washed with 100 ml. of water, dried over magnesium sulfate and evaporated to dryness *in vacuo*. The dark metallic-colored hydrochloride (0.7 g.) was dissolved in methylene chloride and stirred with 2 g. of anhydrous sodium acetate until the dark metallic color of the solution changed to red (about 1 hr). The filtered solution was evaporated to dryness *in vacuo*, leaving 0.60 g. (59%) of red crystals, m.p. 115–120°. Recrystallization from toluene–petroleum ether (b.p. 30–60°) gave red crystals (about 50% recovery) as a toluene solvate, m.p. 60–70°, resolidifying and remelting at 139–141°; $\lambda_{max(\mu)}^{Nujol}$ 5.60 (azlactone C=O); 6.20, 6.29 (C=C, C=N, aryl); 12.2 (*p*-disubstituted benzene); 13.2 (monosubstituted benzene of toluene). The compound traveled as a single spot (R_f 0.24) in system A,[30] giving an intense yellow fluorescence under ultraviolet light.

Anal. Calcd. for $C_{30}H_{18}Cl_2N_2O_2 \cdot 1/2\ C_7H_8$: C, 64.9; H, 5.06; Cl, 16.3. Found: C, 65.1; H, 5.38; Cl, 16.2.

A sample dried at 100° (0.5 mm.) then melted at 139–141° and had the analysis:

Anal. Calcd. for $C_{30}H_{18}Cl_2N_2O_2$: C, 61.7; H, 4.67; Cl, 18.3; N, 7.20. Found: C, 61.6; H, 4.82; Cl, 18.3; N, 7.12.

Methyl α-Benzamido-*p*-[bis-(2-chloroethyl)-amino]-cinnamate (XIV).—A solution of 10.0 g. (0.026 mole) of methyl α-benzamido-*p*-[bis-(2-hydroxyethyl)-amino]-cinnamate (X) in 60 ml. of freshly distilled phosphorus oxychloride was heated in an oil-bath at 70–75° for 30 minutes. The red solution was poured into excess crushed ice and stirred for 15 minutes. The mixture was extracted with methylene chloride (2 × 150 ml.). The combined extracts were thoroughly shaken with 50 ml. of saturated aqueous sodium acetate, then dried with magnesium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in 100 ml. of methylene chloride, then clarified with Norit. Addition of 100 ml. of ether and 25 ml. of petroleum ether (b.p. 30–60°) followed by cooling in an acetone-Dry Ice-bath gave 4.9 g. (45%) of light yellow needles, m.p. 138–139°; $\lambda_{max(\mu)}^{Nujol}$ 3.10 (NH); 5.82, 8.32, 8.45 (ester); 6.04, 6.58 (amide); 12.2 (*p*-disubstituted benzene); 13.8 (C–Cl); 14.1 (benzoyl); no COH near 2.9 and 9.5; no azlactone C=O near 5.6. The compound traveled on paper as a single spot (R_f 0.63) in system A.[30]

Anal. Calcd. for $C_{21}H_{22}Cl_2N_2O_4$: C, 59.9; H, 5.28; Cl, 16.9; N, 6.66. Found: C, 60.0; H, 5.35; Cl, 16.8; N, 6.66.

***p*-[Bis-(2-chloroethyl)-amino]-phenylpyruvic Acid (XII).**—To 30 ml. of reagent methanol, saturated with hydrogen chloride at 5°, was added 1.0 g. (2.4 mmoles) of methyl α-benzamido-*p*-[bis-(2-chloroethyl)-amino]-cinnamate (XIV). The solution was refluxed for 18 hr. protected from moisture, then evaporated to dryness *in vacuo*. The crude residue (XIIIb) gave no color with ferric chloride and showed a normal ester carbonyl at 5.73 μ in the infrared.

A solution of the intermediate XIIIb in 25 ml. of 12 *N* hydrochloric acid was heated on the steam-bath for 20 minutes, then quickly chilled to about −10°. The cold mixture was washed with ether (3 × 20 ml.) to remove benzoic acid. The aqueous solution was neutralized with solid sodium acetate, diluted with about two volumes of water and extracted with ether. Dried with magnesium sulfate, the ether solution was diluted with 50 ml. of toluene, then evaporated to dryness *in vacuo*, leaving 0.7 g. (97%) of a green oil that solidified on cooling. A solution of the crude product in 15 ml. of ethyl acetate was diluted with 75 ml. of petroleum ether (b.p. 30–60°). The clear solution was decanted from the dark green gum that separated, then evaporated *in vacuo*, leaving a light green solid. Recrystallization from methylene chloride–petroleum ether (b.p. 30–60°) gave 0.50 g. (70%) of light green crystals, m.p. 137–139°. Further recrystallization from methylene chloride afforded 0.40 g. (55%) of yellow crystals, m.p. 154–155°; $\lambda_{max(\mu)}^{Nujol}$ 2.93 (enolic OH); 3.5–4.5 (carboxyl OH); 6.07 (chelated C=O and COOH); 6.22, 6.55 (aryl); 8.15, 8.42 (COOH); 12.32 (*p*-disubstituted phenyl); free of alcohol C–OH near 9.5.

Anal. Calcd. for $C_{13}H_{15}Cl_2NO_3$: C, 51.3; H, 4.97; Cl, 23.4; N, 4.61. Found: C, 51.4; H, 5.26; Cl, 23.8; N, 4.79.

No suitable solvent for paper chromatography could be found that gave consistent results, although the best results were obtained with system A; in most runs the compound streaked. Occasionally a paper chromatogram was obtained without streaking, in which case the compound moved as a single spot with R_f 0.81.

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[CONTRIBUTION FROM THE COBB CHEMICAL LABORATORY, UNIVERSITY OF VIRGINIA, CHARLOTTESVILLE, VA.]

The Association of Divalent Cations with Anserine[1]

BY R. BRUCE MARTIN

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The substitution of a methyl group in the 1-position of the imidazole ring of carnosine to yield anserine does not alter the chelating properties with cupric ion. However, the tendency to chelate with nickel ion is reduced by methylation. For both dipeptides the ionization of the amide hydrogen is induced by either metal ion at about neutral *p*H values. Plausible structures of the chelate compounds are discussed.

A recent study[2] reported the association of divalent copper, nickel and zinc ions with L-carnosine (β-alanyl-L-histidine). The first two ions promoted the ionization of the amide hydrogen at physiological *p*H values or less. The recent availability of L-anserine (β-alanyl-L-1-methylhistidine) makes possible a test of the proposals which have been made[2,3] for the association and subsequent ionization of the chelate complexes formed. This paper describes the results of titrating mixtures of divalent cations and anserine with standard base. A comparison is made with the results obtained from similar titrations with carnosine.

Experimental

The techniques have previously been described.[3] Carnosine and anserine nitrate were products of the California Corporation for Biochemical Research.

Results and Discussion

The results of the titrations of mixtures of copper(II) or nickel(II) ions with anserine and, for comparison, with carnosine are shown in Fig. 1. The curves for copper(II) ion are nearly identical for both peptides. In the case of nickel(II) ion the curve for anserine is displaced to higher *p*H values indicating a weaker interaction.

(1) This research was supported by a grant from the National Science Foundation (G9796).

(2) R. B. Martin and J. T. Edsall, THIS JOURNAL, **82**, 1107 (1960).

(3) H. Dobbie and W. O. Kermack, *Biochem. J.*, **59**, 246 (1955).

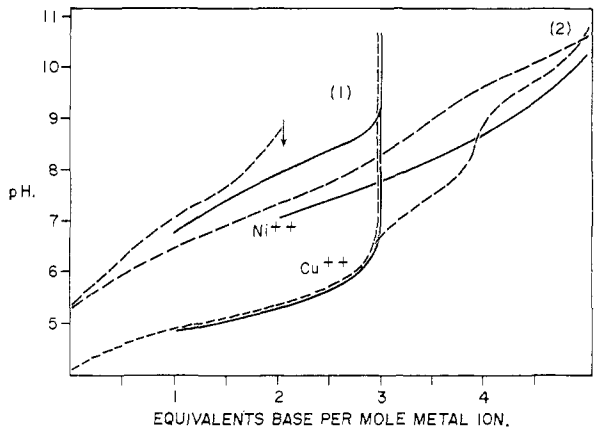


Fig. 1.—Titration curves of 0.02 M carnosine (———) or anserine (- - - -) nitrate and either copper(II) or nickel(II) ions with 0.150 M base. Numerals indicate molar ratios of ligand to metal ion. Arrow indicates onset of precipitation.

In Table I the pK_a values for carnosine and anserine appear in the second column. The first ionization constant is mainly that of the imidazole group and the second that of the α-amino group. The increase in the pK_a of the imidazole group may be accounted for entirely by statistical arguments since a proton may ionize from only one position in anserine and two in carnosine. The expected increase in basicity of anserine due to the effect of the methyl group is apparently compensated by the greater symmetry of the protonated, non-methylated ring in carnosine.

TABLE I

IONIZATION CONSTANTS OF L-CARNOSINE AND L-ANSERINE AT 25° AND 0.16 IONIC STRENGTH REPORTED AS pK_a VALUES

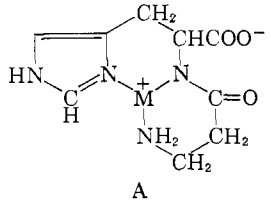
Metal ion	None	Cu^{++}	Ni^{++}
Carnosine	6.86, 9.40	5.0, 5.6	7.35, 8.4
Anserine	7.15, 9.45	5.0, 5.6	7.6, ppt.

The logarithms of the first association constants for the binding of divalent copper, nickel and zinc ions to anserine are about 0.1 log unit greater than those quoted for carnosine.[2] Limited quantities of the expensive anserine prevent a more detailed analysis. The successive formation constants of zinc ion and anserine are greater than expected on statistical arguments as in the case of carnosine[4] and other zinc complexes.

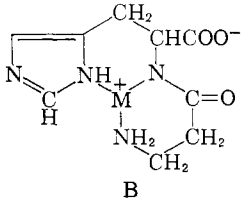
As shown in Fig. 1, titration of equimolar mixtures of L-anserine nitrate and copper(II) ions with base results in the addition of three equivalents of base in order to obtain a pH of about 10 or greater. The acid ionization constants given in Table I for the addition of the second and third equivalent of base are 5.0 and 5.6, respectively, identical with those of carnosine.[2] This is an additional proof that it is the amide hydrogen that ionizes in carnosine. Thus, not only does the amide hydrogen ionize in anserine, but at the same pH as in carnosine. Therefore, the structures of the copper chelates are probably similar.

For equimolar mixtures of L-anserine nitrate with nickel(II) ion, only a pK_1 value of 7.6 is obtained due to precipitation occurring during the addition of the third equivalent of base. However, a 2:1 anserine nitrate–nickel ion mixture titrates 5 equivalents of base per mole of nickel ion as shown in Fig. 1. Therefore, the amide hydrogen ionizes from half of the anserine present in this 2:1 mixture. A 2:1 anserine nitrate–cupric ion mixture also titrates 5 equivalents of base but with different pK values as shown in Fig. 1 and Table I. Precipitation prevented the determination of a pK_1 value for an equimolar cadmium–anserine mixture as has been done for carnosine.[2]

The conclusion is that the amide hydrogen is capable of ionization at less than physiological pH values in both carnosine and anserine in the presence of divalent copper or nickel ions. Two possible structures of carnosine and either divalent copper or nickel ion after the amide ionization are represented in A and B.



A



B

Structure A would seem the more plausible, since it has been the opinion of most investigators that the pyridine and not the pyrrole nitrogen of the imidazole ring is involved in metal complex formation.[5] The identity of the titration curves of anserine and carnosine with cupric ion implies that the same structure must be present for both peptides. However, structure A is not possible for anserine. Therefore, structure B is the favored for the cupric chelate of both anserine and carnosine after ionization of the amide hydrogen. Structure B is similar to the structure proposed by Dobbie and Kermack[3] for the cupric chelate of carnosine.

The ionizations from the nickel(II) ion complex of carnosine occur at lower pH values than for the corresponding anserine complex. Therefore, structure A may be favored for the nickel–carnosine complex and structure B required for the nickel–anserine complex.

(4) A miscount occurs in the first paragraph of page 1108 of reference 2, since a six-membered ring could form by metal ion chelation with the amino nitrogen and the carbonyl oxygen of carnosine. This weakens, but does not destroy, the argument that initial combination by a metal ion takes place predominantly at the imidazole ring. It would be difficult to explain the pK_1 value of the titration curve of the equimolar carnosine and cadmium(II) ion mixture if this were not the case.

(5) N. C. Li, J. M. White and E. Doody, THIS JOURNAL, 76, 6219 (1954).