mixture x 10^{-3}	Molar Conc. of 1-1 Species x 10^{-3}	Reaction Velocity (1/V)
1.22	0	0.0	49¼
	1	0.9	49 3/4
	2	1.9	51
	3	2.9	52
	4	3.9	53¼
0.99	0	0.0	50¼
	1	0.9	52
	2	1.9	53
	3	2.9	54¼
	4	3.9	57
0.73	0	0.0	53 3/4
	1	0.9	55¼
	2	1.9	57¼
	3	2.9	58¼
	4	3.9	62
0.49	0	0.0	58 3/4
	1	0.9	61¼
	2	1.9	63½
	3	2.9	66
	4	3.9	69

according to the general procedure outlined by Friedenwald, et al. (18) appears in Figures 12 and 13. In this method, values of K_I, K_S and α can be determined (see Appendix A). When α is equal to infinity, the inhibition is termed completely "competitive" and when α equals unity, the inhibition is termed completely "non-competitive". Intermediate values of α indicate a combination or mixture of both types of inhibition. The derivation and significance of the α value are shown in the Appendix.

A multiple regression approach was used to fit the data. This approach was used in all α determination studies and the mathematical equations used appear in Appendix A.

Analysis of Figures 12 and 13 indicates that the 1-1 chelate of AOH inhibits the enzyme in a "competitive" manner. Unfortunately, the overall inhibition by this 1-1 chelate species was relatively weak and, therefore, the experimental error was relatively large. Possible reasons for this weak inhibition are: (1) The 1-1 chelate species has only a single plus charge and (2) the terdentate attachment involved in the formation of the 1-1 chelate species leaves only one available coordination site on the metal. The lack of availability of coordination sites is probably an important factor, accounting for the weak inhibition properties; it had been previously reported (34,36) that 2-1 chelate species of Cu^{++}, where there are no coordination vacancies, did not appreciably inhibit the enzyme.

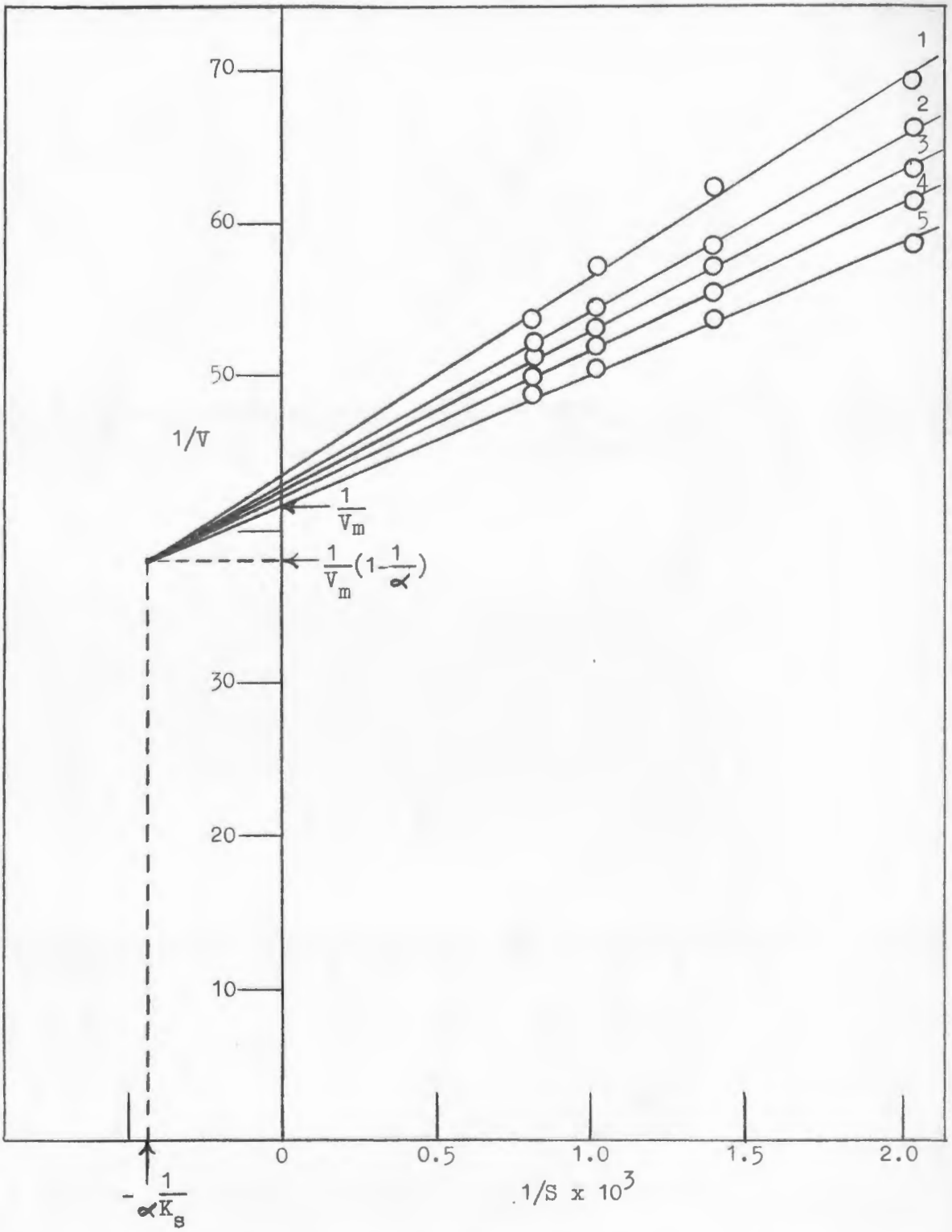


Figure 12. Analysis of inhibition by 1-1 AOH chelate at pH 8.5 using 1/S values. Key: 1, (I) = 3.9×10^{-3}; 2, (I) = 2.9×10^{-3}; 3, (I) = 1.9×10^{-3}; 4, (I) = 0.9×10^{-3}; 5, (I) = 0.

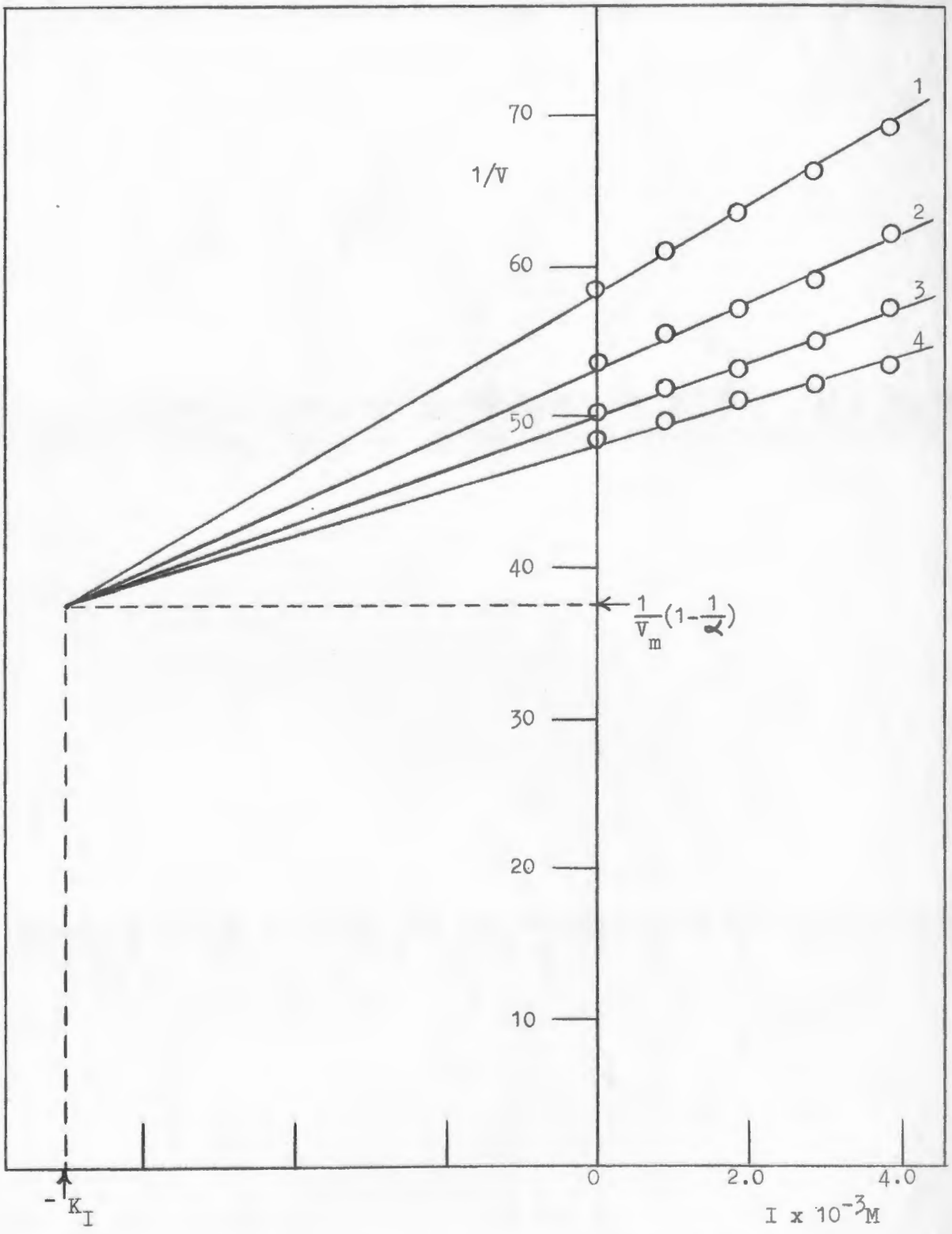


Figure 13. Analysis of inhibition by 1-1 AOH chelate at pH 8.5 using (I) values. Key: 1, (S) = 4.90×10^{-4}M; 2, (S) = 7.30×10^{-4}M; 3, (S) = 9.90×10^{-4}M; 4, (S) = 1.22×10^{-3}M.

Inhibition by the AH System. Analysis of the AH system was more complicated than the AOH system because of the presence of a stable 2-1 chelate species. Furthermore, the ligand-metal interaction of this chelate was much weaker than the AOH system (compare Figures 4 and 6), and, consequently, the selection of ligand-metal ratios and equilibrium pH values was restricted.

Initially, pH 8.0 was selected as the equilibrium pH for inhibition studies because a significant concentration of 1-1 species was present at this pH. It was found that ligand-metal ratios of 2.0-1 or less, could not be used at pH 8.0 because of the presence of relatively high concentrations of free cupric ion. In fact, at lower ratios, cupric hydroxide precipitation was observed when the solutions were allowed to stand for several days. When the ligand-metal ratio was raised to 2.1-1 at pH 8.0, it was found that no precipitation occurred and no time dependence was observed during inhibitory studies. For the above reasons, a 2.1-1 equilibrium chelate solution at pH 8.0 was chosen for the initial inhibition analysis of the AH system.

It was found that the concentration of 1-1 chelate species could be linearly correlated to inhibition, by plotting V_o/V versus 1-1 concentration at pH 8.0. Figure 14 shows the results of this analysis. The 2-1 species did not seem to contribute to the inhibition.

Because of the fact that the 1-1 chelate species seemed to be solely responsible for inhibition, under the above con-

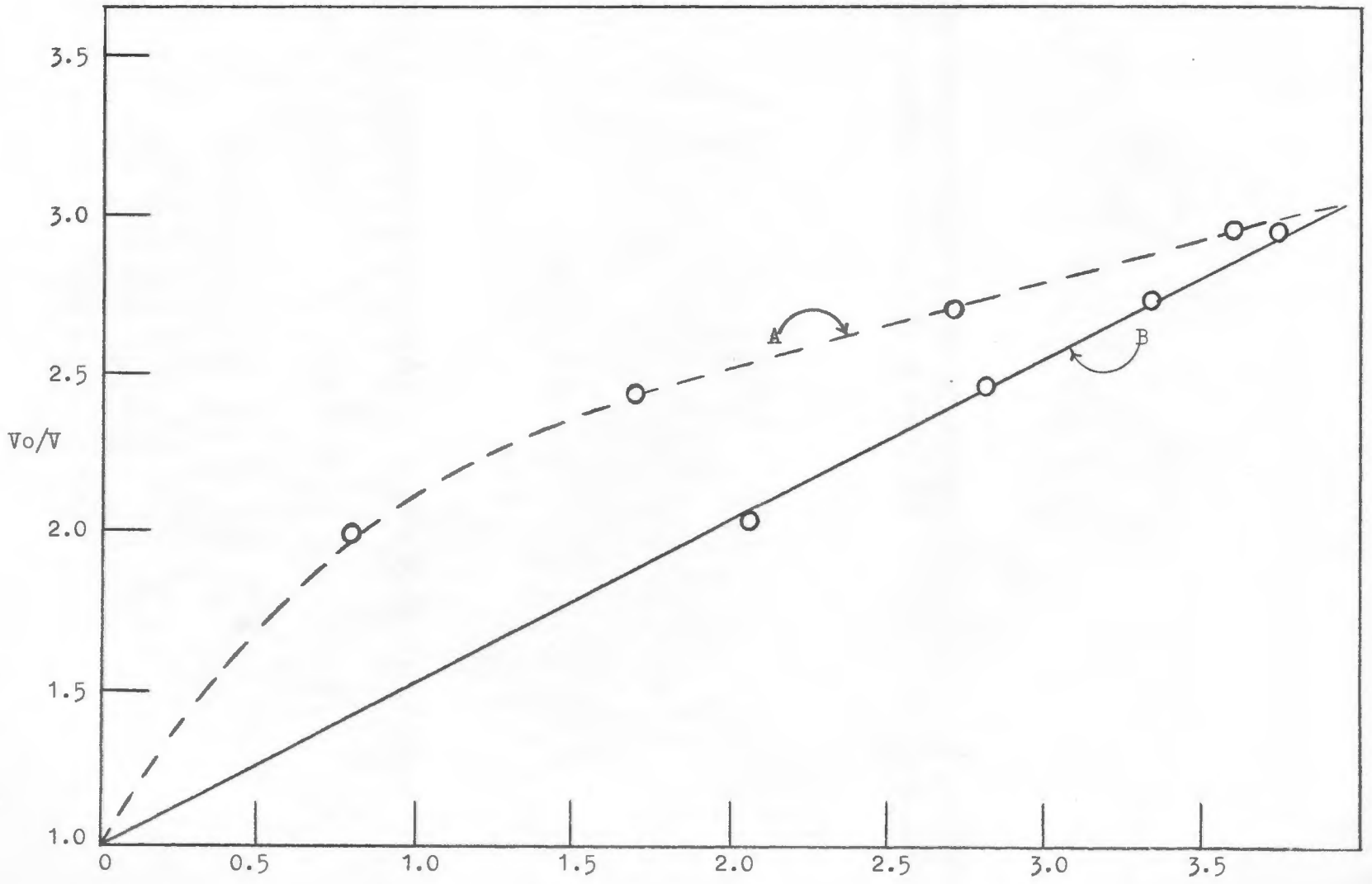


Figure 14. Inhibition by 1-1 and 2-1 AH chelates at pH 8.0. Abscissa taken as 10^{-3}M for 2-1 species and 10^{-4}M for 1-1 species. A, 2-1 species; B, 1-1 species. The ligand-metal ratio is 2.1-1.

ditions, an analysis to determine values of K_1 and α was attempted in a manner similar to that described for the AOH system. The graphical results of this study appear in Figures 15 and 16 and the concentrations of all species present in the equilibrium mixtures are shown in Table VII. Analyses of this data appeared to indicate a primarily "non-competitive" type of inhibition.

To further elucidate the nature of inhibition by the 1-1 AH chelate, the inhibition by solutions containing ligand-metal ratios of 2.2-1 and 2.3-1 was examined at pH 8.0. At these higher ratios, less free cupric ion and smaller amounts of 1-1 species were present but the concentration of 2-1 species increased. The results of these studies, including concentrations of species present in the equilibrium mixture, are shown in Table VIII. The correlation of inhibition with 1-1 chelate species was quite good, even if the very low concentration of 1-1 species is taken into account (Figure 17).

At pH values higher than 8.0, it was not possible to correlate either 1-1 or 2-1 chelate concentrations with inhibition (see Figure 18). At elevated pH values, the expected straight line relationship was not obtained when either the 1-1 or 2-1 chelate concentration was plotted against Vo/V. It was found, however, that plots of the ratio of 1-1/2-1 versus Vo/V yielded straight lines. Some possible explanations for this behavior are: (1) The double charge present in Cu^{++} chelates of AH changes either the site or mechanism (i.e., the

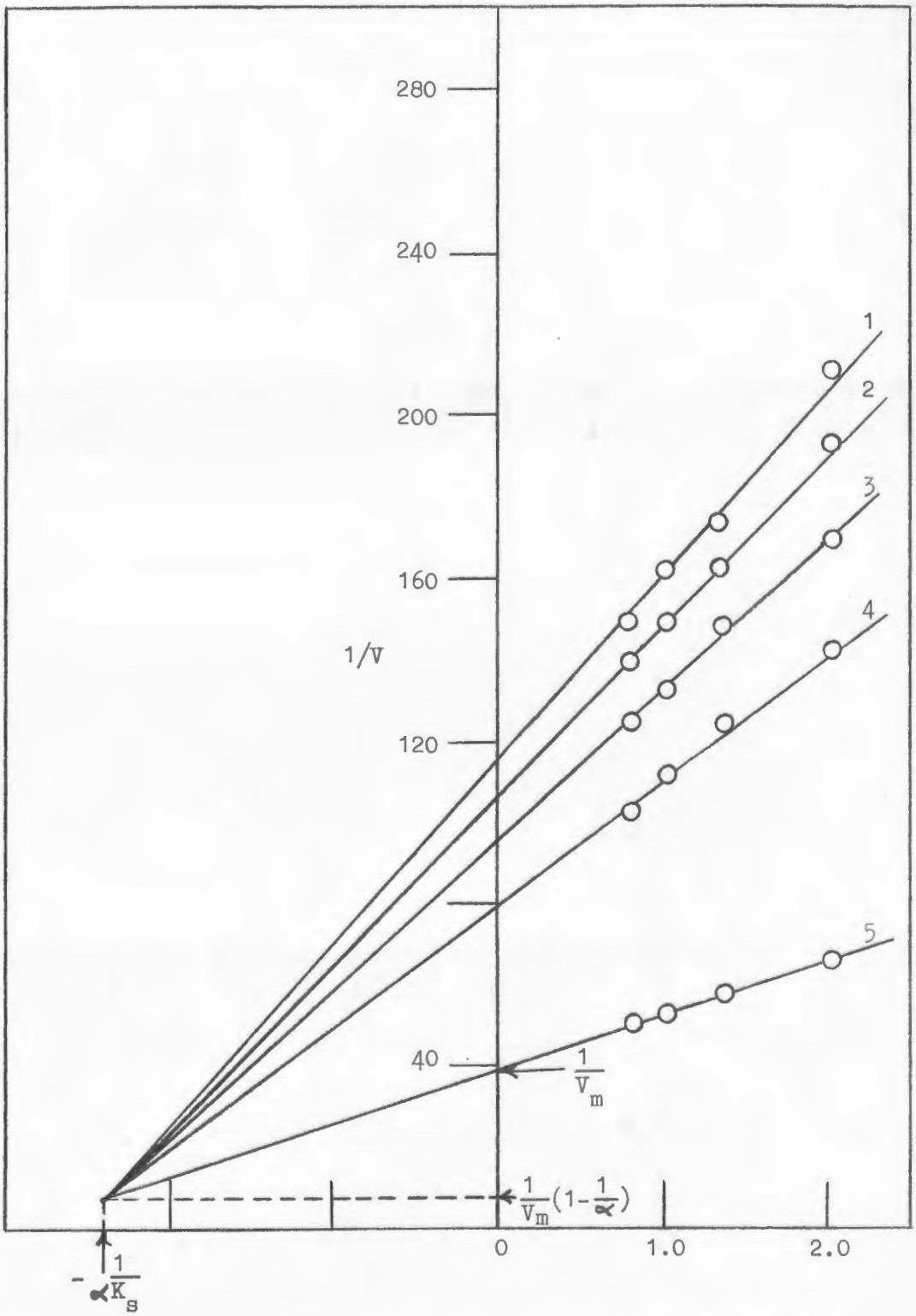


Figure 15. Analysis of inhibition by 1-1 AH chelate at pH 8.0 using 1/S values. Key: 1, (I) = 3.73×10^{-4}M; 2, (I) = 3.34×10^{-4}M; 3, (I) = 2.82×10^{-4}M; 4, (I) = 2.06×10^{-4}M; 5, (I) = 0.

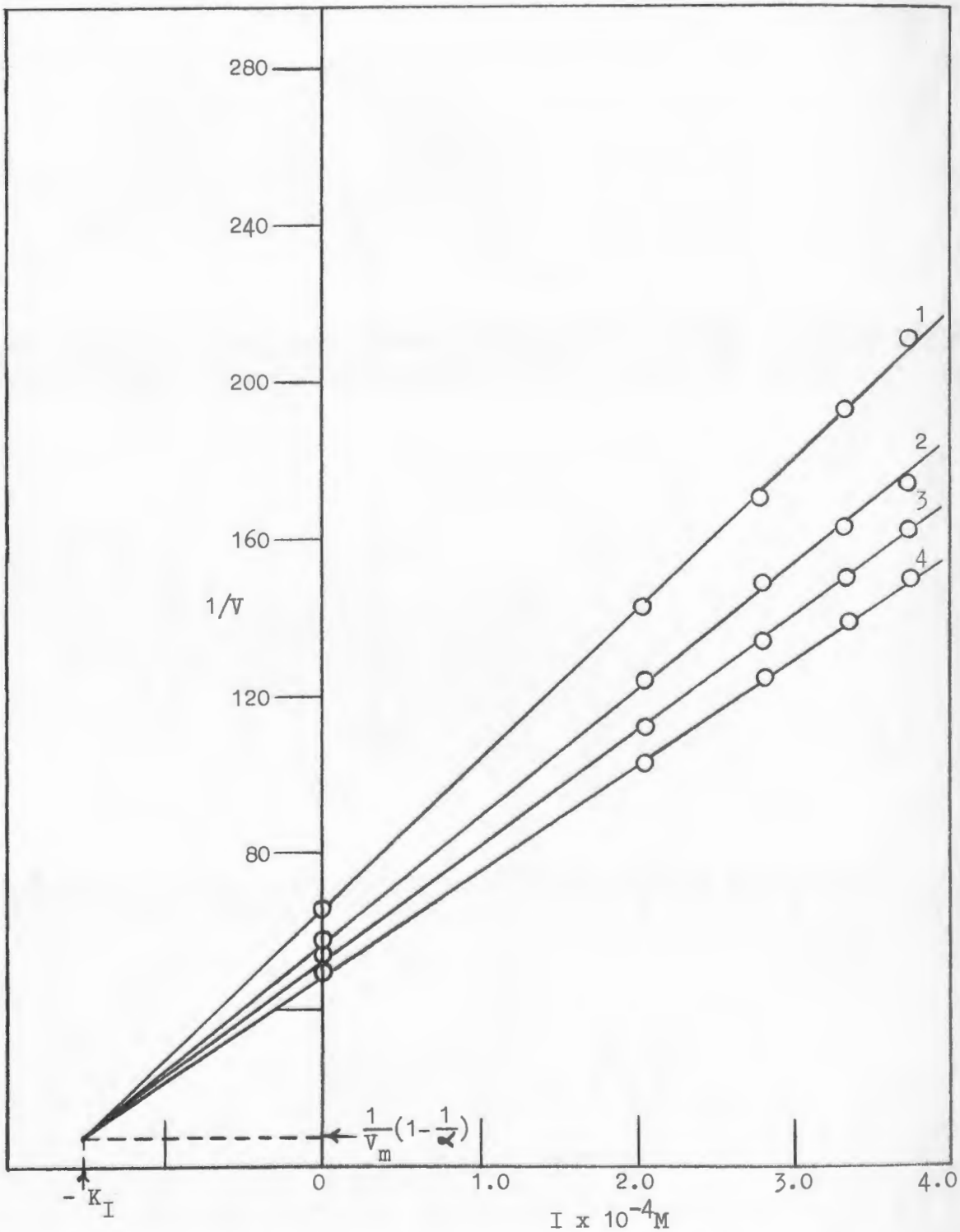


Figure 16. Analysis of inhibition by 1-1 AH chelate at pH 8.0 using (I) values. Curves 1-4 have the same meanings as in Figure 13.

TABLE VII

INHIBITION BY 1-1 AH CHELATES IN A 2.1-1 LIGAND-METAL RATIO SOLUTION AT pH 8.0 WITH VARYING SUBSTRATE CONCENTRATIONS

Molar Conc. of Substrate x 10^{-3}	Molar Conc. of Chelate Mixture x 10^{-3}	Molar Conc. of Free Cupric Ion x 10^{-7}	Molar Conc. of 1-1 Species x 10^{-4}	Molar Conc. of 2-1 Species x 10^{-3}	Reaction Velocity (1/V)
1.22	0	0.00	0.00	0.00	50½
	1	2.50	2.06	0.79	101
	2	2.17	2.82	1.72	124½
	3	1.96	3.34	2.67	138
	4	1.79	3.73	3.63	149
0.99	0	0.00	0.00	0.00	53
	1	2.50	2.06	0.79	111
	2	2.17	2.82	1.72	133
	3	1.96	3.34	2.67	148
	4	1.79	3.73	3.63	161
0.73	0	0.00	0.00	0.00	57½
	1	2.50	2.06	0.79	125
	2	2.17	2.82	1.72	148½
	3	1.96	3.34	2.67	162
	4	1.79	3.73	3.63	170½
0.49	0	0.00	0.00	0.00	65½
	1	2.50	2.06	0.79	142
	2	2.17	2.82	1.72	169
	3	1.96	3.34	2.67	192
	4	1.79	3.73	3.63	211

TABLE VIII

INHIBITION BY 1-1 AH CHELATE IN 2.2-1 and 2.3-1 LIGAND-METAL RATIO SOLUTIONS AT pH 8.0 WITH A SUBSTRATE CONCENTRATION OF 1.22×10^{-3} M

Molar Conc. of Chelate Mixture $\times 10^{-3}$	Molar Conc. of Free Cupric Ion $\times 10^{-7}$	Molar Conc. of 1-1 Species $\times 10^{-4}$	Molar Conc. of 2-1 Species $\times 10^{-3}$	Vo/V
	Ligand-Metal Ratio=2.2-1			
0	0.00	0.00	0.00	1.00
1	1.73	1.75	0.83	2.09
2	1.34	2.25	1.78	2.39
3	1.11	2.55	2.75	2.67
4	0.95	2.75	3.73	2.92
	Ligand-Metal Ratio=2.3-1			
0	0.00	0.00	0.00	1.00
1	1.24	1.50	0.85	2.04
2	0.87	1.84	1.82	2.37
3	0.68	2.02	2.80	2.64
4	0.56	2.13	3.79	2.80

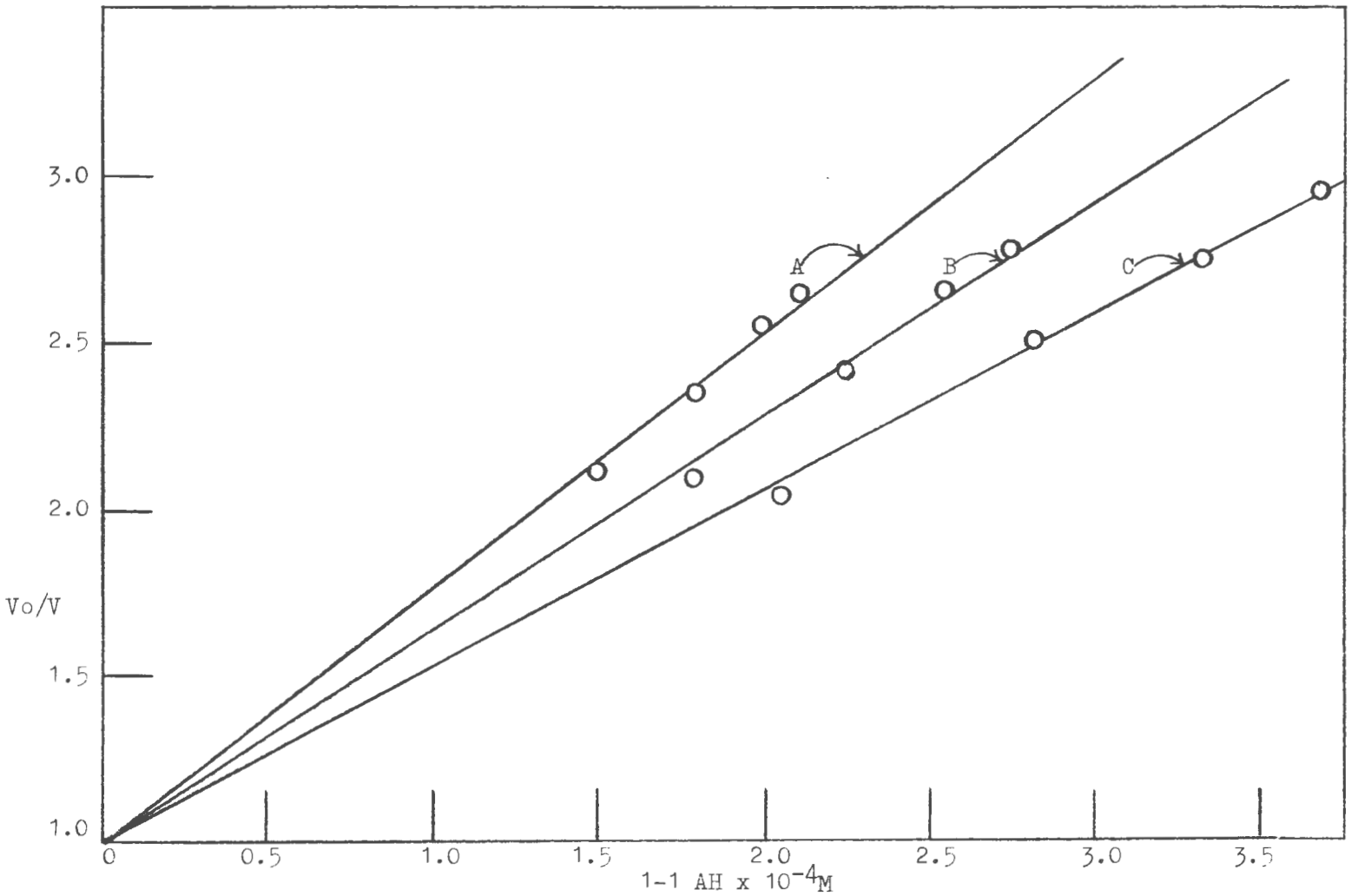


Figure 17. Inhibition by 1-1 AH chelate in several ligand-metal ratios at pH 8.0. A, ligand-metal ratio 2.3-1; B, ligand-metal ratio 2.2-1; C, ligand-metal ratio 2.1-1.

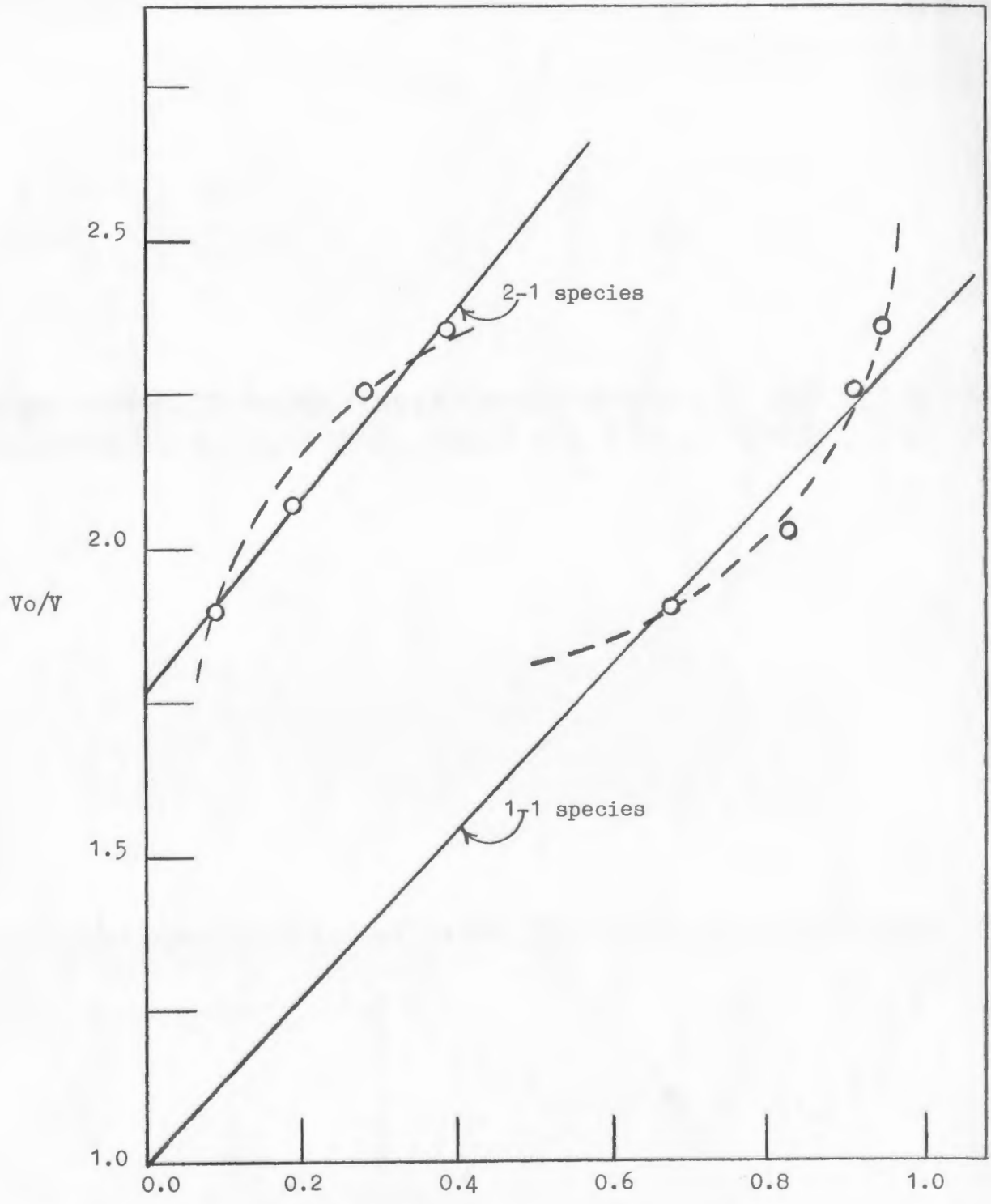
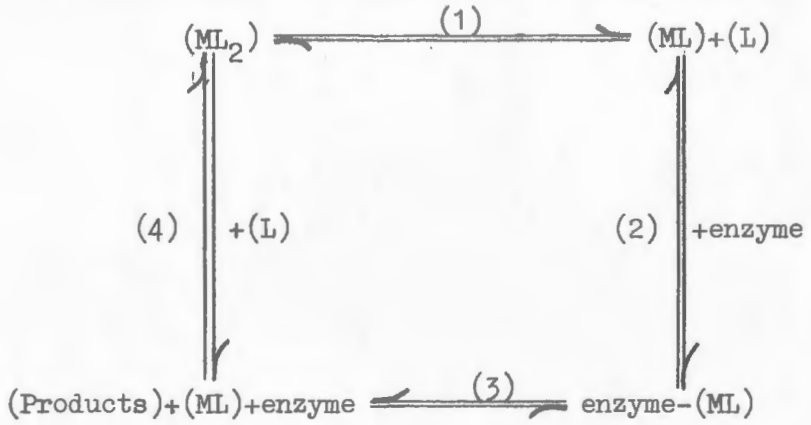


Figure 18. Inhibition by 1-1 and 2-1 AH chelates at pH 8.5. For 1-1 species, abscissa represents 10^{-4}M; for 2-1 species, abscissa represents 10^{-3}M.

mechanism no longer follows Michaelis and Menten kinetics) of binding on the enzyme surface at elevated pH values and/or (2) the concentration of 1-1 chelate species was so small compared to 2-1 chelate that an equilibrium was achieved between the enzyme and the chelate solution according to the following scheme:



Because of this behavior, it was not possible to determine α and K_I values for the AH system at elevated pH values.

Inhibition by the HP System. Analysis of the HP system was conducted in a manner similar to that used for the AH system. Here, again, both a 1-1 and 2-1 chelate species were formed with stability constants much weaker than the AOH interaction, (compare Figures 4 and 8). Consequently, the selection of ligand-metal ratios and equilibrium pH values were again restricted.

Initially, a ligand-metal ratio of 2.0-1 was examined at pH 8.0. This ratio and pH resulted in rather large concentrations of 1-1 species and apparently insignificant amounts of free cupric ion in the equilibrium mixture. It was found, however, that although the equilibrium mixture at this pH exhibited no time dependence, a plot of 1-1 chelate species versus Vo/V yielded a straight line with a positive y-intercept. The "extra" inhibition, not accounted for by the 1-1 species, could possibly be attributed to free cupric ion present at pH 8.0. Since possible interference by free cupric ion was suspected at pH 8.0, the 2.0-1 ligand-metal ratio was examined at pH 8.25. At this slightly higher pH, the influence of free cupric ion might be eliminated.

The results of the experiment at pH 8.25 with a ligand-metal ratio of 2.0-1 are shown in Figure 19. Examination of this data revealed that, apparently, a critical concentration of 1-1 species was necessary before any inhibition occurred. Once this critical concentration was reached, the inhibition followed a straight line relationship for 1-1 concentration versus Vo/V.

In order to substantiate this unusual observation, ligand-metal ratios of 2.1-1 and 2.3-1 were prepared. These mixtures contained less free cupric ion and less 1-1 chelate species but more 2-1 chelate species at equilibrium. The result of this study, including all pertinent concentrations, appears in

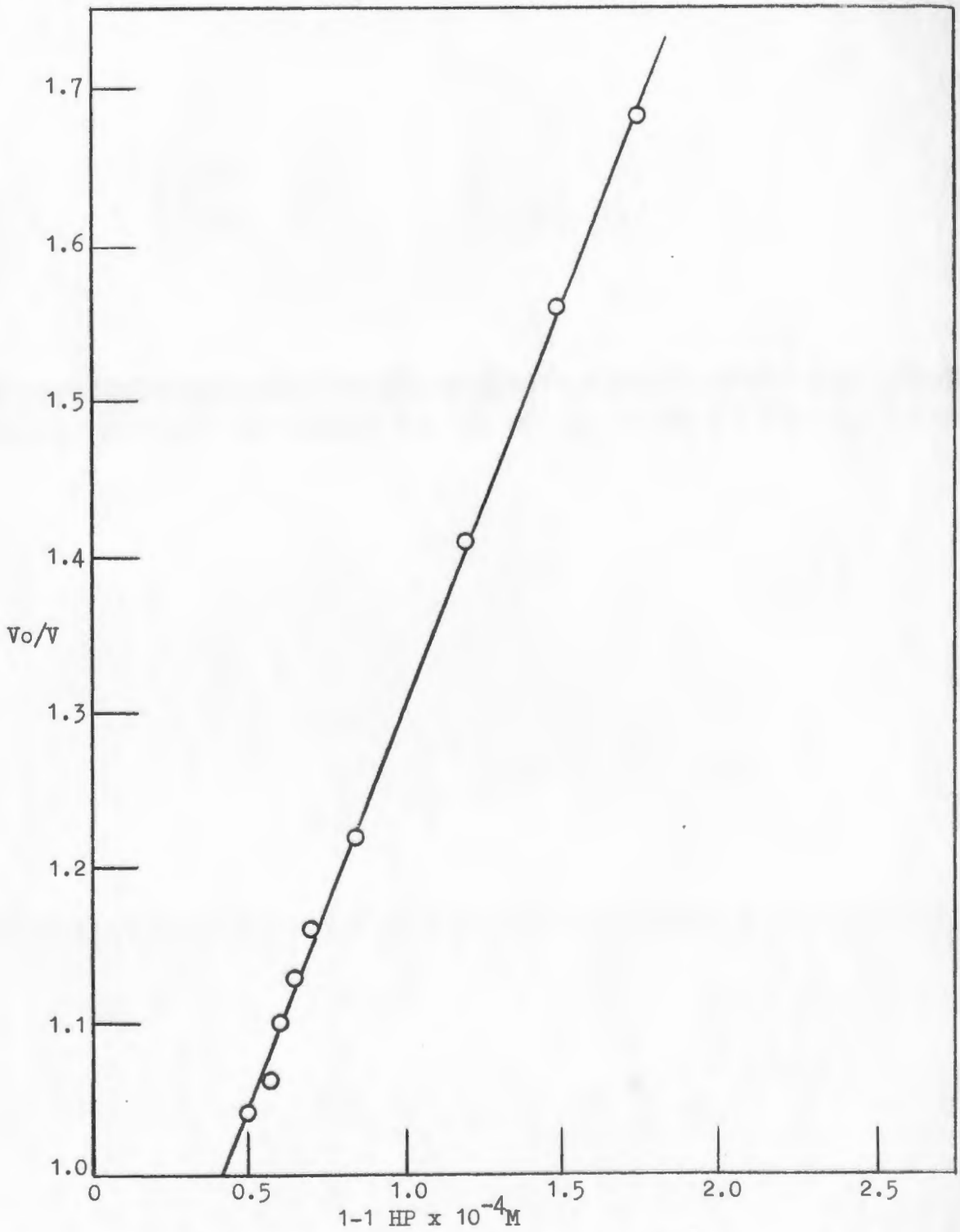


Figure 19. Inhibition by 1-1 HP chelate in several ligand-metal ratios at pH 8.25.

Table IX (see also Figure 19). Analysis of this data indicated a consistent correlation between 1-1 chelate concentration and Vo/V among the solutions of different ligand-metal ratios. For example, low concentrations of the 2.3-1 ligand-metal ratio solution, which contained, at equilibrium, amounts of the 1-1 chelate species almost identical with the "yield value" or critical concentration, showed practically no inhibitory activity.

When the pH was increased to 8.5, not only did the "yield value" disappear, but, as in the case of AH, correlation of 1-1 species and inhibition could not be made. Possibly another mechanism of binding to the enzyme surface or an actual physiochemical change of the enzyme was responsible for this odd behavior. It should be noted that previous studies of the acetylcholinesterase system by Bolton (34,36) were conducted at pH values of 7.0 and 8.0 and, consequently, none of the unusual behavior observed in our studies at equilibrium pH values of 8.5 and higher, had been encountered.

Although the reason for the occurrence of a "yield value" in this study is not known, one possible explanation could be made on the basis of steric hindrance. The HP ligand was the only ring compound studied and the chelates formed were double ring structures. Possibly the relatively large size of the 1-1 chelate prevented easy attachment to the enzyme site involved in binding. At higher pH values the charge of the binding site on the enzyme surface might have changed, thereby eliminating

TABLE IX

INHIBITION BY 1-1 HP CHELATE IN SEVERAL LIGAND-METAL RATIO SOLUTIONS AT pH 8.25 WITH A SUBSTRATE CONCENTRATION OF 1.22×10^{-3}M

Molar Conc. of Chelate Mixture $\times 10^{-3}$	Molar Conc. of Free Cupric Ion $\times 10^{-7}$	Molar Conc. of 1-1 Species $\times 10^{-4}$	Molar Conc. of 2-1 Species $\times 10^{-3}$	Vs/V
	Ligand-Metal Ratio = 2.0-1			
0	0.00	0.00	0.00	1.00
1	1.699	0.85	0.91	1.22
2	1.701	1.22	1.88	1.41
3	1.702	1.50	2.85	1.56
4	1.703	1.74	3.83	1.68
	Ligand-Metal Ratio = 2.1-1			
0	0.00	0.00	0.00	1.00
1	0.570	0.502	0.95	1.05
2	0.391	0.595	1.94	1.10
3	0.300	0.641	2.94	1.13
4	0.245	0.670	3.93	1.16
	Ligand-Metal Ratio = 2.2-1			
0	0.00	0.00	0.00	1.00
1	0.242	0.240	2.97	1.004
2	0.140	0.250	1.96	0.97
3	0.099	0.256	2.96	1.01
4	0.077	0.258	3.96	1.03

this steric effect, resulting in the disappearance of the "yield value."

Correlation of the Inhibition Studies. In this section, an attempt will be made to discuss various factors which may affect inhibitory activity within each ligand system. Also, any deviations from expected results, not previously discussed, will be cited.

Relation of Inhibition to Stability Constants. The chelate stability constants for the three ligands under consideration, appear in Table X. It is difficult to make a complete comparison of the extent of inhibition with formation constants because of the yield value encountered with the HP system. The AOH system exhibited the weakest inhibitory action among the three ligands and it had the largest formation constant. If the availability of the metal ion is essential for inhibition, the weak inhibitory properties of the AOH ligand might be expected because of the (1) high stability constant and (2) shielding of the metal in the terdentate attachment. Table X also contains the formation constants for the ethylenediamine and glycine ligand systems reported by Bolton (34,36). It would have been convenient if all systems could have been examined at the same pH, but because of various factors previously discussed, this was not possible. Examination of Table X reveals a possible relationship between the formation constants (K_1) and the in-

TABLE X

RELATION OF CHELATE STABILITY CONSTANTS (K_1) TO INHIBITION CONSTANTS (K_I)

Ligand	log K_1	K_I	K_s		pH	Charge
AOH	14.20	6.95×10^{-3}	1.91×10^{-4}	10.8	8.5	(+)
AH	9.29	1.5×10^{-3}	3.39×10^{-4}	1.25	8.0	(++)
HP	8.00	.....	.....	....	...	(+)
			Literature (34)			
Ethylene-diamine	10.34	1.9×10^{-4}	.....	1.0	8.0	(++)
Glycine	8.20	1.9×10^{-4}	.....	1.3	8.0	(+)

hibitory constants (K_I). (The calculation of K_I values is discussed in the Appendix, in the section dealing with α determinations.) It appears that high K_1 values result in low K_I values. Bolton, however, calculated identical values for K_I when he compared ethylenediamine and glycine, although the chelate stability constants are quite different. Thus, although there appears to be some relationship between K_1 and K_I, other factors are undoubtedly involved.

Relation of Inhibition to Charge. Although the charge associated with the chelate species may have an effect on binding, it is not possible to measure this effect at a given equilibrium pH. Also, comparisons of charge and inhibition among all three ligands at pH 8.0 may not be meaningful, since the mechanism of chelate attachment to the enzyme surface may be different for each system. It will be demonstrated that charge may become a very significant factor in the extent of binding at elevated pH values.

pH Effects on Inhibition. When equilibrium pH was altered, the extent of inhibition changed in all systems studied. In the AOH system, where the concentration of 1-1 species did not change over a large pH range, studies were made at several equilibrium pH values. These results appear in Figure 10 and indicate a strong dependence of inhibition on equilibrium pH. The data shows a dramatic change in inhibitory activity between

pH 8.50 and 8.75. Possibly the pK_a of the group on the enzyme surface involved in the binding process occurs in this pH range. The overall inhibition noted by the AOH system was quite low until elevated pH values were used. Conceivably, the charge effect becomes a predominant factor at higher pH values and supercedes other mechanisms of attachment which may occur at lower pH values.

It is of interest to note that the 1-1 AH chelate is doubly charged and the system was found to be very pH dependent. At pH 8.0, a correlation of 1-1 species with inhibition seemed evident, but at slightly higher pH values this relationship did not exist. For example, plots of 1-1 AH chelate concentration versus Vo/V at pH 8.5 resulted in curved lines. Because of this result, the inhibition data obtained under these conditions could not be analyzed. As was mentioned earlier, attempts to run studies at lower equilibrium pH values resulted in free cupric ion interference.

The possibility that a different mechanism of inhibition or a change of binding site is involved at elevated pH values seems reasonable. The fact that this curvature was not observed in the AOH system, at elevated pH values, may possibly be attributed to the single positive charge on the 1-1 AOH chelates as compared to the doubly positive charge on the 1-1 AH chelates. Furthermore, the fact that the concentration of 1-1 species is quite small in comparison to the 2-1 species at elevated

pH values may cause a significant equilibrium shift (see page 66 .)

Examination of the HP system at different pH values was not possible. At pH values below 8.25, free cupric ion interfered and at pH values of 8.5 or higher the stability of the chelates was questionable. The results obtained at pH 8.5 have not been included because they were not reproducible. The rather unique behavior of the HP system at pH 8.2 was reproducible and will be discussed later.

Relation of Coordination Vacancies and Inhibition. The fact that coordination vacancies must be present on the metal ion in order to observe inhibition, was observed in all three systems. The weak inhibitory activity of the AO-Cu^{+} chelate could be attributed to the presence of only one coordination vacancy on the Cu^{++}. The absence of inhibitory activity by the 2-1 AH and HP chelates may be attributed to the absence of any coordination vacancies. It is possible, however, that if the metal ion itself is attaching to the enzyme surface, the 2-1 chelates present a sterically blocked cupric ion and prevent the attachment from occurring (except at elevated pH values with doubly charged chelates, sic AH system).

Relation of Configuration and Inhibition. The strained, terdentate attachment of the AO-Cu^{+} chelate has been described. The large formation constant calculated for this chelate would

probably result in very little rotation within the molecule and, consequently, the metal ion would not be readily available for attachment to the enzyme surface.

The AH system presented an unstrained six member chelate ring in the 1-1 species. Thus, the copper ion in this compound was available for attachment to the binding site on the enzyme surface. However, this availability of copper ion was superceded at elevated pH values, probably because of the double charge on the chelate molecule.

The HP system was interesting in that it is the only ligand examined which contained a ring. This ring configuration may be the basis of a possible explanation for "yield" value (or critical concentration requirement) observed at pH 8.25. When the 1-1 HP chelate forms, a double ring compound results which could present a steric hindrance in attaching to the enzyme surface. Possibly, a critical concentration of the 1-1 HP species was necessary to overcome this steric effect, before any inhibition could be observed. Initially, the configuration of the hydroxy group in HP was thought to be of possible value in increasing inhibition because it could be directed towards the esteratic site on the enzyme. Unfortunately, the limited pH range in which this compound could be studied, prevented the design of experiments which could possibly have elucidated effects of this hydroxy group.

IV. SUMMARY

An attempt at the systematic analysis of acetylcholinesterase inhibition by copper chelates, is presented. Previous analyses of enzyme-chelate interactions have been limited to pH values of 7.0 and 8.0, resulting in "non-competitive" inhibition by 1-1 chelate species.

It is shown that the 1-1 chelate species of 1,3 diaminopropane di-HCl inhibits in a "non-competitive" manner at pH 8.0. At higher pH values, the relationship between enzyme reaction velocity and concentration of 1-1 species is no longer linear, indicating a different mechanism of inhibition is occurring.

It is shown that the 1-1 chelate species of 1,3 diaminopropanol-2 inhibits in an essentially "competitive" manner at pH 8.5. Further, a relationship between inhibition and pH is developed for the 1,3 diaminopropanol-2 system resulting in increased inhibition in the pH range 8.50-8.75. Analysis of data indicates a change in charge at the binding sites on the enzyme surface in this pH range.

It is shown that 1-1 copper chelates of hydroxy-L-proline inhibit the enzyme at pH 8.25, after a critical concentration of 1-1 species is reached. At lower pH values, free cupric ion interfers and the chelate species dissociates at pH values above 8.25. The yield value encountered in the hydroxy-L-proline system precludes the use of first-order enzyme kinetic theory

to analyse the data. The yield value may be attributed to steric factors resulting from the formation of a double ring system in the 1-1 chelate.

The effects of free cupric ion are examined in all systems. Results indicate the concentration of free cupric ion is dependent on the formation constant and the pH of the equilibrium mixture. It is further shown that concentrations of cupric ion below 1×10^{-6} M exhibit no inhibitory properties.

The relationship between inhibition and coordination vacancies is examined in all systems. In agreement with previous results, the presence of vacancies on the metal ion is essential for inhibition. The AOH chelate, with only one coordination vacancy on the metal ion, is a weak inhibitor. 2-1 chelates of both AH and HP, with no coordination vacancies on the metal ion, did not appear to inhibit the enzyme significantly.

The effect of chelate charge is examined in all three systems. The charge on the 1-1 AOH chelate, (a single positive charge) is analysed as a function of pH and the data has been discussed earlier. The instability of the 1-1 HP chelate (also with a single positive charge) did not allow equilibrium pH changes and consequently no correlation between inhibition and charge can be made. The 1-1 AH chelate (with a double positive charge) is shown to exhibit non-ideal behavior at elevated pH values. The presence of the double positive charge or a change in the mechanism of binding to the enzyme surface may be factors responsible for this behavior.

BIBLIOGRAPHY

1. Wilson, I.B., Biochem. et Biophys. Acta, 7, 466 (1951).

2. Brecher, A.S., and Baker, B.S., Biochem. Pharmacol., 14, 638 (1956).

3. Salas, M.L., Vinuela, E., Salas, M., and Sols, A., Biochem. et Biophys., 19, 371 (1956).

4. Ottolenghi, A., Bernheim, F., and Wilbur, K.M., Arch. Biochem. Biophys., 56, 157 (1955).

5. Barber, A.A., and Ottolenghi, A., Proc. Soc. Exptl. Biol. Med., 96, 471 (1956).

6. Canzanelli, A., Sossen, R., and Rapport, D., Am. J. Physiol., 188, 547 (1957).

7. Beyer, R.E., Arch. Biochem. Biophys., 79, 269 (1959).

8. Wilson, I.B., "The Enzymes", V. 4, Edited by Boyer, P.D., Lardy, H. and Myrback, K., Academic Press, N.Y. and London, 1960, Ch. 30.

9. Mendel, B., and Rudney, H., J. Biochem., 37, 59 (1943).

10. Wilson, I.B. and Bergmann, F., J. Biol. Chem., 186, 683 (1950).

11. Wilson, I.B. and Bergmann, F., J. Biol. Chem., 185, 479 (1950).

12. Wilson, I.B., J. Biol. Chem., 197, 214 (1952).

13. Michaelis, L., and Menten, M.L., Z. Biochem., 49, 333 (1913).

14. Lineweaver, H., and Burk, D., J. Amer. Chem. Soc., 56, 658 (1934).

15. Straus, O.H., and Goldstein, A., J. Gen. Physiol., 26, 559 (1943).

16. Goldstein, A., J. Gen. Physiol., 27, 529 (1944).

17. Hunter, A., and Downs, C.E., J. Biol. Chem., 157, 427 (1945).

18. Friedenwald, J.S., and Maengwyn-Daview, G.D., "A Symposium on the Mechanism of Enzyme Action", McElroy, W.D., and Glass, B., Eds., Johns Hopkins Press, Baltimore, Md., 1954, p. 154.

19. Morgan, G.T., and Drew, H.D.K., J. Chem. Soc., 117, 1456 (1920).

20. Chaberek, S., and Martell, A.E., "Organic Sequestering Agents", John Wiley & Sons, Inc., N.Y., 1959, Ch. 1.

21. Schwarzenbach, G., and Ackermann, H., Helv. Chim. Acta, 30, 1798 (1947).

22. Schwarzenbach, G., and Biedermann, W., Helv. Chim. Acta, 31, 459 (1948).

23. Schwarzenbach, G., and Ackermann, H., Helv. Chim. Acta, 31, 1029 (1948).

24. Chaberek, S., and Martell, A.E., J. Am. Chem. Soc., 74, 5052 (1952).

25. Ackermann, H., and Schwarzenbach, G., Helv. Chim. Acta, 32, 1543, (1949).

26. Carlson, G.A., McReynolds, J.P., and Verhoek, F.H., J. Am. Chem. Soc. 67, 1334 (1945).

27. Schwarzenbach, G., and Biedermann, R., Helv. Chim. Acta., 31, 331 (1948).

28. Schwarzenbach, G., Ackermann, H., and Ruckstuhl, P., Helv. Chim. Acta, 32, 1175 (1949).

29. Bjerrum, J., "Metal Ammine Formation in Aqueous Solution", P. Hasse & Son, Copenhagen, 1942.

30. Bolton, S.M., and Beckett, A., J. Pharm. Sci., 53, 55 (1964).

31. Wagner-Jauregg, T., Hackley, B.E., Jr., Lies, T.A., Owens, O.D., and Proper, R., J. Am. Chem. Soc., 77, 922 (1955).

32. Courtney, R.C., Gustafson, R.L., Westerback, S.J., Hytiainen, H., Chabarek, S., and Martell, A.E., J. Am. Chem. Soc., 79, 3030 (1957).

33. Wilson, I.B., Ginsburg, S., and Quan, C., Arch. Biochem. Biophys., 77, 286 (1958).

34. Bolton, S.M., and Ellin, R.I., J. Pharm. Sci., 51, 533 (1962).

35. Bolton, S.M., J. Pharm. Sci., 54, 373 (1965).

36. Chaberek, S., and Martell, A., "Organic Sequestering Agents", John Wiley & Sons, Inc. New York, 1959.

37. Bolton, S.M., J. Pharm. Sci., 54, 583 (1965).

38. Breckenridge, J.G., and Hodgins, J.W.R., Can. J. Research, 17B, 331 (1939).

39. Gonick, E., Doctoral Dissertation, The Pennsylvania State College (1951).

40. Mario, E., and Bolton. S.M., Anal. Chem., 37, 165 (1965).

41. Schwarzenbach, G., Maissen, B., and Ackermann, H., Helv. Chim. Acta, 35, 2333 (1952).

42. Albert, A., J. Biochem., 50, 531 (1950).

43. Li, N.C., and Doody, E., J. Am. Chem. Soc., 76, 221 (1954).

44. Wilson, C.L., and Wilson, D.W. "Comprehensive Analytical Chemistry", p. 351, Elsevier Co., N.Y., 1960.

45. Jenkins, E.N., Analyst, 79, 209 (1954).

46. Schwarzenbach, G., Maissen, B., and Ackerman, H., Helv. Chim. Acta., 35, 2333 (1952).

47. Kirk, P.L., and Schmidt, C.L.A., J. Biol. Chem., 81, 237 (1929).

48. Job, P., Ann. Chim., 9, 10, 113 (1928).

49. Hine, J., "Physical Organic Chemistry", 2nd ed., McGraw Hill, New York 1962.

50. Cohn, E.J., Edsall, J.T., "Proteins, Amino Acids, and Peptides", ACS Monograph, Rheinhold, New York, 1962.

51. Taft, R.W., "Steric Effects in Organic Chemistry", Wiley & Sons, New York, 1956.

52. Bertsch, C.R., Fernelius, W.C., Block, B.P., J. Phys. Chem., 62, 444-50 (1958).

53. Ibid, pp. 503-4.

54. Hares, G.B., Doctoral Dissertation, Penn. State Coll. 1952.

APPENDIX

APPENDIX A

Multiple Regression Approach (AOH)

The equation used to determine the constants describing the inhibition is of the following form:

$$1/Vo = 1/V_m + \frac{K_s}{V_m}(1/S) + 1/ K_I V_m(1) + \frac{K_s}{K_I V_m}(\frac{I}{S})$$

This may be rewritten for convenience as follows:

$$y = a + bx + cz + dw$$

where $a=1/V_m$, $b=\frac{K_s}{V_m}$, $c=1/ K_I V_m$, $d=\frac{K_s}{K_I V_m}$.

Considering y as a linear function of x, z, and w, the usual least squares multiple regression approach can be used to obtain the best estimates of a, b, c, and d.

The normal equations are:

$$(y-\bar{y})x = b\ (x-\bar{x})^2 + c\ x(z-\bar{z})^2 + d\ x(w-\bar{w})^2$$

$$(y-\bar{y})z = b\ (x-\bar{x})z + c\ (z-\bar{z})^2 + d\ (w-\bar{w})z$$

$$(y-\bar{y})w = b\ (x-\bar{x})w + c\ (z-\bar{z})w + d\ (w-\bar{w})^2$$

$(x-x)z = 0$ in the present case.

Using the data of Table VI, the following estimates were obtained:

$a = 41.9$ $b = 8.00\times10^{-3}$ $c = 5.57\times10^{2}$ $d = 1.15$

Solving for the approximate constants, we obtain;

$1/V_m=41.9$ $K_s=1.91\times10^{-4}$ $K_I=6.95\times10^{-3}$ $\alpha=10.8$

APPENDIX B

Fortran IV Listing for Quadratic Solution

```
      REALMT
      DIMENSIONANAME(10),MT(20),AT(20)
00026 FORMAT(14HNO CONVERGANCE)
00013 FORMAT(    10A8)
00014 FORMAT(    15X,10A8)
00016 FORMAT(  15X,3HH+=E12.6)
00100 FORMAT(  6E12.6)
00105 FORMAT( 15X,4HAT =F7.5,6X,4HMT =F7.5)
00110 FORMAT(     3F10.2  )
00149 FORMAT( 12)
00150 FORMAT(2F10.6)
00200 FORMAT(1PE17.7)
00250 FORMAT(1PE17.7,4E17.7)
00350 FORMAT(1PE17.7,3E17.7  )
00010 FORMAT(1H1)
00012 FORMAT(    15X,4HM++=E12.6,6X,3HMA=E12.6//)
      WRITE(3,10)
01001 READ(1,13)ANAME
      WRITE(3,14)ANAME
      READ(1,100)FKA1,FKA2,FKA3,FK1,FK2,FK3
      READ(1,149)N
      READ(1,150)(AT(I),MT(I),I=1,N)
      READ(1,110)STPH,FPH,DELPH
      Z=STPH
00152 CONTINUE
      H=1./(1.*(10.**Z))
      WRITE(3,16)H
      AL=1.+H/FKA3+H**2/(FKA3*FKA2)+H**3/(FKA3*FKA2*FKA1)
      DO1000I=1,N
      A=0
      B=1
      C=(AL+FK1*(MT(I)-AT(I)))/(FK1*AL)
      D=-AT(I)/(FK1*AL)
      X=1.0E-05
00040 FX=B*X**2+C*X-D
      FPX=2.*B*X+C
      FPPX=2.*B
      X=X-FX/FPX
      CON=FX*FPPX/(FPX**2)
      IF(CON.LT.1.)GOTO20
      WRITE(3,26)
      GOTO1000
00020 ER=FX/FPX*X)
      IF(ABS(ER).GT..00001)GOTO40
      WRITE(3,105)AT(I),MT(I)
      WRITE(3,250)AL,A,B,C,D
      WRITE(3,350)X,FX,ER,FPX
      AM=MT(I)/(1.+FK1*X)
      AMA=FK1*AM*X
      WRITE(3,12)AM,AMA
      STOP
      END
```

APPENDIX C

Fortran IV Listing for Cubic Solution

```
C     THIS PROGRAM USES THE BOLZANO BISECTION METHOD TO FIND THE POSITIVE
      REALMT
      DIMENSIONANAME(10),MT(40),AT(40)
00013 FORMAT(    10A8)
00014 FORMAT(    15X,10A8)
00016 FORMAT(  15X,3HH+=E12.6)
00100 FORMAT(  6E12.6)
00105 FORMAT( 15X,4HAT =F7.5,6X,4HMT =F7.5)
00110 FORMAT(     3F10.2  )
00149 FORMAT( 12)
00150 FORMAT(2F10.6)
00100 FORMAT(1PE17.7)
00250 FORMAT(1PE17.7,4E17.7)
00350 FORMAT(1PE17.7,2E17.7    )
00010 FORMAT(1H1)
00012 FORMAT(    15X,4HM++=E12.6,6X,3HMA=E12.6,6X,4HMA2=E12.6//)
      WRITE(3,10)
01001 READ(1,13)ANAME
      WRITE(3,14)ANAME
      READ(1,100)FKA1,FKA2,FK1,FK2
      READ(1,149)N
      READ(1,150)(AT(I),MT(I),I=1,N)
      READ(1,110)STPH,FPH,DELPH
      Z=STPH
00152 CONTINUE
      H=1./(1.*(10.**Z))
      WRITE(3,16)H
      AL=1.+H/FKA2+H**2/(FKA1*FKA2)
      DO1000I=1,N
      A=1
      B=(AL+FK2*(2.*MT(I)-AT(I)))/(FK2*AL)
      C=(AL+FK1*(MT(I)-AT(I)))/(AL*FK1*FK2)
      D=-(AT(I))/(AL*FK1*FK2)
      CALLREGFAL(B,C,D,X,FX,ER)
      WRITE(3,105)AT(I),MT(I)
      WRITE(3,250)AL,A,B,C,D
      WRITE(3,350)X,FX,ER
      AM=MT(I)/(1.+FK1*X+FK1*FK2*X**2)
      AMA=(FK1*AM*X)
      AMA2=(FK2*AMA*X)
      WRITE(3,12)AM,AMA,AMA2
01000 CONTINUE
      IF(Z.GE.FPH)GOTO1001
      Z=Z+DELPH
      GOTO152
```

```
      SUBROUTINEREGFAL(A,B,C,X,F,ER)
      H=1.0E-05
      EPS=1.0E-15
      X=0.0
      NPATH=1
      F=((X+A)*X+B)*X+C
00003 Y=X+H
      F1=((Y+A)*Y+B)*Y+C
      IF(F*F1)5,5,4
00004 X=X+H
      F=F1
      IF(NPATH.EQ.1)GOTO3
00005 H=H/2.
      NPATH=2
      IF(H-EPS)1,1,3
00001 ER=F1/((3.*Y+2.*A)*Y+B)
      RETURN
      END
```

APPENDIX D

α Determinations According to the Method Described by Maengwyn-Davies and Friendenwald (18)

The Michaelis-Menten theory is based on the assumption that the enzyme forms reversibly dissociable complexes with substrate, activator, and inhibitor. Equilibrium of such complex formation is presumed to be reached instantaneously. The theory in its simplest form is established by the following argument: (a) The total concentration (E_T) of potentially active enzymatic Loci for a given enzyme can be expressed as the sum of the concentrations of those Loci that are coupled with substrate (ES) and those not so coupled (E).

$$(E_T) = (ES) + (E) \qquad (1)$$

(b) The velocity V of enzymatic catalysis is proportional to the concentration of the enzyme-substrate complex (ES) and reaches a maximum V_m when all available enzyme Loci are so coupled.

$$V \sim (ES) \qquad \frac{V}{V_m} = \frac{(ES)}{(E_T)} \qquad (2)$$

(c) The enzyme-substrate coupling is a reversible association, subject to first-order mass law equation:

$$(ES) \rightleftharpoons (E)+(S) \qquad K_s = \frac{(E)(S)}{(ES)} \qquad (3)$$

Using these assumptions, Michaelis-Menten obtained the equation:

$$\frac{V_m}{V} = \frac{(ET)}{(ES)} = 1 + \frac{K_s}{(S)} \qquad (4)$$

Lineweaver and Burk pointed out that this equation is linear for 1/V versus 1/S when the theory is applicable. The intercept of this line with the 1/S axis occurs at the point having the numerical value $-1/K_s$.

In expanding the theory to include the presence of varying amounts of a dissociable inhibitor, the total enzyme (E_T) equals:

$$(E_T) = (E) + (ES) + (EI) + (EIS) \qquad (5)$$

Applying mass action theory to the various possible dissociations, one obtains:

$$(ES) \rightleftharpoons (E)+(S) \qquad K_S=\frac{(E)(S)}{(ES)} \qquad (E)=(ES)\frac{K_s}{(S)} \qquad (6)$$

$$(EI) \rightleftharpoons (E)+(I) \qquad K_I=\frac{(E)(I)}{(EI)} \qquad (EI)=(E)\frac{(I)}{K_I}=(ES)\frac{(I)K_s}{(S)K_I} \qquad (7)$$

$$(EIS) \rightleftharpoons (EI)+(S) \qquad \alpha K_S=\frac{(EI)(S)}{(EIS)} \qquad (EIS)=(EI)\frac{(S)}{\alpha K_s}=ES\frac{(I)}{\alpha K_I} \qquad (8)$$

$$(EIS) \rightleftharpoons (ES)+(I) \qquad \beta K_I=\frac{(ES)(I)}{(EIS)} \qquad (EIS)=ES\frac{(I)}{\beta K_I} \qquad (9)$$

The evaluation of α can be done as follows:

Substituting into the right-hand side of Equation 5, the values

indicated in Equations 6 to 9 yields:

$$\frac{V_m}{V} = \frac{(E_T)}{(ES)} = 1+\frac{K_s}{(S)}+\frac{(I)K_s}{(S)K_I}+\frac{1}{\alpha K_I} \tag{10}$$

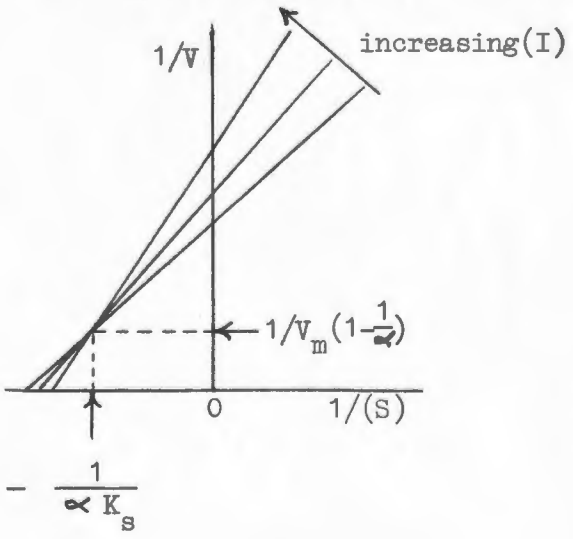
$$\frac{V_m}{V} = \frac{(E_T)}{(ES)} = \left[1+\frac{(I)}{\alpha K_I}\right]\left[1+\frac{\alpha K_s}{(S)}\cdot\frac{(I)+K_I}{(I)+\alpha K_I}\right] \tag{10a}$$

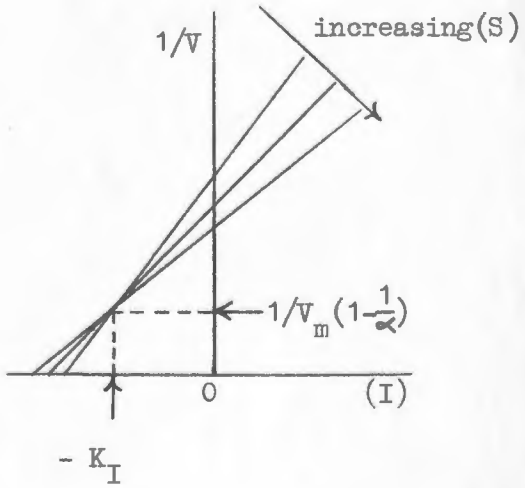
$$= \left[\frac{K_s}{1+(S)}\right]\left[1+\frac{(I)}{\alpha K_I}\cdot\frac{(S)+\alpha K_s}{(S)+K_s}\right] \tag{10b}$$

When (I)=0, Equation 10 reduces to that of Michaelis-Menten:

$$\frac{V_m}{V} = \frac{(E_T)}{(ES)} = 1+\frac{K_s}{(S)}$$

When (I) is constant, Equation 10 is linear in 1/V versus 1/S. From Equation (10b) it can be seen that for constant (S) the Equation is linear for 1/V versus (I). The first test of the applicability of the theory to a particular set of data consists in the demonstration of these linearities.





Consider two arbitrary values of inhibitor concentration (I_1) and (I_2). Either of these, inserted into Equation 10, yields a linear equation in 1/V versus 1/(S). If we equate the right-hand sides of these equations with each other and solve for (S), we will obtain the value of (S) corresponding to the intersection.

$$1+\frac{K_s}{(S)}+\frac{(I_1)K_s}{(S)K_I}+\frac{(I_1)}{\alpha K_I}=1+\frac{K_s}{(S)}+\frac{(I_2)K_s}{(S)K_I}+\frac{(I_2)}{\alpha K_I} \tag{11}$$

$$\frac{(I_1)}{K_I}\left[\frac{K_s}{(S)}+\frac{1}{\alpha}\right]=\frac{(I_2)}{K_I}\left[\frac{K_s}{(S)}+\frac{1}{\alpha}\right] \tag{12}$$

Since (I_1) and (I_2) are arbitrarily chosen and K_I is not infinite, it follows that at the intersection:

$$\frac{K_s}{(S)}+\frac{1}{\alpha}=0 \tag{13}$$

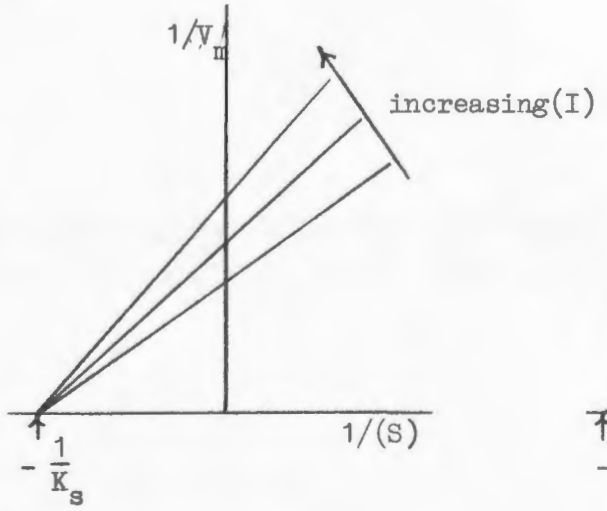
And,

$$1/(S)=-\frac{1}{\alpha K_s}\ ;\ 1/V=1/V_m\left[1-\frac{1}{\alpha}\right] \tag{14}$$

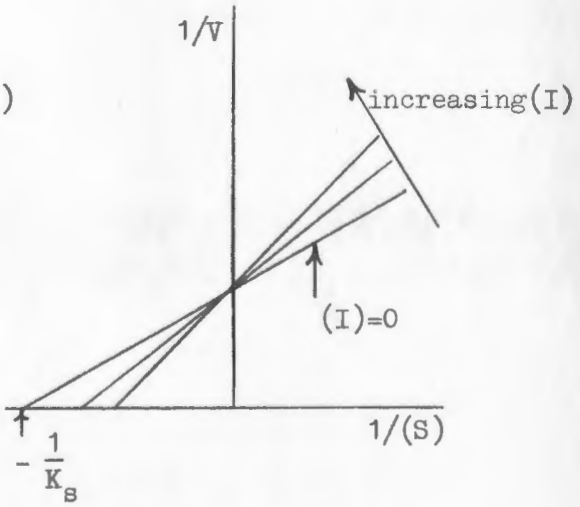
The intersection is independent of (I), consequently, the family of lines meet in a point.

Similarly, on the 1/V versus (I) plot, the family of lines corresponding to different fixed values of (S) will also meet in a point $(I)=-K_I$; $1/V=1/V_m(1-\frac{1}{\alpha})$. From these two intersections together with a lineweaver-Burk plot in the absence of inhibitor, all four constants: K_s, K_I, α, and V_m can be evaluated. For

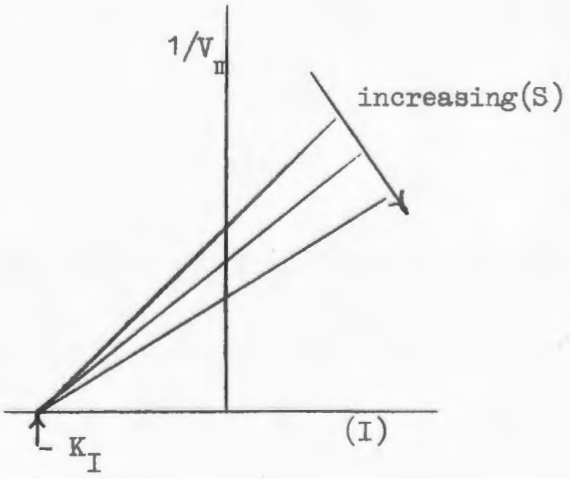
the special cases $\alpha = 1$ and $\alpha = \infty$, i.e., for perfectly non-competitive and perfectly competitive inhibition, the intersections on both plots are shown in the following examples:



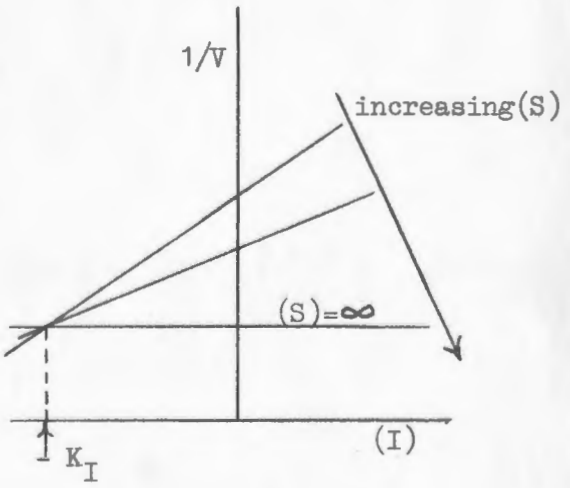
Non-competitive $\alpha = 1$



Competitive $\alpha = \infty$.



Non-competitive $\alpha = 1$



Competitive $\alpha = \infty$.

Solving for K_2 in terms of K_1

$$K_2 = \frac{1}{(L)} \cdot \frac{(\bar{n}-1) + \frac{\bar{n}}{K_1(L)}}{(2-\bar{n})} \qquad (7)$$

Using a K_2 value of 1.41×10^7 obtained from the data at $\bar{n} = 1.5$, a value for K_1 is calculated. This K_1 value is then used to reestimate K_2 and the method is continued until consistent values for K_1 and K_2 are obtained. After repeated calculations, $K_1 = 7.60 \times 10^8$ and $K_2 = 1.63 \times 10^7$ yielded fairly consistent results. Since the data is not exact, $\bar{n}$ is calculated at various free ligand values and compared to observed $\bar{n}$ values. The following tabulation contains the results of this check method and indicates that the values of K_1 and K_2 found are reasonably accurate.

Calculated $\bar{n}$	Observed $\bar{n}$	Free Ligand
0.342	0.303	6.67×10^{-10}
0.392	0.383	8.31×10^{-10}
0.462	0.461	1.07×10^{-9}
0.531	0.593	1.39×10^{-9}
1.120	1.061	1.51×10^{-8}
1.286	1.314	3.72×10^{-8}
1.510	1.504	6.75×10^{-8}
1.600	1.602	9.37×10^{-8}