Acid Dissociation Constants of Some Histidine-containing Peptides and Formation Constants of Their Metal Complexes

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Glycyl-L-histidine, L-histidylglycine, glycylglycyl-L-histidine, glycyl-L-histidylglycine, and L-histidylglycylglycine were synthesized by the carbobenzoxy azide procedure. The acid dissociation constants of these peptides and their related compounds were determined from the potentiometric titration curves of 0.01 M solutions at 21 °C. The dissociation of one ionization group is greatly influenced by the charge on the other groups. The formation constants of metal complexes of L-histidylglycine, L-histidylglycylglycine, and L-3-benzylhistidine were calculated by the least-squares method from the titration curves of the solution containing the ligand (0.01 M) and metal in the molar ratio 2: 1, assuming the formation of 1: 1 and 2: 1 normal complexes (ML^+ and ML_2), and the results were compared with those of L-histidine-metal complexes. For any metal ion, the order of formation constants was found to be histidine≈3-benzylhistidine>histidylglycine>histidylglycylglycine, in agreement with that of the basicity of amino and imidazole groups. In these metal complexes, metal ion is considered to bind through amino, imidazole, and carboxyl or carbonyl groups of histidine residue.

Histidine residue of the protein molecule plays a particularly important role in the interaction with metal ion, due to the presence of three coordinating groups, but detailed studies on the complex formation of the peptides with metal ions as a model system of the protein-metal interaction have been mostly confined to a limited number of simple peptides not containing histidine residue. Recently, however, some valuable information has been given on the complex formation of histidine-containing peptides.[1-5] Because of its significance in the study of protein-metal interaction, we have undertaken an extensive study on the complex formation of various histidine-containing peptides in order to obtain more information. This paper deals with the syntheses and the acid dissociation constants of glycyl-L-histidine, L-histidylglycine, glycylglycyl-L-histidine, glycyl-L-histidylglycine, and L-histidylglycylglycine (abbreviated as H–Gly–His–OH, H–His–Gly–OH, H–Gly–Gly–His–OH, H–Gly–His–Gly–OH, and H–His–Gly–Gly–OH, respectively) and the formation constants of some metal complexes of these peptides.

Experimental

Syntheses. *L-Histidylglycine*: H–His–Gly–OH was synthesized according to the method given by Photaki *et al.*,[6] with some modifications.

Carbobenzoxyglycyl-L-histidine Methyl Ester (Z–Gly–His–OCH_3): To a solution of 0.02 mol of carbobenzoxyglycine hydrazide[7] in 20 ml of 2 M hydrochloric acid and 100 ml of *N,N*-dimethylformamide (DMF) was added a cold concentrated aqueous solution of 0.02 mol of sodium nitrite under cooling with an ice-bath. After 30 min, 0.02 mol of L-histidine methyl ester dihydrochloride was added and the solution was neutralized with triethylamine. The solution was then allowed to stand under cooling for 24 hr. The solvent was evaporated and the residue was dissolved in water. The pH of the solution was adjusted to 8—9 with the addition of 1 M sodium hydroxide, and the resulting precipitate was collected and washed with water; yield 80%.

Carbobenzoxyglycyl-L-histidine (Z–Gly–His–OH): To a solution of 0.01 mol of Z–Gly–His–OCH_3 in 20 ml of methanol was added 10 ml of 1 M sodium hydroxide. After 30 min the solution was neutralized by the addition of 1 M hydrochloric acid and evaporated to dryness. The residue was dissolved in methanol. After removing the insoluble material, the solution was allowed to stand. The crystals of Z–Gly–His–OH precipitated within about 30 min. After 1 hr, the product was collected and used in the subsequent procedure without further purification; yield 95%.

Glycyl-L-histidine: To a solution of 0.005 mol of Z–Gly–His–OH in 20 ml of methanol, 10 ml of water and 1 ml of glacial acetic acid was added 100 mg of 10% Pd/C, and hydrogen was bubbled for 3 hr. The catalyst was removed by filtration. The filtrate was concentrated and applied to a column of Dowex 50-X2, H-form. Water was passed until no chlorine ion was detected in the effluent. H–Gly–His–OH was eluted with 1 M ammonium hydroxide and the effluent was concentrated to dryness. Dehydrated ethanol was added and evaporated. The procedure was repeated to obtain H–Gly–His–OH as amorphous powder; yield 95%.

Carbobenzoxyglycylglycyl-L-histidine Methyl Ester (Z–Gly–Gly–His–OCH_3), Carbobenzoxyglycyl-L-histidylglycine Ethyl Ester (Z–Gly–His–Gly–OC_2H_5), and Carbobenzoxy-L-histidylglycylglycine Ethyl Ester (Z–His–Gly–Gly–OC_2H_5): These peptides were prepared in the same way as in the syntheses of Z–Gly–His–OCH_3, from carbobenzoxydipeptide hydrazides[7] with amino acid ester hydrochlorides; yield 80%.

Carbobenzoxyglycylglycyl-L-histidine (Z–Gly–Gly–His–OH) and Carbobenzoxyglycyl-L-histidylglycine (Z–Gly–His–Gly–OH): To a solution of 0.01 mol of carbobenzoxytripeptide ester in 20 ml of DMF was added 10 ml of 1 M sodium hydroxide. After 30 min the solution containing carbobenzoxytripeptide was neutralized with 1 M hydrochloric acid and used in the subsequent procedure.

Glycylglycyl-L-histidine and Glycyl-L-histidylglycine: These peptides were prepared from the solutions of carbobenzoxytripeptides in the same way as in the synthesis of H–Gly–His–OH; yield 90%.

L-Histidylglycylglycine Ethyl Ester (H–His–Gly–Gly–OC_2H_5): To a solution of 0.005 mol of Z–His–Gly–Gly–OC_2H_5 in 50 ml of DMF was added 100 mg of 10% Pd/C, and hydrogen was bubbled for 3 hr. The catalyst was removed and the solution containing H–His–Gly–Gly–OC_2H_5 was used in the subsequent reaction.

L-Histidylglycylglycine: To a solution of H–His–Gly–Gly–OC_2H_5 was added 5 ml of 1 M sodium hydroxide. After 1 hr, the solution was neutralized with 1 M hydrochloric acid and concentrated. NaCl was removed in the same way

TABLE 1. HISTIDINE-CONTAINING PEPTIDES AND THEIR DERIVATIVES

Compound	Molecular formula	Mol. wt.	Mp, °C	Anal. Calcd % C	Calcd % H	Calcd % N	Found % C	Found % H	Found % N
Z-Gly-His-OH[a]	$C_{16}H_{18}O_5N_4 \cdot 1\frac{1}{3}H_2O$	370.37	(b)	51.88	5.63	15.13	51.75	5.76	14.94
Z-His-Gly-OH	$C_{16}H_{18}O_5N_4$	346.34	232	55.48	5.24	16.18	55.22	5.40	16.13
H-Gly-His-OH	$C_8H_{12}O_3N_4 \cdot H_2O \cdot \frac{1}{3}C_2H_5OH$	245.59	(c)	42.38	6.58	22.82	42.75	6.34	22.79
H-His-Gly-OH	$C_8H_{12}O_3N_4$	212.21	201	45.28	5.70	26.40	45.29	5.71	26.69
Z-Gly-His-$NHNH_2$	$C_{16}H_{20}O_4N_6$	360.37	146	53.32	5.59	23.32	53.43	5.58	22.80
Z-His-Gly-$NHNH_2$	$C_{16}H_{20}O_4N_6$	360.37	116	53.32	5.59	23.32	53.38	5.59	23.20
Z-Gly-Gly-$NHNH_2$	$C_{12}H_{16}O_4N_4$	280.28	160	51.42	5.75	19.99	51.53	5.64	19.71
Z-Gly-Gly-His-OCH_3	$C_{19}H_{23}O_6N_5 \cdot \frac{1}{2}H_2O$	426.42	(b)	53.51	5.68	16.42	53.46	5.77	16.31
Z-Gly-His-Gly-OC_2H_5	$C_{20}H_{25}O_6N_5$	431.44	173	55.67	5.84	16.23	55.84	5.83	16.45
Z-His-Gly-Gly-OC_2H_5	$C_{20}H_{25}O_6N_5$	431.44	182	55.67	5.84	16.23	55.92	6.06	16.13
H-Gly-Gly-His-OH	$C_{10}H_{15}O_4N_5 \cdot 1\frac{1}{3}H_2O$	293.28	(c)	40.95	6.05	23.87	41.28	5.95	23.47
H-Gly-His-Gly-OH	$C_{10}H_{15}O_4N_5 \cdot \frac{1}{2}H_2O$	278.27	224	43.15	5.81	25.17	43.23	5.94	25.47
H-His-Gly-Gly-OH	$C_{10}H_{15}O_4N_5 \cdot \frac{1}{2}H_2O$	278.27	205	43.15	5.81	25.17	43.13	5.90	25.17

a) Sample desalted by Dowex 50-X2.
b) Not determined.
c) Hygroscopic.

as in the case of H-Gly-His-OH; yield 90%.

Other Materials. L-3-Benzylhistidine (3-Bzl-His-OH) (from Peptide Institute, Protein Research Foundation) and commercial ligands and metal salts were used. Metal ion solutions prepared from metal chlorides or metal sulfate were standardized with EDTA.

Potentiometric Titration. Potentiometric titration was carried out with a Radiometer Titrator TTT 1 and a Titrigraph according to the prescription given by Albert and Serjeant.[8] A solution (10 ml) containing a ligand (10^{-2}M) and hydrochloric acid (0—2.0×10^{-2} M), or a ligand (10^{-3} M), hydrochloric acid (1.0—2.0×10^{-3} M) and a metal ion (0.5×10^{-3} M) was titrated with 0.1 M potassium hydroxide. During the course of titration, the temperature was maintained at 21 °C and a stream of CO_2 free nitrogen was passed through the titration vessel.

Results and Discussion

Syntheses. Histidine-containing peptides were synthesized by the carbobenzoxy azide method.[6,7] A slight modification was necessary in some cases. In the synthesis of H-His-Gly-Gly-OH, catalytic reduction was carried out prior to the hydrolysis of Z-His-Gly-Gly-OC_2H_5, because a side reaction took place in the hydrolysis. The analytical data of the peptides synthesized are shown in Table 1, together with their melting points. The results of the electrophoresis and thin layer chromatography confirmed the purity of these peptides.

Acid Dissociation Constants. The potentiometric titration curves for histidine-containing peptides and their related compounds are shown in Figs. 1 and 2. Acid dissociation corresponding to three coordinating groups, namely amino, imidazole, and carboxyl groups, are observed in all these ligands. The equilibria in the dissociation are expressed as follows.

$$LH_3^{2+} \rightleftharpoons LH_2^+ + H^+$$
$$K_{COOH} = [LH_2^+][H^+]/[LH_3^{2+}] \quad (1)$$

$$LH_2^+ \rightleftharpoons LH + H^+$$
$$K_{Im} = [LH][H^+]/[LH_2^+] \quad (2)$$

$$LH \rightleftharpoons L^- + H^+$$
$$K_{NH_2} = [L^-][H^+]/[LH] \quad (3)$$

The values of K_{Im} and K_{NH_2} were calculated simultaneously as shown below, since the acid dissociations of amino and imidazole groups overlap in most of the ligands. In the range where a value, which represents moles of KOH added per mole of completely protonated ligand, is between 1 and 3, total concentration of the ligand and the electroneutrality are expressed by

$$[L]_T = [LH_2^+] + [LH] + [L^-] \quad (4)$$

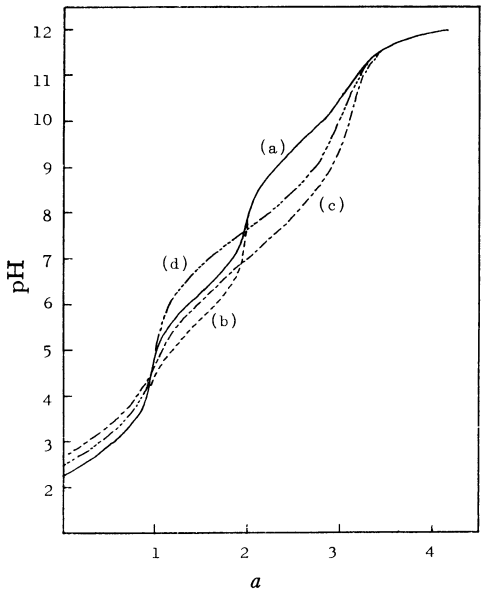


Fig. 1. Titration curves of histidine-containing dipeptides and related compounds;
(a) histidine·HCl+HCl, (b) 3-Bzl-His-OH+2HCl, (c) H-His-Gly-OH+2HCl, (d) H-Gly-His-OH+2HCl; ligand concentration: 10^{-2} M.

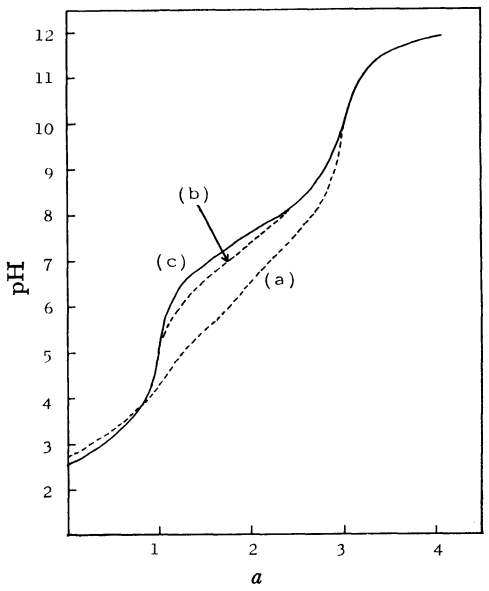


Fig. 2. Titration curves of histidine-containing tripeptides;
(a) H–His–Gly–Gly–OH+2HCl, (b) H–His–Gly–OH +2HCl, (c) H–Gly–Gly–His–OH+2HCl; ligand concentration: 10^{-2} M.

$$[LH_2^+] + [H^+] + [K^+] = [OH^-] + [Cl^-] + [L^-]. \quad (5)$$

From Eqs. (2)~(5), we obtain

$$[H^+]([L]_T - P)/P = K_{Im} + ([L]_T + P)K_{Im}K_{NH_3}/P[H^+] \quad (6)$$

where $[L]_T$=total concentration of ligand
$[L^-]$=concentration of completely deprotonated ligand
$[LH]$=concentration of monoprotonated ligand
$[LH_2^+]$=concentration of diprotonated ligand
$[LH_3^{2+}]$=concentration of triprotonated ligand
$P=[L^-]([H^+]^2/K_{Im}K_{NH_3}-1)$.

The values of K_{Im} and K_{NH_3} were calculated by the least-squares treatment of Eq. (6). The constants of L-histidine methyl ester (H–His–OCH_3) were similarly calculated. The values of K_{COOH} were calculated by the usual method. The results are shown in Table 2.

The value of pK_{COOH} increases in the order histidine ≈3-Bzl–His–OH < H–Gly–His–OH < H–Gly–Gly–His–OH < H–His–Gly–OH < H–His–Gly–Gly–OH ≈ H–Gly–His–Gly–OH. This indicates that the dissociation of the carboxyl group is greatly influenced by the electrostatic effect[9] of the positive charges on amino and imidazole groups. The basicity of the imidazole group decreases in the order H–Gly–Gly–His–OH > H–Gly–His–OH > H–Gly–His–Gly–OH > histidine > H–His–Gly–OH > 3-Bzl–His–OH > H–His–Gly–Gly–OH. This order agrees with that expected from the electrostatic effect of the amino and the carboxyl groups, except for the case of 3-Bzl–His–OH, *viz.*, the basicity of the imidazole group is strengthened by the negative charge but weakened by the positive charge. A weaker basicity of the imidazole group of 3-Bzl–His–OH than expected is explicable in terms of the decrease of the electron-delocalization in the imidazole ring. Similarly, it seems that the values of pK_{NH_3} are mainly governed by the electrostatic effect based on the carboxyl and

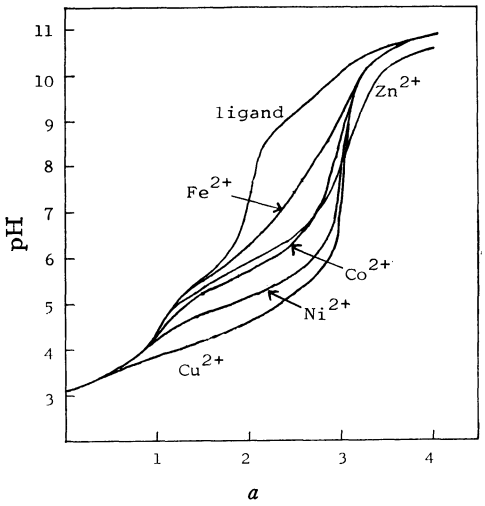


Fig. 3. Titration curves of 3-Bzl–His–OH in the presence of metal ion; ligand (10^{-3}M) to metal ratio=2: 1.

TABLE 2. ACID DISSOCIATION CONSTANTS OF HISTIDINE-CONTAINING PEPTIDES AND ITS RELATED COMPOUNDS AT 21 °C CALCULATED FROM THE TITRATION CURVES OF 0.01 M SOLUTIONS

	Present study			Results of other workers		
	pK_{COOH}	pK_{Im}	pK_{NH_3}	pK_{COOH}	pK_{Im}	pK_{NH_3}
Histidine	2.22	6.17	9.28	1.82[b]	6.00[b]	9.16[b]
3-Bzl–His–OH	2.25	5.59	9.26			
H–His–Gly–OH	2.94	6.01	7.87	2.96[c]	5.58[c]	7.50[c]
H–Gly–His–OH	2.75	6.85	8.33	2.66[c]	6.77[c]	8.24[c]
H–His–Gly–Gly–OH	3.17	5.52	7.62			
H–Gly–His–Gly–OH	3.19	6.63	8.17	3.26[d]	6.92[d]	7.96[d]
H–Gly–Gly–His–OH	2.84	6.99	8.23	2.84[c]	6.87[c]	8.22[c]
Z–Gly–His–OH[a]	3.33	6.94	—			
Z–His–Gly–OH[a]	3.81	6.82	—			
H–His–OCH_3	—	5.10	7.18	—	5.39[e]	7.37[e]

No activity coefficient corrections were made.
a) Ligand concentration: 10^{-3} M.
b) Ref. 12, Ligand concentration is 0.01 M at the commencement of the titration, 25 °C.
c) Ref. 16, μ=0.16, 25 °C.
d) Ref. 17, μ=1.0, 25 °C.
e) Ref. 14, μ=0.16, 25 °C.

imidazole groups. The higher values of pK_{Im} and pK_{COOH_3} of carbobenzoxy dipeptides, in which amino groups are blocked, than those of corresponding free dipeptides can be accepted in view of the influence of electrostatic effect. The lower values of pK_{Im} and pK_{NH_3} of H–His–OCH_3 than those of histidine are explicable in terms of the absence of the electrostatic effect of the carboxyl group.

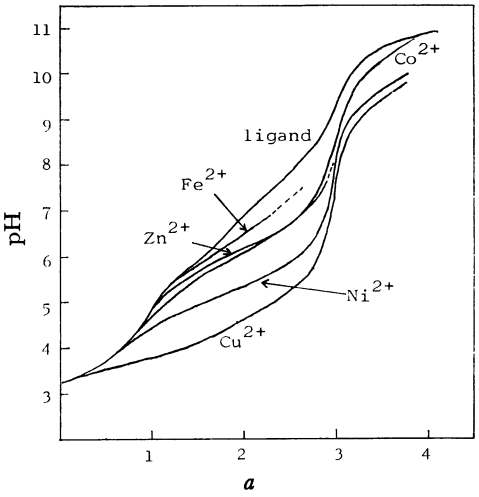


Fig. 4. Titration curves of H–His–Gly–OH in the presence of metal ion; ligand (10^{-3}M) to metal ratio= 2: 1. Dotted lines indicate precipitations.

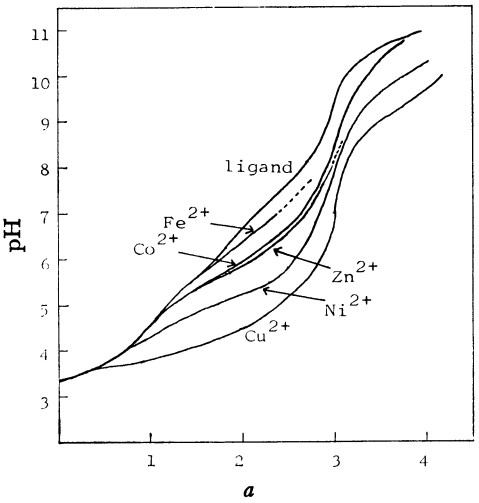


Fig. 5. Titration curves of H–His–Gly–Gly–OH in the presence of metal ion; ligand (10^{-3}M) to metal ratio= 2: 1. Dotted lines indicate precipitations.

Complex Formation of H–His–Gly–OH and H–His–Gly–Gly–OH with Some Transition Metal Ions. The titration curves of the solutions containing H–His–Gly–OH, H–His–Gly–Gly–OH, and 3-Bzl–His–OH and metal ions with the ratio of 2 to 1 are shown in Figs. 3, 4, and 5. They are very similar to those of histidine and metal ions. The inflection of pH was observed at $a=3$, indicating the formation of 2: 1 (ligand to metal) complexes in all cases. The following equilibria are postulated in the complex formation which occurred in the range where a value is between 1 and 3. The formation constants are denoted by K_1 and K_2.

$$M^{2+} + L^- \rightleftarrows ML^+ \quad K_1 = [ML^+]/[M^{2+}][L^-] \quad (7)$$

$$ML^+ + L^- \rightleftarrows ML_2 \quad K_2 = ML_2/[ML^+][L^-] \quad (8)$$

The formation constants were calculated by the least-squares treatment of the equation of Irving and Rossotti.[10,11]

$$\bar{n}/(\bar{n}-1)[L^-] = (2-\bar{n})[L^-]K_1K_2/(\bar{n}-1) - K_1 \quad (9)$$

The calculated values do not vary significantly with pH values in most cases. In the systems H–His–Gly–OH–Fe(II) and H–His–Gly–Gly–OH–Fe(II), only log K_1 was approximately obtained from the formation curves, assuming $\log K_1 = -\log[L^-]$ at $\bar{n}=0.5$, since the precipitation occurred in the course of the titrations and the accurate values were not obtained by least-squares treatment. In the systems 3-Bzl–His–OH–Cu-(II) and histidine-Cu(II), the occurrence of complicated complex formation is suggested, since consistent values were not obtained. The results are shown in Table 3. The validity of these constants was approved in most cases, by the coincidence of the experimental and calculated formation curves, obtained from the formation constants determined (Figs. 6, 7, and 8). The results might indicate that the formation of species other than normal complexes can be neglected. In the case of 3-Bzl–His–OH–Cu(II) and histidine-Cu(II) sys-

Table 3. Formation constants of divalent metal complexes of histidine containing peptides and their related compounds at 21 °C obtained from mixtures of ligand (0.001 M) and metal in molar ratio 2:1

No activity coefficient corrections were made.

	Fe(II)		Co(II)		Ni(II)		Cu(II)		Zn(II)	
	$\log K_1$	$\log K_2$	$\log K_1$	$\log K_2$	$\log K_1$	$\log K_2$	$\log K_1$	$\log K_2$	$\log K_1$	$\log K_2$
Histidine	5.39[a]	3.35[a]	6.92[b]	5.52[b]	8.96[b]	6.83[b]	10.56[b]	8.25[b]	6.63[b]	5.63[b]
	5.25	4.44	6.88	5.83	7.89	6.94	10.14[c]	7.49[c]	6.36	5.77
3-Bzl–His–OH	5.65	4.59	6.87	6.17	7.95	7.39	10.17[c]	8.03[c]	6.54	6.00
H–His–Gly–OH	4.20	(e)	5.52	4.23	6.88	5.94	9.13	6.78	4.86	4.68
H–His–Gly–Gly–OH	3.92	(e)	5.09	4.11	6.50	5.73	8.60	6.47	4.93	4.71

a) Ref. 18, $\mu=0.2$, 15 °C.
b) Ref. 12, Ligand concentration is 0.01 M at the commencement of titration, 25 °C.
c) Consistent value could not be obtained.
d) Obtained from formation curve.
e) Not determined.

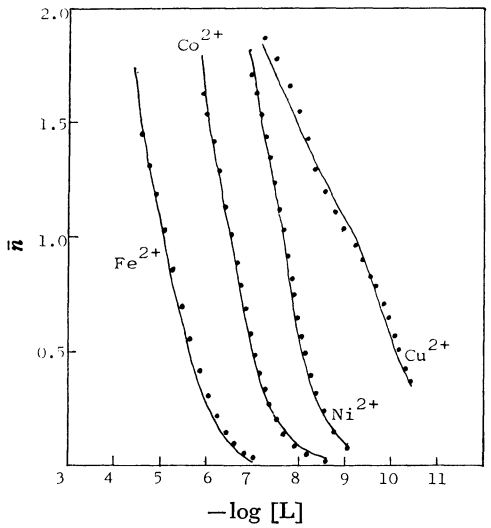


Fig. 6. Formation curves of metal complexes of 3-Bzl–His–OH. The curves were obtained directly from the experimental data. The value of each point was calculated by the use of the values in Table 3.

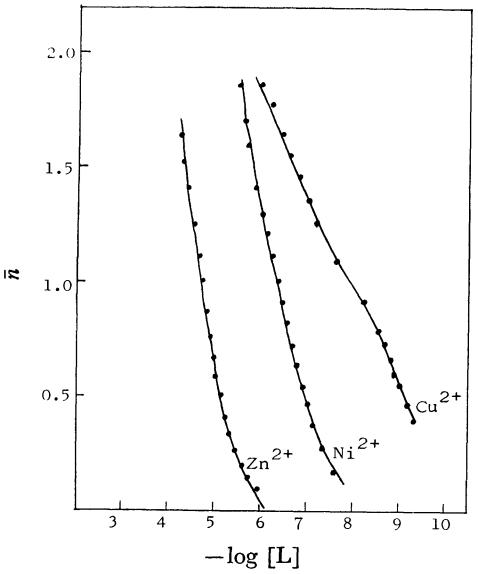


Fig. 7. Formation curves of metal complexes of H–His–Gly–OH.

tems, experimental and calculated formation curves did not coincide. In histidine–Cu(II) system the discrepancy has been explained on the basis of the formation of the protonated complex, in which Cu(II) is assumed to bind to imidazole and carboxyl groups, in addition to the normal complex.[11–14] Accordingly, the formation of a similar protonated complex can possibly be presumed also in 3-Bzl–His–OH–Cu(II) system. In this connection, the coincidence between experimental and calculated formation curves in the systems H–His–Gly–OH–Cu(II) and H–His–Gly–Gly–OH–Cu(II) suggests that the formation of the protonated complex might be neglected in these systems. The low stability of the protonated complex is explained in the light of the structure of these ligands, *viz.*, the existence of imidazole and carboxyl groups with long distance. It is clear from Table 3 that the Irving-

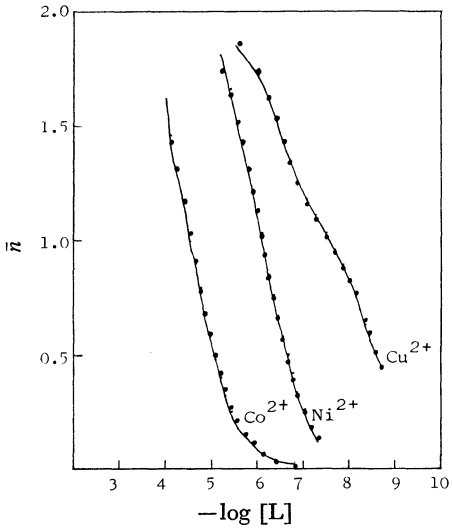
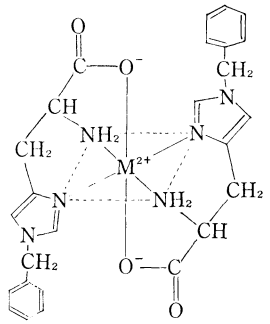
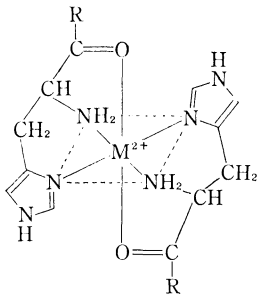


Fig. 8. Formation curves of metal complexes of H–His–Gly–Gly–OH.

Williams' order,[19] Fe(II)<Co(II)<Ni(II)<Cu(II)> Zn(II), is satisfied for all ligands. Further, with any given metal ion, the order of the stability constants with respect to the four ligands is 3-Bzl–His–OH≈ histidine>H–His–Gly–OH>H–His–Gly–Gly–OH, agreeing with that of the basicity of amino and imidazole groups. This indicates that metal ions strongly bind to amino and imidazole groups in 3-Bzl–His–OH, H–His–Gly–OH, and H–His–Gly–Gly–OH, as in the case of histidine. The third coordination site may be oxygen atom of carboxyl or carbonyl groups. The probable structures of the 2: 1 complexes, ML_2, may be expressed as **I** and **II**. In the region where *a* value is larger



(**I**)



(**II**)

R {NHCH$_2$COO$^-$: H–His–Gly–OH
{NHCH$_2$CONHCH$_2$COO$^-$: H–His–Gly–Gly–OH

than 3, additional dissociation of proton from amide group occurs in the systems H–His–Gly–OH and H–His–Gly–Gly–OH with Cu(II) and Ni(II) and the dissociation causes remarkable change in the structure of the complex. Information was briefly given on this point.[5]

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