Stability of Fused Rings in Metal Chelates. X. Structures and Stability Constants of the Copper(II) Complexes of Tripeptides Composed of Glycine and/or β-Alanine

Osamu YAMAUCHI, Yasuo NAKAO, and Akitsugu NAKAHARA

Institute of Chemistry, College of General Education, Osaka University, Toyonaka, Osaka 560

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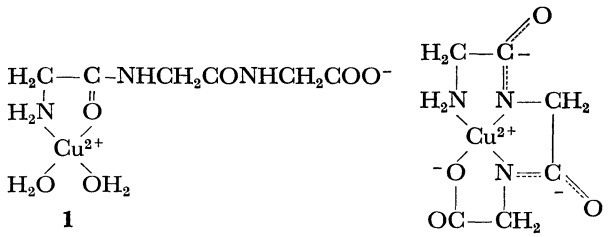
On the basis of comparative studies of the equilibria involving copper(II) and triglycine (G·G·G), glycylglycyl-β-alanine (G·G·β-A), glycyl-β-alanylglycine (G·β-A·G), or β-alanylglycylglycine (β-A·G·G), the structures of the complexes formed at various pH values and the structure-stability relationship between the fused-ring chelates have been discussed in reference to the equilibrium constants determined by potentiometric titration at 25° (μ=0.1 (KNO_3)) and the properties of the copper(II) chelates isolated. For each tripeptide, the stability constant K_1 for the 1:1 complex and the constants for the deprotonation reactions K_{c_1} and K_{c_2} were calculated by the method of non-linear least-squares. For G·G·G, G·G·β-A, and G·β-A·G, the equilibrium indicating the formation of a protonated complex was detected at pH 3.5—4.5. The relatively small difference in the log K_1 values (5.25—5.60) and the $-\log K_{c_1}$ values (5.23—5.36) and the larger difference in the $-\log K_{c_2}$ values (5.54—6.73) have been interpreted in terms of the steric requirements around copper(II) in the fused-ring chelates. Relative stabilities of the fused-ring systems in the deprotonated chelates have been inferred from the $K_1K_{c_1}K_{c_2}$ values to be in the order 5-6-5(G·β-A·G–Cu(II))≃6-5-5(β-A·G·G–Cu(II))>5-5-6(G·G·β-A–Cu(II))>5-5-5(G·G·G–Cu(II)).[1]

In view of the importance of the metal-protein interactions in biological systems, attention has been paid to various fundamental aspects of the protein-metal bonding, such as the bonding modes and the extents or strengths of the interactions in simplified systems. The complex formation between peptides and metal ions has been extensively investigated.

However, there still exist some ambiguities about the nature of the metal–peptide binding in aqueous solution, which is a prerequisite for understanding the interactions in the biological fluid. In this connection we have extended our studies on the copper(II) complexes of the dipeptides[2] and the amino acid amides[3] to the tripeptides composed of glycine and β-alanine, in order to reveal their behavior toward copper(II) ion.

Valuable findings characterizing the triglycine-copper(II) complexes in solution have accumulated by detailed potentiometric,[4–8] thermodynamic,[9,10] kinetic,[11,12] and spectral studies.[6,7,13] The coordination sites in the complexes in aqueous solution have been inferred from these findings and the results of X-ray crystal structure analyses[14,15] to be the amino nitrogen and the peptide oxygen in the acid region (structure **1**), and the amino nitrogen, the peptide nitrogens and the carboxyl oxygen in the neutral and alkaline regions (structure **2**). Probably because of the steric requirements around copper(II), the triglycine complex isolated from the alkaline solution has a dimeric structure,[15] where the carboxyl group coordinates to the copper(II) above (or below) the plane of the chelate ring considered and one of the peptide nitrogens axially interacts with that copper(II).



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We considered it to be of basic importance to investigate how and to what extent the steric requirements in the consecutive chelate rings affect the stability in solution of the copper(II)–tripeptide complexes and, if possible, to infer the bonding modes in the fused-ring chelates. Since replacement of a glycyl group of triglycine with a β-alanyl group is expected to introduce a six-membered ring which would affect the stability of the fused-ring system, we have investigated the solution equilibria of the copper(II) complexes of several tripeptides containing glycine and/or β-alanine, and attempted to elucidate the structure-stability relationship between them.

1) Such notations are used throughout the text to express arrangement and size of the individual rings involved in the fused-ring system.

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12) H. Hauer, E. J. Billo, and D. W. Margerum, *ibid.*, **93**, 4173 (1971).

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TABLE 1. ELEMENTAL ANALYSIS AND SOME PROPERTIES OF TRIPEPTIDE-COPPER(II) CHELATES

Complex	Elemental analysis						Absorp. max.		
	C(%)		H(%)		N(%)		Dec. temp.[b] (°C)	($\times 10^3$ cm^{-1}) (log ε)	pH[c]
	Calcd	Found	Calcd	Found	Calcd	Found			
Na[Cu–G·G·G]·$2H_2O$[a]	23.34	23.24	3.93	3.63	13.61	13.57	250—263	17.9 (2.16)	9.0
Na[Cu–G·G·β-A]·$4H_2O$	23.43	23.75	5.07	4.90	11.71	11.31	235—245	18.0 (2.02)	8.6
K[Cu–G·β-A·G]·$2H_2O$	24.80	25.08	4.17	4.52	12.40	12.29	240—253	18.4 (1.77)	7.5
Na[Cu–β-A·G·G]·$5H_2O$	22.31	22.37	5.36	4.96	11.15	11.18	255—260	17.8 (1.92)	8.8

a) The corresponding chelate used by Freeman *et al*[15] for X-ray study was a monohydrate.
b) Determined on a micro melting point apparatus.
c) Values of 5.0×10^{-3}M aqueous solutions of the chelates.

For the present purpose, we chose triglycine (G·G·G), glycylglycyl-β-alanine (G·G·β-A), glycyl-β-alanylglycine (G·β-A·G), and β-alanylglycylglycine (β-A·G·G) as typical ligands, and carried out potentiometric titration and preparation of the copper(II) chelates. Determination of the equilibrium constants and the discussion of the structures and relative stabilities of the complexes in solution constitute the main subjects.

Experimental

Ligands. *G·G·G*: This was purchased from Nakarai Chemicals Co., Ltd. and checked by elemental analysis and amino acid analysis with a Yanagimoto amino acid analyzer LC-5.

G·G·β-A: To a cooled and stirred solution of glycyl-β-alanine[16] (7.8 g) in 4 M sodium hydroxide (13.4 ml) was added, alternately, chloroacetyl chloride (9.1 g) and 4 M sodium hydroxide (20.4 ml) at such a rate that the reaction mixture could be maintained alkaline at a temperature below 5 °C. After addition was over, stirring was continued for 10 min, and the pH of the solution was adjusted to *ca.* 2 with concd. hydrochloric acid. The colorless precipitate formed was collected by filtration and recrystallized from acetone to give chloroacetylglycyl-β-alanine, mp 158—159 °C (uncor.). Found: C, 37.75; H, 4.83; N, 12.20%. Calcd for $C_7H_{11}N_2O_4Cl$: C, 37.76; H, 4.99; N, 12.58%.

Chloroacetylglycyl-β-alanine was treated with aqueous ammonia as for chloroacetyl-β-alanine,[16] and the product was recrystallized from water–ethanol to give G·G·β-A, which was checked by melting point[16] and elemental analysis.

G·β-A·G: This was prepared by catalytic reduction[16] of carbobenzoxyglycyl-β-alanylglycine prepared from β-alanylglycine[17] and carbobenzoxyglycyl chloride,[18] and checked by melting point and elemental analysis.

β-A·G·G: This was prepared and checked analogously.[16]

(1) Copper(II) Chelates: All the chelates were prepared by the method described for sodium glycylglycylglycinatocuprate(II) monohydrate[15] and dried over silica gel. The analytical data and the spectral properties of the four chelates isolated are summarized in Table 1.

(2) Measurements of Spectra: Visible spectra were measured for 5.0×10^{-3} M aqueous solutions with a Shimadzu QR-50 spectrophotometer.

(3) pH Titrations: *(a) Reagents*: 0.1 M Potassium hydroxide, 0.1 M nitric acid, and 0.01 M copper(II) nitrate were prepared in the same manner as described.[3] All the reagents used were of analytical grade, distilled and deionized water being used.

(b) Apparatus: A Radiometer PHM 4d pH meter equipped with a G202B glass electrode and a K401 calomel electrode was used after standardization with a Horiba and a Radiometer standard buffer solution (pH 4.01 and 6.48 at 25 °C).

(c) Procedure: The procedure was essentially the same as that reported.[3] An aqueous solution containing nitric acid and equimolar amounts of a ligand and copper(II) nitrate (*ca.* 0.004 M) was titrated with 0.1 M potassium hydroxide, the pH being measured at 25±0.05 °C under a nitrogen atmosphere. The ionic strength was adjusted to 0.1 with potassium nitrate. The ligands were titrated in the absence of copper(II) nitrate under the same conditions. Conversion of pH to $-\log[H^+]$ and determination of the apparent ion product of water were made as reported.

Results

Titration Curves and Equilibria. The titration curves obtained for the tripeptides in the absence and the presence of copper(II) are given in Figs. 1 and 2, respectively. All the peptides give similar titration curves with four protons dissociating from the protonated peptide molecule in the presence of copper(II). The same reaction sequence can thus be reasonably assumed for all the systems. The G·G·G–Cu(II) system has been expressed by the following equilibria in acid and neutral solutions corresponding to *a* values (moles of KOH added per mole of ligand) of 1—4:[4-8]

$$Cu^{2+} + H_2L^- \overset{K_1}{\rightleftharpoons} Cu(H_2L)^+ \quad (1)$$

$$Cu(H_2L)^+ \overset{K_{c_1}}{\rightleftharpoons} Cu(HL) + H^+ \quad (2)$$

$$Cu(HL) \overset{K_{c_2}}{\rightleftharpoons} CuL^- + H^+ \quad (3)$$

where Cu^{2+} and H_2L^- refer to free copper(II) ion and free ligand, respectively, and equilibria (2) and (3) express the dissociation of protons from the peptide groups in the complex. The equilibrium constants are

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17) Y. Nakao, H. Ishibashi, and A. Nakahara, This Bulletin, **43**, 3457 (1970).
18) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

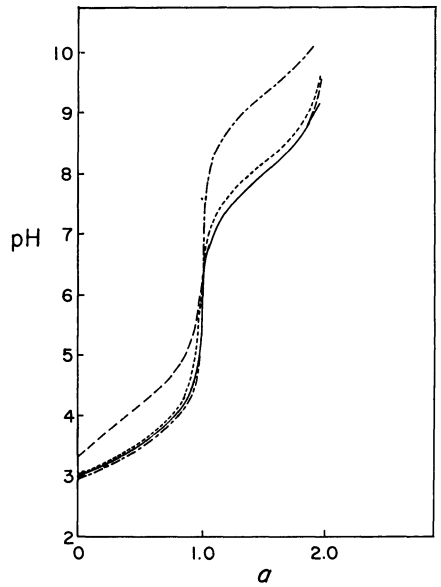


Fig. 1. Titration curves for the tripeptides.
———: G·G·G, – – – –: G·G-β-A, – – – –: G·β-A·G,
— — —: β-A·G·G.
a: moles of KOH added per mole of ligand.

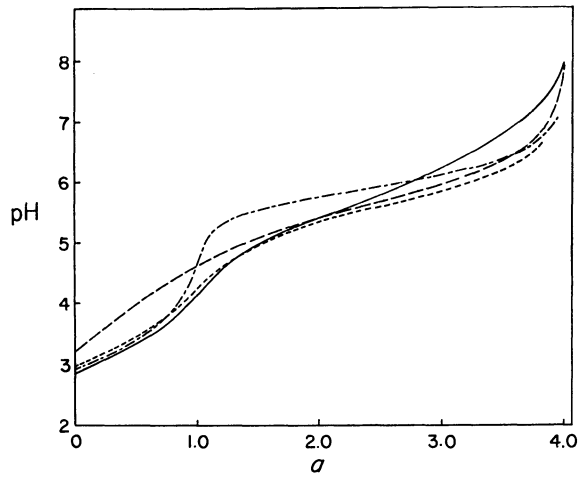


Fig. 2. Titration curves for the 1:1 tripeptide–copper(II) systems.
———: G·G·G-Gu(II), – – – –: G·G·β-A-Cu(II),
– – – – –: G·β-A·G-Cu(II), —·—·—: β-A·G·G-Cu(II).

defined by the following equations.

$$K_1 = \frac{[Cu(H_2L)^+]}{[Cu^{2+}][H_2L^-]} \quad (4)$$

$$K_{c_1} = \frac{[H^+][Cu(HL)]}{[Cu(H_2L)^+]} \quad (5)$$

$$K_{c_2} = \frac{[H^+][CuL^-]}{[Cu(HL)]} \quad (6)$$

In the pH range 3.5—4.5, pH depressions due to complex formation with copper(II) are small but not negligible for the G·G·G–, G·G·β-A–, and G·β-A·G–Cu(II) systems, whereas hardly any depression is observed for the β-A·G·G–Cu(II) system. In order to achieve better fit to the titration curves in this region, it was found necessary to add the following equilibria when the tripeptides have glycine residue as the NH_2-terminus:

$$Cu^{2+} + H_3L \overset{K^H}{\rightleftharpoons} Cu(H_3L)^{2+} \quad (7)$$

$$Cu(H_3L)^{2+} \overset{K_a^H}{\rightleftharpoons} Cu(H_2L)^+ + H^+ \quad (8)$$

where H_3L is the zwitterionic species of the tripeptides and $Cu(H_3L)^{2+}$ the protonated complex. The equilibria involving such a protonated complex have been reported for G·G·G at μ=3.0 ($NaClO_4$) by Österberg and Sjöberg,[8] and they seem to exist also in the other tripeptide–Cu(II) systems in 0.1 M KNO_3.

Method of Calculation. On the basis of equations for the mole balance for total ligand and total metal ion concentrations C_L and C_M and for the electroneutrality of solution, the equilibrium constants K^H, K_1, K_{c_1}, and K_{c_2} were calculated by the method of non-linear least-squares with the aid of a NEAC 2200/500 computer. Constant K_a^H for the equilibrium (8) was calculated by

$$K_a^H = \frac{K_{a_2}K_1}{K^H} \quad (9)$$

where K_{a_2} refers to the acid dissociation constant of the amino group.

Details of the computer calculations are the same as those reported previously.[3] The Newton-Raphson iterations for calculating the free ligand and free metal ion concentrations at each data point were continued until satisfactory convergence had been attained. When the residuals $|C_L(\text{observed}) - C_L(\text{calcd})|$ and $|C_M(\text{observed}) - C_M(\text{calcd})|$ failed to converge to within $10^{-5} \times C_L(\text{observed})$ and $10^{-5} \times C_M(\text{observed})$, respectively, after 150 iterations, the calculation was abandoned, and the computer proceeded to the next data point. When the total number of such non-convergent data points amounted up to 10, the initial constants were replaced by another set of estimates. Least-squares refinements were complete after 5—10 cycles when the initial estimates were nearly satisfactory, but 30 or more cycles were necessary when they were poor. Usually, 70—100 data were subjected to the least-squares treatment, which afforded the constants that could satisfactorily reproduce the titration curves. The difference between the observed and calculated titers of 0.1 M potassium hydroxide was less than 0.03 ml for all the data points with the titers ranging from 2.0 to 6.5 ml. The acid dissociation constants for each ligand were calculated from 40—50 data points.

Equilibrium Constants. Table 2 shows the equilibrium constants for the tripeptides and the two dipeptides included for comparison. The excellent fit of the theoretical titration curves to the observed ones over all the data points used substantiates the equilibria considered and the relevant constants in the 1:1 tripeptide–Cu(II) systems. When the theoretical curves were described by the three constants K_1, K_{c_1}, and K_{c_2} calculated from the data corresponding to a=1—4, serious deviations (*ca.* 8% when expressed in the titer) from the experimental curves were observed at $a<1$ for G·G·G–, G·G·β-A–, and G·β-A·G–Cu(II) systems. In the case of the β-A·G·G–Cu(II) system, calculation of the reliable values of K^H was not successful probably because of the very low stability of

Table 2. Equilibrium constants (25±0.05 °C; μ=0.1 (KNO_3))[a]

Ligand	pK_{a_1}	pK_{a_2}	log K^H	$pK_a^{Hb)}$	log K_1	pK_{c_1}	pK_{c_2}	log $K_1K_{c_1}K_{c_2}$
G·G·G	3.26±0.002	7.93±0.009	1.7[c]	4.4	5.25±0.003	5.23±0.003	6.73±0.002	−6.71
G·G·β-A	4.08±0.005	7.93±0.005	1.9	4.6	5.25±0.004	5.27±0.006	6.08±0.002	−6.10
G·β-A·G	3.34±0.005	8.09±0.002	1.8	4.2	5.60±0.003	5.36±0.004	5.74±0.003	−5.50
β-A·G·G	3.23±0.003	9.29±0.007	<1		5.28±0.018	5.32±0.027	5.54±0.011	−5.58
G·G[d]	3.14±0.006	8.09±0.006	negligible		5.50±0.001	4.10±0.001	[e]	
β-A·G[d]	3.22±0.009	9.45±0.009	negligible		5.45±0.001	4.09±0.001	[e]	

a) Variances are expressed in standard deviations estimated from the elements of the inverted matrix and the assumed error (0.01 ml) for the titers of 0.1 M potassium hydroxide. b) Calculated by eq. (9). c) The value reported by Österberg and Sjöberg[8] is 1.58 at 25° (μ=3.0($NaClO_4$)). d) Previously reported.[3] e) Not measured.

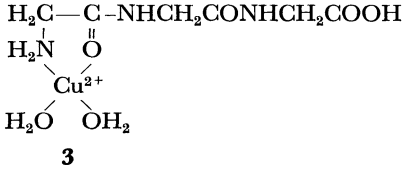
the protonated complex. Log K^H and hence the pK_a^H (=$-\log K_a^H$) values may not be sufficiently accurate, and we may safely use two significant figures for each. Some characteristic features of chelate stability can be seen from Table 2. The log K_1 values for G·G·G, G·G·β-A, and β-A·G·G are nearly the same, the value for G·β-A·G being slightly higher. A somewhat different trend is observed for the log K^H values which indicate the importance of the size of the NH_2-terminal chelate ring and the pK_a of the amino group. It is also evident from the values for G·G·G and G·G·β-A that the nature of the COOH-terminus has practically no influence on the stability constants.

Replacement of a glycine residue of G·G·G with a β-alanine residue does not seem to affect the log K_1 and pK_{c_1} (=$-\log K_{c_1}$) values greatly, but the steric effects associated with it are most clearly reflected in the pK_{c_2} (=$-\log K_{c_2}$) values, which vary from 5.54 of β-A·G·G to 6.73 of G·G·G.

The relative abundances of the species present at various pH in the tripeptide–copper(II) systems were calculated from the equilibrium constants and plotted against pH (Figs. 3(a)—(d)).

Discussion

The sequence and mode of coordination to copper(II) of the amino, peptide, and carboxyl groups of peptides have been discussed by many investigators. As regards the structures of the protonated copper(II) complexes $Cu(H_3L)^{2+}$ in acid solution, the log K^H values suggest the structure like **3** where the amino group in the coordination sphere of copper(II) is



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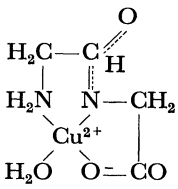
assumed with the carboxyl group left un-ionized. Such a structure seems to explain the higher stability of the protonated G·G·G–, G·G·β-A–, and G·β-A·G–Cu(II) complexes, which have a stable five-membered ring as compared with the less stable six-membered ring in the β-A·G·G–Cu(II) complex. The structure is not inconsistent with the crystal structure for $Cu(H_2L)^+$ [14] formed upon dissociation of a proton from $Cu(H_3L)^{2+}$ and is also in line with the NMR studies by Li *et al.*[19] and Kim and Martell[13] on the pH-dependent disappearance of the signals of the three CH_2-groups in the G·G·G–Cu(II) complex. On the other hand, no appreciable formation of the protonated complexes was detected for glycylglycine(G·G) and β-alanylglycine(β-A·G). Their titration curves could be reproduced without serious deviations by K_1 and K_{c_1} over the range covering a=0—3. Since the pK_a values of G·G do not differ much from those of G·G·G, the lack of the intermediate step similar to reaction 7 in the G·G–Cu(II) system suggests that the complex formation therein must be achieved, even before dissociation of the peptide proton, by the coordination of the amino, peptide, and carboxyl groups (structures **4a** and **4b**). This mode of coordination may be due to the quite favorable locations of these groups in the ligand molecule for constructing a fused-ring chelate. The reason for the behavior of β-A·G may in part be the same as that for β-A·G·G.



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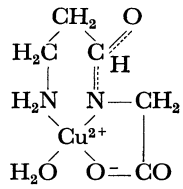


4b

We have disclosed by X-ray crystal structure analysis[20] that chloroglycylglycinatocopper(II) monohydrate, which corresponds to the tripeptide complexes of the type $Cu(H_2L)^+$, has a dimeric structure very similar to that of the G·G·G–Cu(II) complex. Although the crystal structure generally gives evidence to the structures in solution, it seems unlikely that the carboxyl groups maintain the dimeric structure even in dilute aqueous solution.[2,3] In this connection, Bair and Larsen[21] inferred from spectral studies on the isolated chelates that the binding sites in the G·G–Cu(II) chelate of the type $Cu(HL)^+$ (HL^- refers to the free G·G ion) were the amino and peptide nitrogens, although contribution of the carboxyl group remained

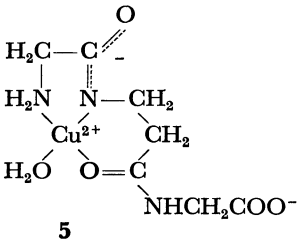
19) N. C. Li, R. L. Scruggs, and E. D. Becker, *J. Amer. Chem. Soc.*, **84**, 4650 (1962).

20) M. Shiro, Y. Nakao, O. Yamauchi, and A. Nakahara, *Chem. Lett.*, **1972**, 123.

21) M. L. Bair and E. M. Larsen, *J. Amer. Chem. Soc.*, **93**, 1140 (1971).

unknown.

The complexes of the type Cu(HL), where one of the peptide hydrogens is removed, have been expressed by a fused-ring structure like **5** which is also consistent with our finding about the pK_{c_1} values. We see from Table 2 that the pK_{c_1} values for the four tripeptides are very close to each other. If we assume the coordination by the tripeptides to be bidentate, we should expect a



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higher pK_{c_1} value for β-A·G·G from the usual rule for the steric effects of the ring size on stability, because pK_{c_1} can be taken as a measure of the steric accessibility of the peptide group to copper(II). The degrees of formation of Cu(HL) can be compared at a constant pH by the $K_1K_{c_1}$ values according to the following equation[2]

$$\frac{[Cu(HL)]}{[Cu^{2+}][H_2L^-]} = \frac{K_1K_{c_1}}{[H^+]} \qquad (10)$$

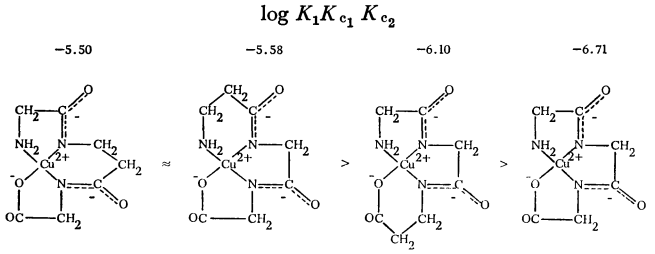
The small difference between the ligands (less than 0.28 log unit) is similar to that in the results obtained for the dipeptide–copper(II) systems and may be considered as indicative of approximately equal stability of 5-5-membered systems (G·G·G– and G·G·β-A–Cu(II)) and 6-5-(or 5-6-)membered systems (β-A·G·G– and G·β-A·G–Cu(II)).

In contrast to the log K_1 and pK_{c_1} values, a striking difference is observed between the pK_{c_2} values. The value for G·G·G is the highest, which may reasonably be interpreted as resulting from the steric strain due to the fused 5-5-5-membered ring (structure **2**), where the accumulated ring strains can not be relieved by a six-membered ring existing in the complexes of the other three β-alanine-containing tripeptides, whose pK_{c_2} values are more than 0.6 log unit lower.

The relative stability of the complexes of the type CuL^- at a constant pH inferred from the $K_1K_{c_1}K_{c_2}$ values in the following equation clearly indicates the effects of the overall steric requirements around copper(II) on the stability of the chelates:

$$\frac{[CuL^-]}{[Cu^{2+}][H_2L^-]} = \frac{K_1K_{c_1}K_{c_2}}{[H^+]^2} \qquad (11)$$

We see that the values decrease in the order G·β-A·G≈



Scheme 1. Relative stabilities of the tripeptide–Cu(II) chelates of the type CuL^-

β-A·G·G>G·G·β-A>G·G·G (Scheme 1), being the manifestation of accumulation of, or release from, the strains in the rigid fused-ring systems composed of three individual rings. The low stability of the probably monomeric G·G·G–Cu(II) formed in neutral and alkaline solutions might give rise to the formation of a dimer with a less hindered structure in the crystalline state.[15] Hence, it may be expected that the

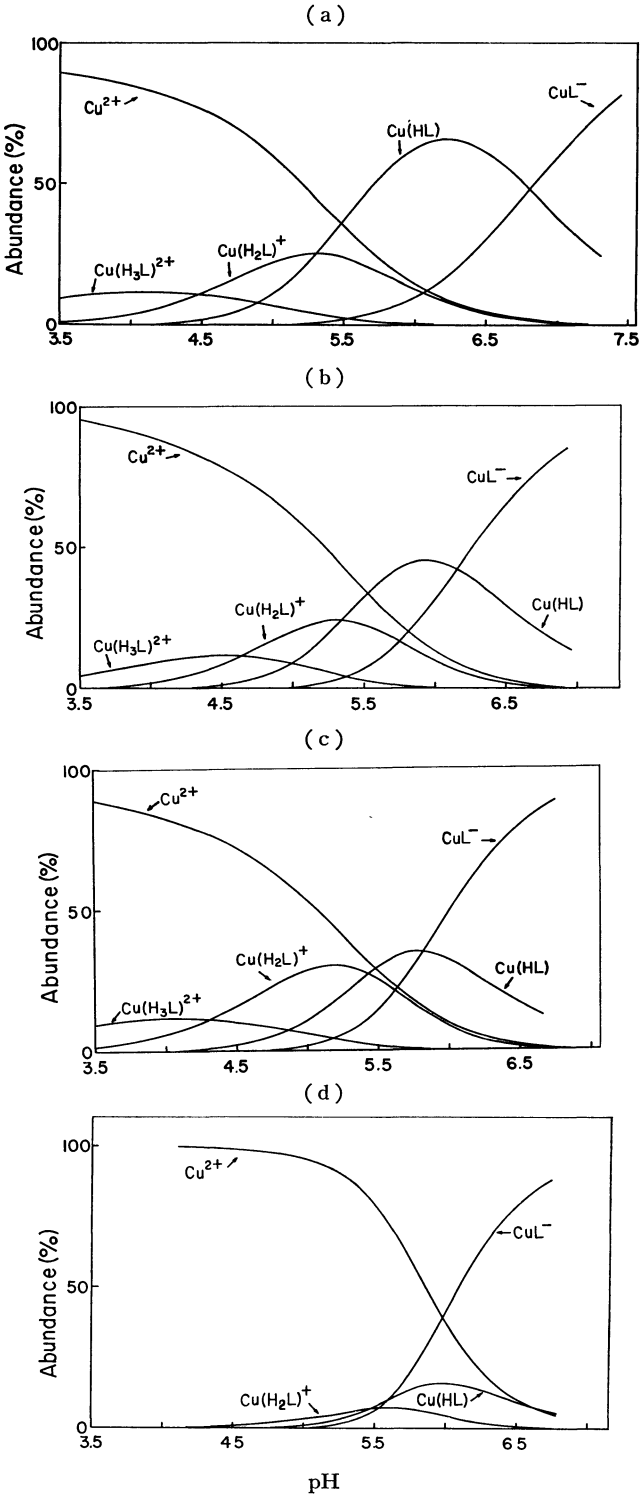


Fig. 3. Calculated abundances of the coordinated species in the 1:1 tripeptide–copper(II) systems.
(a): G·G·G–Cu(II) system, (b): G·G·β-A–Cu(II) system, (c): G·β-A·G–Cu(II) system, (d): β-A·G·G–Cu(II) system.

chelates are monomeric also in the crystalline state when the steric strains are cancelled by a β-alanine residue. It is of particular interest to note in this connection that the recent studies[22] on temperature dependence of magnetic susceptibilities of the chelates isolated as crystals indicated no copper(II)-copper(II) interactions. This supports the view that in the crystalline state the chelates of the β-alanine-containing tripeptides may have monomeric structures. On the other hand, the dimeric G·G·G–Cu(II) chelate exhibits such interactions owing to the short distance (3.077 Å)[15] between the copper(II) ions constituting the dimeric structure.

Figures 3(a)—(d) show the pH dependence of the relative amounts of the species present in the 1:1 tripeptide–copper(II) systems. In acid solution, the protonated complexes are present over considerably wide pH ranges. The four coordinated species $Cu(H_3L)^{2+}$, $Cu(H_2L)^{+}$, Cu(HL), and CuL^{-} are present at pH 5—6 in the systems of the tripeptides with glycine at the NH_2-terminus. In the G·G·G–Cu(II) system, there is a distinct peak for Cu(HL) at pH 6.2 ascribable as being due to the difficulty of deprotonation from the peptide group nearest the carboxyl terminus. Thus, formation of the species CuL^{-} in this system occurs at higher pH values as compared with other systems. However, the predominant species at pH above 7 is CuL^{-} in all the systems investigated, which may be taken as evidence for the bonding modes in the biological fluid.

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22) W. Mori, Y. Nakao, O. Yamauchi, M. Kishita, and A. Nakahara; Proceedings of the 21st Annual Symposium of Coordination Chemistry, Nagoya, p. 285 (1971).