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Metal Complexes of Carnosine*

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Stability constants of complexes formed by carnosine and twelve metal ions are determined. Evidence is presented for the formation of chelates MHL^{2+} and ML^{+} from divalent metal ions and the ligand HL. The nickel(II) and copper(II) chelates further promote the ionization of a peptide hydrogen. The order of stability constants for the chelates ML^{+} is Cu(II) > Hg(II) > Ni(II) > Mn(II) > Cd(II) ~ Mg(II) ~ Ca(II) ~ Sr(II). With Co^{2+}, Pb^{2+}, and Zn^{2+}, precipitation permitted determination of only the protonated complex constant. Silver(I) gave an insoluble complex, while Ca(II), Cd(II), Mg(II), and Sr(II) formed only the normal chelate compound ML^{+}.

Since the dipeptide carnosine (β-alanyl-L-histidine) was extracted from muscle by Gulewitsch and Amiradzibi (1900a,b), considerable effort has gone into the elucidation of its biochemical function, but none has been found. However, Hanson and Smith (1949) found a protease in swine kidney and in rat liver, pancreas, and kidney, which attacks the peptide bond in carnosine and which was named carnosinase. They discovered that the enzyme was stabilized by the metals Mn(II) and Zn(II). This enzyme was highly purified by Rosenberg (1960a) and he confirmed the observations of Hanson and Smith in that the enzyme was stabilized by Mn(II). Carnosinase was found to be stabilized by the divalent metal ions in the order: Mn > Ca > Mg ~ Cd > Zn (Rosenberg, 1960b). It was also found that carnosinase was activated by the divalent metals in the order Mn > Cd > Zn > Co (Rosenberg, 1960c). To explain these results Rosenberg postulated the existence of two sites on the enzyme, one for stabilization, the other for activation. The nature of the interaction of stabilization is believed to be chelate-ring formation.

In view of the above observation involving activation and stabilization of the peptidase and advancement by Smith and co-workers (Smith, 1949a,b; Smith and Bergman, 1941; Smith and Lumry, 1949) of the theory that metal ions are chelated simultaneously to enzyme protein and substrate, a knowledge of the carnosine metal-formation constants is desirable.

Experimental

Reagents.—A sample of L-carnosine of specific rotation $[\alpha] = +21.3°$ in water, $c = 2$, was purchased from Mann Research Laboratories, New York. To the stock solution of L-carnosine was added exactly one equivalent of hydrochloric acid in order to form the protonated ligand. Metal-ion solutions were prepared from Fisher Reagent Grade metal nitrates, except in the case of manganese and mercury which were the sulfate and chloride, respectively. The metal solutions were standardized by titration with EDTA[1]

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[1] Abbreviations used in this work: EDTA, ethylenediaminetetraacetic acid.

using known methods (Schwarzenbach, 1956; Welcher, 1958).

Potentiometric Measurements.—Measurements of hydrogen-ion concentration were carried out in a 100-ml jacketed titration cell which was fitted with a magnetic stirrer and a tightly fitting rubber stopper through which were inserted nitrogen inlet and outlet tubes, microburet delivery tube, and glass and calomel extension electrodes. A Beckman Model GS *p*H meter was used to determine hydrogen-ion concentration. The electrodes were calibrated by titrating acetic acid with standard carbonate-free sodium hydroxide and comparing the meter reading with the hydrogen-ion concentration calculated from the data tabulated by Harned and Owen (1950). In the *p*H regions below 3.5 and above 10.5, the *p*H was calibrated by adding known amounts of hydrochloric acid and carbonate-free sodium hydroxide, respectively. All potentiometric measurements were made at 25.05 ± 0.05° and at an ionic strength of 0.10 M in KNO_3. The concentration of ligand was of the order of 0.005 M.

Calculations.—All calculations were made with the aid of an IBM 1620 computer at the computation center of Illinois Institute of Technology, using programs written by the author (G.R.L.). To insure accuracy, individual hand calculations were used to check the results obtained from all new programs.

Acid-Dissociation Constants.—The acid-dissociation constants K_n^H of L-carnosine monohydrochloride were calculated algebraically using the standard procedures for a simple one-step dissociation (the titration curves of carnosine hydrochloride (H_2L^+) consist of two separate buffer regions of one equivalent each). The same method was also used to calculate the *pK* of the Mn(II) chelate. However, this and all subsequent chelate ionization constants are reported as the association constant, i.e., in the preceding case as $MnL^+ + H^+ \rightleftharpoons MnHL^{2+}$ (K_{ML}^H).

In order to calculate the association constant (1) of hydrogen ion with the ionized peptide bond of the nickel complex, the equation developed by Doran *et al.* (1964) for two overlapping constants was fitted to a least squares analysis. This equation was also

$$MA + H^+ \rightleftharpoons ML^+$$

$$K_{MA}^H = [ML^+]/([MA][H^+]) \quad (1)$$

where $HA^- = L^-$

used for the calculation of the association of hydrogen ion with the normal mercury complex, which is analogous to that of manganese(II).

For the calculation of the ionization constant K_{ML}^H of the initial peptide-metal complex, the equations developed by Schwarzenbach *et al.* (1947) were placed into the slope-intercept form and solved simultaneously with the computer. In this way a large number of points could be used in the calculations.

Chelate-Stability Constants.—For the calculation of K_{ML}, equations analogous to those used for the preceding are used. However, equation (3) is valid only if no prior chelation takes place, i.e., for Cd(II), Mg(II), Ca(II), and Sr(II). By the combination of equation (2) with second ionization constant of carnosine and

$$K_{ML} = [ML^+]/[M^{2+}][L^-] \quad (2)$$

the usual mass balance and electroneutrality relationships, one obtains:

$$K_{ML} = \frac{T_M - X[L^-]}{X[L^-]^2} \quad (3)$$

where $X = [1 + [H^+]/K_2^H]$ and T_M is the total concentration of metal species present.

$$[L^-] = \frac{(2 - a)T_M + [OH^-] + [H^+]}{X - Y}$$

where $Y = [H^+]/K_2^H$.

K_{MHL} may be calculated in a manner analogous to the preceding. Combination of an equation analogous to (2), where $[L^-]$ is replaced by [HL], with the first ionization constant of carnosine (K_1^H) and with mass and electroneutrality relationships, leads to:

$$K_{MHL} = \frac{T_M - X[HL]}{X[HL]^2} \quad (4)$$

where $X = 1 + [H^+]/K_1$, and

$$[HL] = \frac{(1 - a)T_M + [OH^-] + [H^+]}{X - Y}$$

where $Y = [H+]/K_1^H$

When a protonated complex is formed, K_{ML}, the formation constant, may be calculated from the following relationship:

$$K_{ML} = K_{MHL}/K_{ML}^H K_2^H$$

where

$$K_{ML}^H = \frac{[MHL^{2+}]}{[ML^+][H^+]}$$

Results

Since the carnosine was obtained as the free base, one equivalent of hydrochloric acid was added to the stock solution to form the hydrochloride. An aliquot of this solution, when diluted and brought to a constant ionic strength of 0.10 M with potassium nitrate, was titrated with base. An examination of the potentiometric equilibrium curve (Fig. 1) shows the presence of two buffer regions. The first buffer region is due to the imidazolium proton, the second to the amino group.

When base was added to equimolar ratios of carnosine with one of the ions of Mg(II), Ca(II), Sr(II), and Cd(II), the first equivalent region of the potentiometric curve (Fig. 1) was identical with that of the free ligand, indicating that no coordination had taken place. In the second buffer region there was a significant lowering of the titration curve from that of the free ligand, indicating formation of the normal complex, ML^+. In the case of the Pb(II), Co(II), and Zn(II) ions there was significant deviation from the first buffer region of the free ligand curve, indicating the formation of protonated complexes, MHL^{2+}. Precipitation at the beginning of the second buffer region prevented further potentiometric studies of the normal complexes of these metal ions. The manganese(II) curve indicates definite interaction with the ligand in both buffer regions, with the formation of protonated and normal complexes. The behavior of Ni(II) with this ligand is unusual in that it is similar to that of Cd(II) in forming a normal complex (NiL^+) and no protonated complex, yet undergoes the dissociation of a third proton to give the species, NiA, which involves the dissociation of the amido hydrogen of the peptide linkage. The copper(II) titration curve has a single three-equivalent buffer region which indicates the formation of protonated and normal complexes, $CuHL^{2+}$ and CuL^+, as well as a metal chelate CuA in which the amido peptide hydrogen is dissociated. The potentiometric curve of mercury(II) is similar to that of Cu(II), but precipitation after the addition of the second equivalent of base prevented our determining whether mercury(II) could cause the ionization of the peptide hydrogen. Addition of silver(I) to carnosine caused immediate precipitation which did not dissolve after the addition of three equivalents of base.

TABLE I
METAL CHELATE STABILITY CONSTANTS[a]

Metal Ion	log K_{MHL}	log K_{ML}	log K_{MHL}^{H}	log K_{ML}^{H}
Ag(I)		Precipitate		
Ca(II)		3.22 ± 0.05		
Cd(II)		3.19 ± 0.02		
Co(II)	3.69 ± 0.02			
Cu(II)	5.01 ± 0.06	9.72 ± 0.06	4.65 ± 0.02	5.14 ± 0.02
		8.65[b]		5.55[b]
Hg(II)	5.27 ± 0.02	8.08	6.55 ± 0.02	
Mg(II)		3.10 ± 0.04		
Mn(II)	3.14 ± 0.02	4.40 ± 0.02	8.10 ± 0.02	
Ni(II)		5.42 ± 0.05		9.14 ± 0.02
Pb(II)	3.40 ± 0.02			
Sr(II)		3.34 ± 0.02		
Zn(II)	3.39 ± 0.02			

[a] t = 25°; μ = 0.10. [b] Dobbie and Kermack (1955), 20°.

The stability constants obtained from the potentiometric data calculated in accordance with the relationships given above, are collected in Table I.

DISCUSSION

The first buffer region of the free ligand curve is due to the imidazolium proton of the histidine ring, as may be seen from a comparison of the pK values of imidazole, histidine, and carnosine (Table II). It may be seen that the pK values of imidazole and histidine are comparable with the first pK of carnosine. If the first pK is assigned to the imidazole ring then the second may be assigned to the β-amino group of the β-alanyl moiety, as is indicated in Table II.

TABLE II
pK VALUES OF CARNOSINE AND RELATED LIGANDS AT 25°

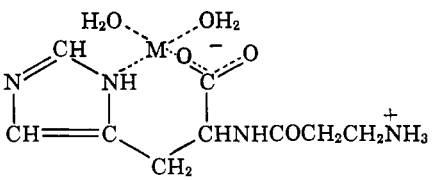
	pK_1 (Imidazolium)	pK_2 ($-NH_3^+$)
Carnosine	6.76[a] ± 0.01	9.36 ± 0.01
	6.86[b]	9.40[b]
Histidine	6.05[c]	9.17[c]
Imidazole	7.12[d]	

[a] This investigation, μ = 0.10 [b] Martin and Edsall (1960), μ = 0.16, 25°. [c] Li *et al.* (1955), μ = 0.15, 25°. [d] Tanford and Wagner (1953), μ = 0.15, 25°.

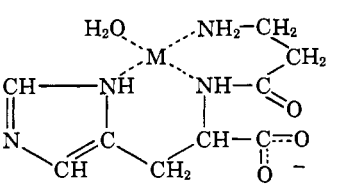
The formation of a protonated complex, evidenced by the decrease in pH over the free ligand, probably involves chelation through the imidazole ring of histidine and the carboxyl group, while the proton resides on the remote amino group of β-alanine as indicated by structure I. This is the case for Zn(II), Co(II), Pb(II), Mn(II), and Hg(II). With Mn(II) and Hg(II) the presence of a second buffer region indicates further chelation to the completely dissociated complex. In order to achieve additional coordination by the ligand it is probable that structural rearrangement occurs; i.e., the coordinated carboxyl group may be displaced by the amino group of β-alanine to form the six-membered chelate ring indicated by structure II. A similar observation has been made by Martin *et al.* (1960) in connection with stability studies on tri- and tetraglycine complexes.

In the case of Cd(II) and the alkaline earths, no protonated complexes are formed and very weak 1:1 complexes are formed at high pH. These complexes probably involve the same donor groups as those formed from the protonated complexes described above.

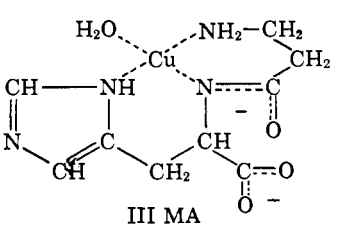
With nickel(II) there is a buffer region which requires three equivalents of base, although the initial part of the curve coincides with that of the pure ligand. Thus while a protonated chelate is not formed initially, there is no doubt but that a metal chelate analogous to structure II is formed, and that this compound undergoes further ionization. The last dissociation must involve simultaneous ionization of the peptide proton and coordination of the peptide nitrogen with nickel(II) to give a metal chelate similar to structure III. The dissociation of a peptide hydrogen in nickel chelates has been observed previously (Manyak *et al.*, 1955; Martin *et al.*, 1960; Martin and Edsall, 1960). Martin *et al.* (1960) showed that it was the peptide hydrogen rather than the water in the coordination sphere which ionized.



I MHL^{2+}



II ML^{+} (MHA^{+})



III MA

With copper(II) the reaction is rather more complex, since there is a single buffer region covering three equivalents of base, at much lower pH than that of the free ligand curve. This indicates that formation of protonated complex and of the normal 1:1 complex and ionization of the peptide hydrogen take place simultaneously or in overlapping steps. A probable structure of the copper complex from which the peptide hydrogen ion has been displaced is indicated by structure III. The structure involving coordination through the amide nitrogen atom is favored over that through the amide oxygen, since Doran *et al.* (1964)

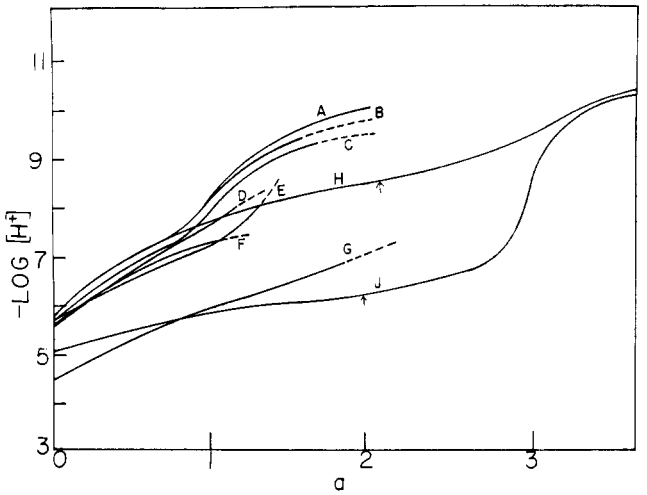


FIG. 1.—Potentiometric equilibrium curves of carnosine in the presence of molar equivalent concentrations of metal ions. A, no metal ion; B, Cd(II); C, Mn(II); D, Zn(II); E, Co(II); F, Pb(II); G, Hg(II); H, Ni(II); J, Cu(II); μ = 0.10 (KNO_3); t = 25.05 ± 0.05°; a = moles of base added per mole of ligand.

have shown with molecular models that chelation is sterically difficult in the latter tautomeric form for the analogous Cu(II)-histidyl histidine chelate.

Additional evidence for the conversion of compounds II and III may be found in the color changes to yellow for the Ni(II) chelate, and to purple for the Cu(II) chelate, as indicated by arrows in Figure 1. These color changes are similar to those observed by Manyak *et al.* (1955) for the analogous Ni(II) and Cu(II)-triglycine complexes. Further, the Cu(II) and Ni(II) chelates of carnosine are the only ones of those investigated for which equilibrium was attained slowly in the buffer region between two and three equivalents of base. A similar slow reaction, characteristic of coordination rearrangement, was also observed for Cu(II) and Ni(II) tri- and tetrapeptides by Martin *et al.* (1960), whereas rapid equilibrium, without the color change, was observed for the complexes of dipeptides.

Further investigation of the reactions of peptides with Ni(II) and Cu(II) is being carried out in these laboratories by spectroscopic measurements in the infrared and in the visible-ultraviolet regions (M. Kim and A. E. Martell, submitted for publication).

The stability constants (K_{ML}) are in the same order as ordinarily observed for amino acids chelates of divalent metal ions, i.e., Cu > Hg > Ni > Zn > Co > Cd > Mn > Mg ~ Sr ~ Ba. However, it is curious that the stabilities of the transition-metal chelates of the protonated forms, with the exception of that of Cu(II), are all of the same order of magnitude, i.e., $\sim 10^3$. These complexes may be considered as involving initial coordination through the carboxylate group and the imidazole ring.

The relative metal chelate stabilities listed in Table I indicate that carnosine is indeed an unusual ligand. The high stability of the Cu(II) complexes ML^+ and MHL^{2+} would be expected on the basis of the proposed structures. On the other hand the very low stabilities of the analogous Ni(II) chelates are certainly unexpected and unusual. The high stability of the Cu(II) chelate formed through interaction with the peptide linkage is similar to what has already been observed for the Cu(II) chelates of glycine peptides (Martin *et al.*, 1960; Martin and Edsall, 1960; Murphy and Martell, 1957; Dobbie and Kermack, 1955; Doran *et al.*, 1964). Similarly, the formation of analogous Ni(II) chelates with peptide linkages as donor groups has been observed to be considerably less stable than the Cu(II) complexes (Martin *et al.*, 1960; Martin and Edsall, 1960; Manyak *et al.*, 1955), in agreement with the findings in this investigation. The relatively low stabilities of the normal chelates of Co(II), Cd(II), and Mn(II) are about the order of magnitude that would be expected in comparison to those of Ni(II), but not with those of the Cu(II) ion. The coordination tendencies of the alkaline earth ions with this ligand seem unusually high when compared to those of the first-row transition metals [except copper(II)].

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