[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, YALE UNIVERSITY SCHOOL OF MEDICINE]

Manganous Complexes of Several Amino Acids[1]

BY HARRY KROLL[2,3]

The binding of divalent manganese by several amino acids in the presence of excess manganous ions has been investigated, and the formation constants of these complexes have been determined. The predominating form of the metal complex in solution is a 1:1 combination of metal ion and amino acid. The relationship between the structure and metal binding properties of the α-amino acids is discussed.

The extensive participation of manganous ions[4] in biological systems has placed this ion in a special category insofar as its effects on enzymatic processes are concerned. It was therefore of interest to determine some of the chemical properties of the manganous ion in the presence of natural compounds which are known to be acted upon by enzymes activated by small amounts of this metal. This report deals with an evaluation of the interaction of manganous ions with amino acids and peptides.

The effect of manganous ions on leucine aminopeptidase has been reviewed by Smith and Lumry[5] who have postulated the formation of an enzyme–metal complex which can bind the substrate molecule, an amino acid amide or peptide, by the formation of a bidentate complex. The combination of divalent manganese with proteins and amino acids has been discussed by Main and Schmidt.[6] More recently, stability constants of amino acid complexes of divalent manganese have been described by Mellor and Maley.[7]

After the experimental work to be described had been completed, Albert[8] reported stability constants of the manganous–glycine and manganous–proline complexes by a procedure identical with that used in the present investigation. More recently, Monk[8a] has reported similar data for manganous–glycine and manganous–diglycine complexes. His conclusions are in substantial agreement with our own findings.

Experimental

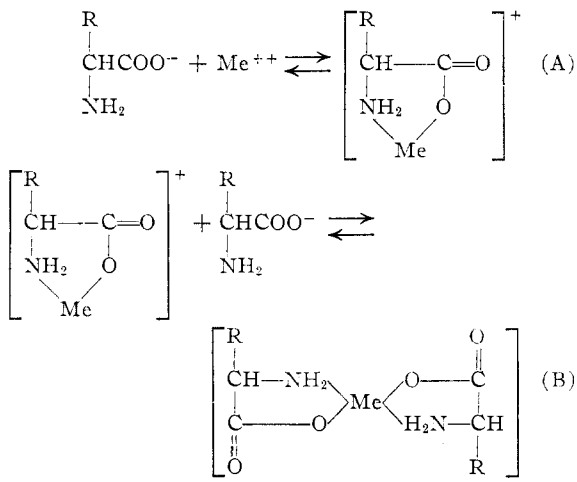
Materials.—All amino acids and peptides were purified preparations available in this Laboratory. Aqueous solutions were prepared from oxygen-free water, and all measurements were carried out under an atmosphere of purified nitrogen.

Procedure.—The method used for the determination of the formation constants of the manganous–amino acid complexes has been described by Schwarzenbach and co-workers.[9]

The titrations were carried out on solutions made up to an ionic strength of 0.1 with potassium chloride and at a temperature of 25°. The glass electrode measurements were made with a Cambridge Research Model *p*H Meter equipped with external electrodes. One hundred milliliters of a solution containing approximately 1×10^{-3} mole/liter of amino acid and 1×10^{-2} mole/liter of manganous chloride was titrated with 0.02 *N* sodium hydroxide. Readings were taken between the *p*H range of 7.0 and 8.2.

Results and Discussion

The formation of a metal complex of an amino acid containing one amino and one carboxyl group takes place by a stepwise process[10] involving the binding of the anion of the amino acid by the metal ion



These equilibria can be described by the mass law expressions

$$K_1 = [MeY^+]/[Me^{++}][Y^-] \quad (1)$$

$$K_2 = [MeY_2]/[MeY^+][Y^-] \quad (2)$$

$$\bar{K} = [MeY_2]/[Me^{++}][Y^-]^2 \quad (3)$$

where HY is the amino acid, Y^- is the amino acid anion, Me^{++} is the divalent metal ion, K_1 and K_2 are the formation constants of reactions *A* and *B*, respectively, and $\bar{K}$ is the stability constant for the over-all process.

The tendency of the manganous ion to enter into complex formation with organic ligands is weak compared to cupric and cobaltous ions. Preliminary titration studies indicated that in the presence of excess manganous ions and in the *p*H range of 7 to 8, the predominating complex was a 1:1 combination of metal ion and amino acid. This is in agreement with a similar conclusion made by Monk.[8a] Since the 1:1 species appears to be the predominant form of the manganous–amino acid complex, the stability constants for this system were evaluated for glycine, L-leucine, L-proline, L-aspartic acid, L-histidine and L-cysteine.

These constants were calculated by a method[8a,9] based on (a) the *p*H of the solution, (b) the equation for electroneutrality, (c) the known total con-

(1) Presented before the Division of Biological Chemistry at the September, 1950, Meeting of the American Chemical Society.

(2) Research Fellow, American Cancer Society, 1949–1951.

(3) Alrose Chemical Co., Providence, R. I.

(4) A. L. Lehninger, *Physiol. Rev.*, **30**, 393 (1950).

(5) E. L. Smith and R. Lumry, *Cold Spring Harbor Symposia*, **14**, 168 (1950).

(6) R. K. Main and C. L. A. Schmidt, *J. Gen. Physiol.*, **19**, 127 (1935).

(7) L. E. Maley and D. P. Mellor, *Nature*, **165**, 453 (1950).

(8) A. Albert, *Biochem. J.*, **47**, 531 (1950).

(8a) C. B. Monk, *Trans. Faraday Soc.*, **47**, 297 (1951).

(9) G. Schwarzenbach, E. Kampitsch and R. Steiner, *Helv. Chim. Acta*, **28**, 828 (1945).

(10) J. Bjerrum, "Metal Amine Formation in Aqueous Solution," P. Haase and Sons, Copenhagen, 1941.

centration of amino acid, (d) the known total concentration of metal ion, and (e) the acid dissociation constants of the amino acids.[11] These data are listed in Table I.

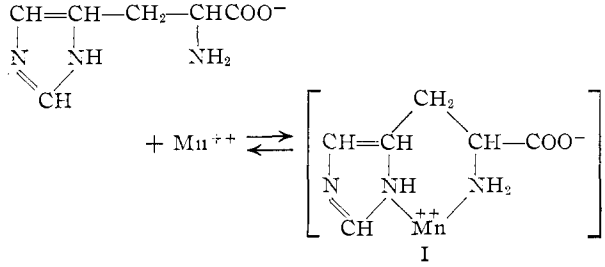
TABLE I

FORMATION CONSTANTS OF SEVERAL AMINO ACIDS AT 25°

Amino acid	$K_1 \times 10^{-3}$	Amino acid bound, *p*H 8.0, %
L-Leucine	0.14	2
Glycine	0.70	11
L-Proline	2.2	4
L-Histidine	3.8	72
L-Aspartic acid	5.5	49
L-Cysteine K_{1a}	*ca.* 0.1	..
L-Cysteine K_{1b}	130	43

There are several contributing factors which may influence the stability of the metal complex and the amount formed at a constant *p*H and temperature. These are (1) a steric factor, (2) the acidity of the α-ammonium group, and (3) the presence of functional groups in the side chain which may react with the metal ion. The steric effect is illustrated by a comparison of the formation constant of the L-leucine complex with that of glycine (*cf.* Table I). Since there is little difference in the acidity of the α-ammonium group of these two acids, the decrease in the stability of the L-leucine–manganous complex must be attributed to the size of the aliphatic side chain. A comparison of the glycine and proline complexes (Table I) shows that although the manganous ion will bind more glycine, the proline complex is more stable. This is due to the greater acidity of glycine which makes available a higher concentration of its conjugate base which is the effective agent in complex formation. However, the stability of the complex is related to the acidity of the dissociating ammonium group, and the metal ion is bound more firmly by a more basic α-amino group. The concept that complexity constants are a measure of a competitive reaction between a hydrogen ion and a metal ion for the complexing molecule has been reviewed by Bjerrum.[12]

In amino acids such as histidine, aspartic acid, cysteine, etc., a second functional group may participate in the complex formation thus introducing two types of metal–amino acid complexes. Histidine can react with divalent manganese to give I



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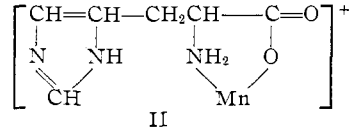
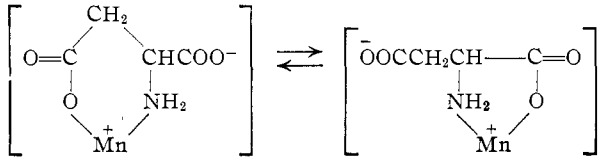


II

or II. Chelate I would be expected to form first at lower *p*H values because of the greater acidity of the ammonium group as compared to the α-ammonium group, which allows the imidazole nitrogen to initiate the metal complex formation. Unless manganese participates in a resonating system in complex I, which has been indicated for the cobaltous complex,[13] there should be an appreciable concentration of II present.

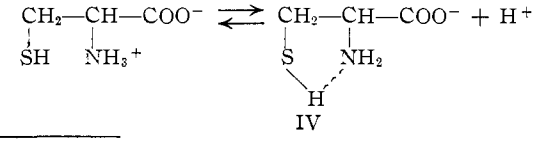
A similar relationship[14] holds for aspartic acid where the two chelate forms are



A comparison of the affinity of manganous ions for histidine and aspartic acid (Table I) indicates that the latter amino acid forms a more stable complex. However, histidine, because of the more acid character of the ammonium nitrogen as compared to the α-ammonium group of aspartic acid, will dissociate to give a higher concentration of the metal complexing conjugate base. As a result, histidine at *p*H 8.0 will bind 1.5 times as much metal ion as aspartic acid, despite the greater stability of the dibasic amino acid–manganous complex. It is apparent that this relationship holds true only in the presence of an excess of metal ions and that in the presence of minute amounts of the metal ion as in biological systems, the amount of metal complex formed will depend on the formation constants of the amino acids present and the *p*H of the solution.

The estimation of the formation constant of the manganese–cysteine complex corresponding to V (K_{1a}) can be made from calculations of the titration data obtained below *p*H 7.5, or by the method of Schwarzenbach and Ackerman.[15] The log K_1 of this constant approximates 2 but an exact evaluation is difficult because of the limitations of the method. It can be shown that at a *p*H above 7.8, the concentration of VI is more than one hundred times that of V, and the use of an equation based on complex formation between the sulfhydryl ion and the amino group (VI) appears to describe this system adequately. The magnitude of the formation constant K_{1b} indicates the existence of a metal complex of relatively high stability, and satisfactorily accounts for the known affinity of cysteine for heavy metal ions.

In the dissociation of cysteine to the amino acid IV, interaction between the sulfhydryl hydrogen and amino nitrogen would be expected.



IV

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 84.

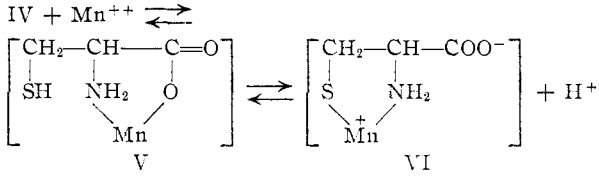
(12) Bjerrum, *Chem. Revs.*, **46**, 381 (1950).

(13) J. Z. Hearon, D. Burk and A. L. Schade, *J. Nat. Cancer Institute*, **9**, 337 (1949).

(14) The referee has indicated that the increased stability of the aspartic acid complex may be due to the electrostatic influence of the second carboxyl group.

(15) G. Schwarzenbach and H. Ackerman, *Helv. Chim. Acta*, **30**, 1798 (1947).

Evidence for this is the low pK value for the α-ammonium group in cysteine, 8.16, as compared to cystine, 9.6.[11] This interaction of sulfhydryl and amino group must be in direct competition with the formation of the manganese complex involving the amino group and the carboxylate ion (IV and V). The conversion of V to VI is favored



V VI

by increasing pH, since the higher alkalinity favors the formation of the more stable sulfur–manganese bond. The binding of the manganous ion by the amino group in V increases the acidity of the sulfhydryl group due to the positive inductive effect of the metal ion, thus favoring the conversion of V to VI.

Attempts to measure the ability of glycine amide, L-leucine amide, glycylglycine[8a] and triglycine to form complexes with manganous ions have been unsuccessful. These complexes are formed in very small amounts and are extremely unstable as compared to the parent amino acids. This point is significant in attempting to elucidate the mechanism of manganese-activated proteolytic enzyme systems where the amino acid derivative is used as a substrate, and the amino acid appears as an end product. Unpublished data from this Laboratory indicate that L-leucine and other amino acids will inhibit the hydrolysis of L-leucineamide by a leucine aminopeptidase, and the extent of the inhibition depends on the affinity of the amino acid for the metal ion.

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The Participation of Heavy Metal Ions in the Hydrolysis of Amino Acid Esters[1]

BY HARRY KROLL[2,3]

The rates of hydrolysis of α-amino acid esters in the presence of heavy metal divalent ions were investigated. A mechanism is postulated which involves a second-order reaction between a metal complex of the amino acid ester and hydroxyl ions. The hydrolytic rate is determined by the nature of the side chain in the alpha amino acid ester and the complexing tendency of the metal ion.

In a continuation of our studies on the effects of metal ions in biological systems,[4] it was observed that heavy metal ions accelerated the hydrolysis of amino acid esters in the pH range of 7.5–8.5. This reaction was investigated more closely in order to obtain information which could contribute to an understanding of the nature of metal ion activation of enzyme catalyzed hydrolytic reactions.[5]

Experimental

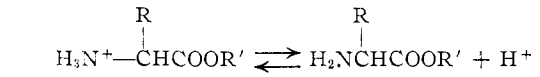
Amino Acid Esters.—The amino acid esters used in this investigation were available in this Laboratory or were prepared by treatment of the appropriate amino acid with methanolic hydrogen chloride. All esters were recrystallized, when possible, from the appropriate solvents. Proline methyl ester hydrochloride and dimethyl aspartate hydrochloride were isolated as viscous oils which could not be crystallized. However, the determination of their nitrogen content showed a satisfactory agreement with the theory.

General Procedure.—All kinetic studies reported were carried out at 25.4°. Four milliliters of a 0.1 M solution of the amino acid ester hydrochloride was pipetted into a 25-ml. volumetric flask, followed by 7.0 ml. of 0.5 M tris-(hydroxymethyl)-aminomethane buffer and by 2.7 ml. of 0.1 N sodium hydroxide. The flask was placed in the water-bath and after five minutes the exact amount of metal ion solution was added plus sufficient water to bring the reaction mixture to volume. After a rapid thorough mixing, a 3-ml. aliquot was withdrawn and titrated immediately to pH 4.0 with 0.05 N hydrochloric acid.

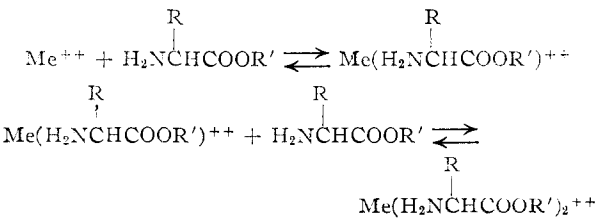
The hydrolytic process was followed by titration of samples at ten-minute intervals. The difference in the amount of 0.05 N hydrochloric acid required to titrate the solution at any given time interval as compared to the value obtained for zero time was used as a measure of the hydrolysis of the amino acid ester. A Cambridge Research Model pH Meter equipped with external glass electrodes was used in the titrations, and all solutions were prepared from oxygen-free distilled water.

Discussion and Results

Amino acid esters in aqueous solutions participate in the equilibrium



Since the process of metal complex formation involves the step-wise addition of the free base of the amino acid ester to the metal ion,[6] it is apparent that, at a pH sufficiently high to allow an appreciable concentration of the free base, a solution of a divalent heavy metal ion and an amino acid ester will contain a series of metal complexes of the type shown below.



At any given pH value, the various species of metal-ester complexes will be determined by the coördination characteristic (affinity) of the metal

(1) Presented before the Division of Organic Chemistry at the Boston Meeting of the American Chemical Society, April, 1951.

(2) Research Fellow, American Cancer Society, 1949–1951.

(3) Alrose Chemical Co., Providence, R. I.

(4) H. Kroll, THIS JOURNAL, **74**, 2034 (1952).

(5) A. L. Lehninger, *Physiol. Rev.*, **30**, 393 (1950).

(6) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haase and Sons, Copenhagen, 1941.