The method of spectrophotometric analysis is therefore considered the best of the three methods used here for testing the results derived from potentiometric titration experiments. It proved to be of particular value in a later study of the combination between cupric ions and diglycylglycine, where the reaction constants of the system could not be evaluated from the titration data alone.

SUMMARY

1. The following methods have been studied with a view to their application to the reactions between metals and polypeptides in aqueous solution: (a) potentiometric titration, (b) copper-electrode potential measurements, (c) electrophoresis, and (d) spectrophotometric observations.

2. When these methods were applied to the study of complex formation between glycine and cupric ions they confirmed the view that two complexes ML^+ and ML_2 are formed.

3. Even in alkaline solution and with an excess of glycine there was no evidence from the electro-

phoretic observations of the formation of a complex with a negative charge such as would be found if one cupric ion combined with more than two glycine anions.

4. Absorption curves in the visible range of the spectrum have been calculated for the copper-glycine complexes.

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REFERENCES

- Albert, A. (1950). *Biochem. J.* **47**, 531.
 Albert, A. (1952). *Biochem. J.* **50**, 690.
 Bjerrum, J. (1941). *Metal Ammine Formation in Aqueous Solution*. Copenhagen: Haase and Son.
 Irving, H. & Rossotti, H. S. (1953). *J. chem. Soc.* p. 3397.
 Monk, C. B. (1951). *Trans. Faraday Soc.* **47**, 285.
 Perkins, D. J. (1952). *Biochem. J.* **51**, 487.
 Tourky, A. R. & Wakkad, S. E. S. E. (1948). *J. chem. Soc.* p. 749.

Complex-Formation between Polypeptides and Metals

2. THE REACTION BETWEEN CUPRIC IONS AND SOME DIPEPTIDES

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The reaction of cupric ions with peptides has been studied qualitatively by a number of workers, often with the object of shedding light on the biuret reaction. Kober & Sugiura (1912) described the colours produced on the addition of alkali to solutions containing cupric ions along with a di-, tri- or tetra-peptide in the metal:peptide molar ratio of 1:1, and tried to correlate these colours with the formation of bonds between the metal and nitrogen atoms. This work was extended by the spectrophotometric investigations of Kober & Haw (1916) with various peptides, some of considerable complexity. More recently, information on the absorption coefficients of the copper complexes of di- to octa-peptides has been supplied by Plekhan, Gavrilov & Poddubnaya (1942), Plekhan & Gavrilov (1948) and Plekhan (1951). These workers, and also Rising, Parker & Gaston (1934) and Wenaas (1937), who isolated crystalline copper complexes of tri- and tetra-glycylglycine, respectively, postulated the existence (in a copper-peptide complex) of links between the metal and the atoms of the peptide

bond. Confirmation of the existence of this linkage in metal-peptide complexes has been obtained by Chouteau & Lenormant (1951) and Chouteau (1951) by means of infrared spectrophotometric measurements.

Monk (1951) studied the effect of the presence of glycylglycine on the solubility of cupric iodate at various pH values. On the assumption that the observed increase in solubility is due to complex-formation between the cupric ions and the peptide and that two complexes are formed as with glycine, he calculated the following stability constants for these complexes:

$$K_1 = [CuG^+]/[Cu^{2+}][G^-] = 10^{6.0}$$

and $K_2 = [CuG_2]/[CuG^+][G^-] = 10^{5.6},$

where G^- represents the glycylglycine anion. These results were confirmed by potentiometric-titration experiments. The solutions studied in this work contained an excess of peptide and were apparently examined in the pH range 4-8.

In the present work the reaction between cupric ions and each of the dipeptides glycylglycine, glycyl-L-leucine, glycyl-L-tyrosine and carnosine (β -alanylhistidine) has been investigated. Complex-formation between cupric ions and glycylglycine has been the most comprehensively studied using the methods described in the previous paper (Dobbie, Kermack & Lees, 1955). Since it appears that in this case the conclusions reached by the method of potentiometric titration are confirmed by the observations made using the other experimental methods, only the former method has been used to study complex-formation involving the other three dipeptides.

EXPERIMENTAL

Materials

The dipeptides were obtained from Roche Products Ltd. and were found to be chromatographically pure, each giving a single spot when run one-dimensionally on Whatman no. 4 paper with (a) phenol-water (4:1, w/v), (b) phenol-*m*-cresol-water (2:2:1, w/v/v), or (c) *n*-butanol-acetic acid-water (4:1:5, by vol.) as solvent.

Methods

The experimental methods used in this work have been described in the previous paper (Dobbie *et al.* 1955) on the combination between cupric ions and glycine. The modified mathematical treatment of the potentiometric-titration data necessary for each peptide will be given in the appropriate sections.

It was necessary to evaluate the ionization constants of the peptides used. This was done by potentiometric titration of peptide solutions with 0.1N-KOH or N-HCl, values for the constants being calculated as described by Albert (1950).

RESULTS

Glycylglycine

The values calculated for the ionization constants of this peptide are $K_a = 10^{-8.37}$ and $K_{a'} = 10^{-3.12}$. Those recorded in the literature are $10^{-8.23}$ and $10^{-3.19}$ (Glasstone & Hammel, 1941).

Potentiometric titrations were done on solutions containing 0.005M-CuCl₂, and glycylglycine in the molar ratios of 1:1, 1:2, 1:3 and 1:4. The titration curves obtained are shown in Figs. 1-4, respectively. Inspection of these curves reveals that when a solution containing equimolar quantities of CuCl₂ (0.005M) and glycylglycine is titrated with alkali, one equivalent of alkali/copper atom is required to raise the pH of the solution from 3.89 to 4.73, and a second to raise it to 7.95. From this it may be inferred that two protons are being liberated in the range 3.89-7.95. The first of these must be associated with the combination of a cupric ion and a peptide anion to give the complex CuG⁺ (where G⁻ represents the glycylglycine anion), while the second may be released from the peptide bond of that complex due to the metal becoming bound to the nitrogen or oxygen atom there. Alternatively, the

buffering between pH 4.73 and 7.95 may be due to the uptake of a hydroxyl group by the metal in the complex CuG⁺. These two explanations will be discussed more fully later on, but meanwhile it may be observed that in a mathematical treatment of the situation, the loss of a proton from the complex CuG⁺ with ionization constant K_o is identical with the uptake of a hydroxyl group with constant $K_b = K_w/K_o$. Each leads to the formation of a neutral complex.

During the addition of a further equivalent of alkali above pH 7.95 the copper in the complex CuG is presumably taking up an OH⁻ group.

The titration curve of a solution containing CuCl₂ and glycylglycine in the ratio of 1:2 shows that four protons/copper atom are released during complex-formation. The loss of the first two, between pH values 3.89 and 4.46 and pH values 4.46 and 6.00, respectively, is presumably due to the formation of the complexes CuG⁺ and CuG already mentioned, while that of the third between pH 6.00 and 9.00 may be due to the uptake of a peptide anion by the complex CuG to give CuG₂⁻. The ionization of this last complex to give CuG₂²⁻ would then account for the fourth proton liberated. If the second and fourth reactions postulated here (namely the formation of CuG and CuG₂²⁻) involve the displacement of peptide-bond protons by the copper, then the outcome of combination between cupric ions and glycylglycine will be the stable dichelate complex shown in Fig. 5.

During the titrations of solutions containing 3 and 4 peptide molecules/copper atom, 5 and 6 protons/copper atom are released respectively. These observations suggest that two peptide molecules are bound by each metal ion with the loss of four protons, while any additional peptide molecule loses the ionizable proton of its NH₃⁺ group in accordance with the pK_a of 8.37.

On the assumption that combination between cupric ions and glycylglycine involves the following steps:

where G⁻ represents the glycylglycine anion, mathematical equations may be derived to obtain, from the potentiometric-titration data, values for the following equilibrium constants:

$$K_1 = [\text{CuG}^+]/[\text{Cu}^{2+}][\text{G}^-],$$

$$K_2 = [\text{CuG}_2^-]/[\text{CuG}][\text{G}^-],$$

$$K_o = [\text{CuG}][\text{H}^+]/[\text{CuG}^+],$$

$$K_{o'} = [\text{CuG}(\text{OH})^-][\text{H}^+]/[\text{CuG}],$$

$$K_{o''} = [\text{CuG}_2^{2-}][\text{H}^+]/[\text{CuG}_2^-].$$

As in the case of combination between cupric ions and glycine, the stability constants K_1 and K_2 may each be expressed at certain stages of the complexing process in terms of $[L^-]$ the concentration of free ligand anions, and $\bar{n}$ the corresponding average number of ligand anions bound per metal ion.

During the addition of the first two equivalents of alkali (BOH) per metal atom to a solution containing peptide of concentration $[L_0]$ and metal of concentration $[M_0]$, $[L^-]$ is given by:

$$\beta[L^-] = \gamma[L_0] - [B^+] - [H^+], \quad (1)$$

where
$$\gamma = \frac{1 + 2K_a/[H^+]}{1 + K_a/[H^+]}$$

and

$$\beta = (\gamma + 1) [H^+]^2 / K_a K_a' + \gamma [H^+] / K_a + \gamma - 1.$$

Fig. 1. *A*, titration curve of solution containing 33.02 mg. glycylglycine (0.005M) + 42.63 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 46 ml. *B*, change with pH in potential of copper electrode immersed in above solution. —, observed; $\odot$, calculated.

Fig. 2. *A*, titration curve of solution containing 66.04 mg. glycylglycine (0.01M) + 42.63 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 45 ml. *B*, change with pH in potential of copper electrode immersed in above solution. —, observed; $\odot$, calculated.

The corresponding value of $\bar{n}$ is given by

$$\bar{n}[M_0] = [L_0] - \alpha[L^-], \quad (2)$$

where
$$\alpha = [H^+]^2 / K_a K_a' + [H^+] / K_a + 1,$$

and hence K_1 may be evaluated from

$$K_1 = \frac{\bar{n}}{(1 - \bar{n}) [L^-]} \frac{1}{1 + K_a/[H^+]}. \quad (3)$$

Fig. 3. Titration curve of solution containing 99.06 mg. glycylglycine (0.015M) + 42.63 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 42.5 ml. —, observed; $\odot$, calculated.

Fig. 4. Titration curve of solution containing 132.08 mg. glycylglycine (0.020M) + 42.63 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 42.5 ml. —, observed; $\odot$, calculated.

Fig. 5. Proposed structural formula for the copper-glycylglycine complex CuG_2^{2-} . (G^- represents the glycylglycine anion.)

During the addition of further equivalents of alkali to a solution in which $[L_0] > [M_0]$, $[L^-]$ may be found from

$$\beta'[L^-] = \delta[L_0] + (2 - \delta)[M_0] - [B^+] + [OH^-], \quad (4)$$

$$\text{where } \delta = \frac{1 + 2K_c/[H^+]}{1 + K_c/[H^+]}$$

and $\beta' = (\delta + 1)[H^+]^2/K_a K_c + \delta[H^+]/K_a + \delta - 1$,

and $\bar{n}$ is given by eqn. 2; hence K_2 may be calculated from

$$K_2 = \frac{(\bar{n} - 1)}{(2 - \bar{n})[L^-]} \frac{1}{1 + K_c/[H^+]}. \quad (5)$$

Eqns. 1 and 4 each contain two unknowns, and a method of trial and error was adopted to evaluate $[L^-]$ throughout any titration.

Values for K_c and $K_{c'}$, which were suggested by inspection of the titration data, were tried in eqns. 1 and 3 and eqns. 4 and 5, respectively. Small successive adjustments were then made to K_c and $K_{c'}$ until values were found which gave the most constant sets of values for K_1 and K_2 when calculated at several points on the titration curve.

For a solution containing equimolar quantities of $CuCl_2$ and glycylglycine, the constant $K_{c'}$ must also be evaluated. The pH of the solution when 2.5 equivalents of alkali have been added is taken as a provisional value of $pK_{c'}$.

When approximate values have thus been obtained for all the equilibrium constants, the expected titration curve of a solution containing $CuCl_2$ and glycylglycine in any ratio may be calculated.

The value of $\bar{n}$ at any pH at which it is expected to be less than 1 is given by

$$\frac{(1 - \bar{n})(X - \bar{n})}{\bar{n}} = \frac{\alpha}{K_1[M_0](1 + K_c/[H^+])},$$

where $X = [L_0]/[M_0]$.

The corresponding value of $[L^-]$ may be calculated from eqn. 2 and the amount of base required to reach the chosen pH from eqn. 1. For a pH at which $\bar{n}$ is expected to be greater than 1, the value of $\bar{n}$ may be found from

$$\frac{(2 - \bar{n})(X - \bar{n})}{(\bar{n} - 1)} = \frac{\alpha}{K_2[M_0](1 + K_c/[H^+])},$$

and the corresponding values of $[L^-]$ and of $[B^+]$ from eqns. 2 and 4, respectively. In the neighbourhood of $\bar{n} = 1$, the accuracy of any value of $[L^-]$ found as above requires to be checked by insertion in the equation

$$\begin{aligned} [L_0] = & \alpha K_1 K_2 \frac{K_c}{[H^+]} [L^-]^3 - ([L_0] - 2[M_0]) K_1 K_2 \\ & \times \frac{K_c}{[H^+]} [L^-]^2 + \alpha K_1 \left(1 + \frac{K_c}{[H^+]}\right) [L^-]^2 + \alpha [L^-] \\ & - ([L_0] - [M_0]) K_1 \left(1 + \frac{K_c}{[H^+]}\right) [L^-]. \end{aligned} \quad (6)$$

The corresponding value of $[B^+]$ may then be found from

$$\begin{aligned} [B^+] = & [L^-] \left(1 - \frac{[H^+]^2}{K_a K_{c'}}\right) + 2[M_0] - \\ & \frac{\alpha [L^-] - ([L_0] - [M_0]) \left(2 + K_1 [L^-] - K_1 K_2 [L^-]^2 \frac{K_c}{[H^+]}\right)}{1 - K_1 K_2 [L^-]^2 \frac{K_c}{[H^+]}}. \end{aligned} \quad (7)$$

The amount of base required to reach any pH at which $\bar{n} = 1$ during the titration of a solution in which $[L_0] = [M_0]$ may be calculated from

$$[B^+] = \gamma'[M_0] + [OH^-], \quad (8)$$

$$\text{where } \gamma' = \frac{1 + 2K_c/[H^+] + 3K_c K_{c'}/[H^+]^2}{1 + K_c/[H^+] + K_c K_{c'}/[H^+]^2}.$$

When the titration curves thus calculated for several solutions with different values of $[L_0]/[M_0]$ are compared with those obtained experimentally it is usually found that though there is reasonable agreement on the whole, one or other of the calculated curves may show significant deviation from the observed. An appropriate modification of one or more of the constants is made until a set is obtained which provides the best fit between the expected and observed titration figures of all the solutions considered.

The values thus found for the stability and ionization constants of the complexes formed in solutions containing cupric ions and glycylglycine are $K_1 = 10^{5.88}$, $K_2 = 10^{3.28}$, $K_c = 10^{-4.25}$, $K_{c'} = 10^{-9.65}$, $K_{c''} = 10^{-10.20}$. These figures were used to calculate the titration curves of solutions in which $[L_0]/[M_0] = 1, 2, 3$ and 4. These curves are compared in Figs. 1-4, respectively, with those observed.

By the method previously described (Dobbie *et al.* 1955) copper-electrode potential measurements were made during the titration of solutions containing $CuCl_2$ and glycylglycine in the molar ratios of 1:1, 1:2 and 1:4. From these readings the corresponding values of free cupric-ion concentrations were obtained. These are compared in Figs. 1, 2 and 4, respectively, with the figures calculated from the equation

$$[M^{2+}] = \frac{[M_0]}{1 + K_1 [L^-] \left(1 + \frac{K_c}{[H^+]}\right) + K_1 K_2 [L^-]^2 \frac{K_c}{[H^+]^2} \left(1 + \frac{K_{c'}}{[H^+]}\right)}, \quad (9)$$

and the values of K_1 , etc., given above.

As before, the copper electrode became unstable in solutions with $[Cu^{2+}]$ in the neighbourhood of $10^{-8} M$ or less. However, it is interesting to note, as mentioned in the previous paper, that stable

readings can be obtained in these solutions at lower cupric-ion concentrations than in solutions containing pure inorganic cupric salts.

The method of electrophoresis on filter-paper strips was used to determine the effective mean charge on the metal at various pH values in a solution containing CuCl_2 and glycylglycine in the molar ratio of 1:2.

The average distances traversed by samples of the solution at different pH values during 5 hr. electrophoresis with a potential gradient of 5 v/cm. are given in Table 1. These figures have been corrected for adsorption on the filter paper and are given relative to glucose which was used as a marker of the electroendosmotic flow of the glycylglycine solutions used to wet the strips. Table 1 also shows how the metal is distributed in the form of free ions and complexes at the pH values of electrophoretic examination. These figures were calculated from the values given above for the stability and ionization constants of the copper-glycylglycine complexes.

It will be seen that the pH at which the copper has no apparent electrophoretic movement is approximately the same as that at which it is expected to be present almost solely in the form of the neutral complex CuG .

Spectrophotometric observations were made at various pH values with solutions containing CuCl_2 and glycylglycine in the molar ratios of 1:1, 1:2, 1:3 and 1:4. Because of the greater number of complex species, the derivation of a characteristic absorption curve for each was less straightforward and more laborious than for glycine and copper.

At any pH in any of the solutions the species are present in the ratio

$$\text{Cu}^{2+}:\text{CuG}^+:\text{CuG}:\text{CuG}_2^+:\text{CuG}_2^{2-}=1:K_1[\text{G}^-]:$$

$$K_1[\text{G}^-]\frac{K_c}{[\text{H}^+]}, K_1K_2[\text{G}^-]^2\frac{K_c}{[\text{H}^+]}, K_1K_2[\text{G}^-]^2\frac{K_cK_c'}{[\text{H}^+]},$$

but generally only three of these species are simultaneously present in appreciable amounts. The absorption curve of Cu^{2+} is readily obtained and that of CuG_2^{2-} may be taken as approximately the same as the absorption curve of the 4:1 glycylglycine- Cu^{2+} solution at a high pH.

The obvious way to find the absorption curves of CuG and CuG_2^+ would be to use the absorption curves observed for a 2:1 glycylglycine-copper solution at two pH values where only the species CuG , CuG_2^+ and CuG_2^{2-} are present in significant amounts in solving the equations:

$$E'\alpha_{\text{CuG}} + E''\alpha_{\text{CuG}_2^+} + E'''\alpha_{\text{CuG}_2^{2-}} = E_1,$$

$$E'\alpha_{\text{CuG}} + E''\alpha_{\text{CuG}_2^+} + E'''\alpha_{\text{CuG}_2^{2-}} = E_2,$$

E' , E'' and E''' designate the extinction coefficients for 0.005 M solutions of the complexes CuG , CuG_2^+ and CuG_2^{2-} , respectively, at one particular wavelength; E_1 and E_2 are the experimentally observed absorption coefficients of the solution at that wavelength at the two pH values, respectively, and α_{CuG} is the fraction of the total copper which exists in the form of the complex CuG at the pH of the first solution.

However, the range of pH in which these equations may be applied is limited, and so the coefficients α_{CuG} and $\alpha_{\text{CuG}_2^+}$ will be very similar, also the coefficients $\alpha_{\text{CuG}_2^+}$ and $\alpha_{\text{CuG}_2^{2-}}$. Consequently, the

Table 1. *Electrophoretic behaviour of copper-glycylglycine complexes*

Change with pH in the electrophoretic movement of the copper in a solution containing 0.005 M- CuCl_2 + 0.01 M glycylglycine. Figures given are distances traversed by the copper-peptide and glucose spots on a filter-paper strip to which a potential of 5 v/cm. was applied. Corrections are made for electroendosmosis and adsorption. All distances are measured as positive in the direction of the cathode. The theoretical change with pH in charge on metal is shown by distribution of metal in form of free ions and glycylglycine complexes. G^- represents the glycylglycine anion.

pH	...	...	...	...	...	...	...	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Average observed movement of copper-peptide spot (cm.)								15.3	4.9	4.5	4.5	5.5	3.1	0.3	-2.5
Average observed movement of glucose spot (cm.)								3.8	4.7	5.3	5.5	6.8	6.1	4.8	3.3
Calculated movement of copper-peptide spot due to electroendosmosis (glucose movement $\times R_F$ of copper-peptide spot)								3.2	2.0	3.9	4.8	6.2	6.1	4.8	3.3
Net movement of copper-peptide spot due to electrophoresis								12.1	2.9	0.6	-0.3	-0.7	-3.0	-4.5	-5.8
Distribution of copper (%)	$\left\{ \begin{array}{l} \text{Cu}^{2+} \\ \text{CuG}^+ \\ \text{CuG} \\ \text{CuG}_2^+ \\ \text{CuG}_2^{2-} \end{array} \right.$							98.6	72.3	7.9	0.1	—	—	—	—
								1.4	17.7	13.9	1.7	0.1	—	—	—
								—	10.0	77.9	94.8	77.5	44.8	29.6	23.1
								—	—	0.3	3.4	22.4	54.9	66.2	47.2
								—	—	—	—	—	0.3	4.2	29.7

values calculated for E' and E'' will be very sensitive to experimental error.

Furthermore, there is the objection that this method employs only two out of all the observed absorption curves.

Perhaps the best theoretical solution would be obtained by applying the method of least squares but, in view of the great number of experimental figures, we have considered it more convenient to adopt the following method of successive approximations.

From consideration of the general changes with pH in the absorption of each solution at one wavelength and the particular value of the absorption coefficient at any pH where one species predominates it was possible to choose a likely value for the absorption coefficients of every complex at that wavelength. By inserting these figures in equations of the type shown above, absorption coefficients were calculated for solutions containing CuCl_2 and glycylglycine in any of the ratios used and at any of the pH values examined. Comparison of these calculated figures with those observed showed discrepancies, which suggested modifications of the values of the assumed constants, and from these modified values new calculated absorption coefficients for the solutions were obtained. If necessary, further adjustments were made until the best agreement at all pH values in all the solutions had been obtained.

When this process had been carried out at several wavelengths it became possible to deduce by

graphical interpolation the approximate values of the extinction coefficients of the species at intermediate wavelengths. Further slight modifications in some of the figures were necessary to get the closest agreement between all of the calculated and observed curves.

The characteristic absorption curves thus obtained for the copper-glycylglycine complexes are given in Fig. 6. When these are combined in the proportions shown in Table 2A the absorption curves expected at various pH values for solutions containing different proportions of CuCl_2 and glycylglycine are obtained. In Table 2B, these calculated curves are compared with those obtained experimentally. Only a selection of the figures actually observed and calculated are given here.

It will be seen that substantial agreement is obtained between the calculated and observed values of the absorption coefficients. For any wavelength, this agreement holds over the pH range 4–11, and for a series of solutions in which the proportion of glycylglycine to copper varies from 1:1 to 4:1. This is achieved by the use of four arbitrary absorption curves, namely those of CuG^+ , CuG , CuG_2^- and CuG_2^{2-} , and in addition that of $\text{CuG}(\text{OH})^-$ which is considered only in the 1:1 solution at a pH > 8.

The fact that this wide range of agreement can be obtained with the restricted number of arbitrary constants shows that the potentiometric measurements and the spectrophotometric observations can both be accommodated quantitatively by the present theory.

Glycyl-L-leucine

The values found for the ionization constants of glycylleucine are $K_a = 10^{-6.38}$ and $K_a' = 10^{-3.13}$ at 20°.

The observed titration curves of solutions containing glycylleucine (0.005M) and CuCl_2 in the molar ratios of 1:1 and 2:1 are shown in Fig. 7. These curves closely resemble those obtained with glycylglycine (Figs. 1 and 2), and complex-formation may be assumed to follow the same course.

By means of the equations derived in the section on glycylglycine, the following values were calculated from the data in Fig. 7 for the stability and ionization constants of the copper-glycylleucine complexes $K_1 = 10^{6.10}$, $K_2 = 10^{3.30}$, $K_c = 10^{-4.80}$, $K_c' = 10^{-9.65}$ and $K_c'' = 10^{-10.00}$.

The theoretical titration curves calculated from these constants are also shown in Fig. 7.

Glycyl-L-tyrosine

The ionization constants of the amino, carboxyl and phenolic hydroxyl groups of glycyltyrosine were found to have the values $K_a = 10^{-8.37}$, $K_a' = 10^{-3.01}$ and $K_a'' = 10^{-10.31}$, respectively, at 20°. Greenstein (1932) reports the values $10^{-8.40}$, $10^{-2.98}$ and $10^{-10.40}$ at 25°.

Fig. 6. Absorption curves of copper-glycylglycine complexes. Curve A, CuG^+ ; curve B, CuG ; curve C, $\text{CuG}(\text{OH})^-$; curve D, CuG_2^- and curve E, CuG_2^{2-} . (G^- represents the glycylglycine anion.)

Table 2. *Copper-glycylglycine complexes*

A: distribution of copper in form of cupric ions and copper-glycylglycine complexes at various pH values in solutions containing 0.005 M-CuCl₂·2H₂O and different concentrations of glycylglycine. *B:* comparison of observed and calculated optical densities of the solutions at various pH values. The figures are log *I*₀/*I* × 10³ and have been adjusted to refer to a solution in which [Cu₀] = 0.005 M at each pH. G⁻ represents the glycylglycine anion.

pH	A. Distribution of metal as					B. Optical densities at (mμ.)									
	Cu ²⁺ (%)	CuG ⁺ (%)		CuG(OH ⁻) (%)		550		600		650		700		750	
					Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	
(a) Glycylglycine concn., 0.005 M (composition of solution as in Fig. 1)															
3.89	88.2	8.2	3.6	—	—	—	—	21	23	39	41	54	55	64	65
4.89	31.9	12.7	55.4	—	—	98	97	207	208	252	253	195	198	121	123
7.95	—	—	98.0	2.0	—	170	165	355	352	417	414	300	297	157	154
8.96	—	—	83.2	16.8	—	175	168	349	348	410	410	302	298	162	158
10.59	—	—	10.3	89.7	—	181	182	326	326	391	391	306	305	178	179
(b) Glycylglycine concn., 0.01 M (composition of solution as in Fig. 2)															
pH	A. Distribution of metal as					B. Optical densities at (mμ.)									
	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG ₂ ⁻ (%)	CuG ₃ ²⁻ (%)	550		600		650		700		750	
4.12	64.5	20.4	15.1	—	—	—	—	75	73	103	102	105	103	94	92
5.16	4.3	10.4	84.8	0.5	—	144	146	307	311	368	369	270	273	148	150
6.90	—	0.2	80.1	19.7	—	180	178	364	364	408	410	284	286	137	149
7.83	—	—	49.3	50.5	0.2	203	199	382	383	402	404	264	271	139	143
9.00	—	—	29.6	66.2	4.2	216	211	388	390	395	398	254	262	137	140
10.43	—	—	18.6	30.2	51.2	198	196	350	351	382	381	274	276	155	155
11.00	—	—	12.0	12.0	76.0	188	189	330	331	375	372	282	282	163	162
(c) Glycylglycine concn., 0.02 M (composition of solution as in Fig. 4)															
4.06	51.4	29.5	19.1	—	—	—	—	90	95	127	130	131	127	112	106
5.60	0.2	4.1	91.5	4.2	—	150	163	330	344	404	402	304	289	163	153
7.23	—	—	40.3	59.6	0.1	199	205	380	388	401	402	272	266	144	141
8.67	—	—	7.2	90.2	2.6	222	227	402	405	390	394	252	250	133	135
10.07	—	—	3.0	55.7	41.3	216	211	378	370	381	381	256	263	138	143
11.07	—	—	0.6	11.8	87.6	194	191	334	327	364	366	275	281	156	164

The observed titration curves of solutions containing CuCl_2 (0.005M) and glycylytyrosine in the molar ratios of 1:1 and 1:2 are shown in Fig. 8. Inspection of the first of these curves shows that 4 equivalents of alkali/copper atom are taken up between pH 3.7 and 11.5. Here it has been assumed that complex-formation takes place as between copper and glycyglycine, with successive formation of the complexes ML^+ and ML . The latter complex then loses the ionizable proton of its phenolic OH group so that a complex ML^- is formed. Finally, in a high pH range the metal in this complex may become associated with a hydroxyl group in a complex designated $\text{ML}(\text{OH})^{2-}$.

Fig. 7. Titration curves of solutions containing A, 23.52 mg. glycyllucine (0.005M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 23.75 ml. B, 47.04 mg. glycyllucine (0.005M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.0025M) in 47.5 ml. —, observed; $\odot$ and $\triangle$, calculated.

Fig. 8. Titration curves of solutions containing A, 29.76 mg. glycylytyrosine (0.005M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 23 ml. B, 59.53 mg. glycylytyrosine (0.01M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 21.25 ml. —, observed; $\odot$, calculated.

K_1 and K_c , the stability and peptide-bond ionization constants of ML^+ , respectively, may be evaluated as for glycyglycine while

$$K_c = [\text{ML}^-][\text{H}^+]/[\text{ML}]$$

and

$$K_{c_1} = [\text{ML}(\text{OH})^{2-}][\text{H}^+]/[\text{ML}^-]$$

are given values consistent with the titration data above pH 7.

The validity of values thus obtained for these four constants may be checked by calculations of the theoretical titration curve which they imply for a 1:1 glycylytyrosine-copper mixture. Below $\bar{n}=1$ this is done as for glycyglycine. When $\bar{n}=1$ the amount of alkali required to reach any pH in this region is given by

$$[\text{B}^+] =$$

$$[\text{OH}^-] + [\text{M}_0] \left(\frac{2 + 3K_c/[\text{H}^+] + 4K_c K_{c_1}/[\text{H}^+]^2}{1 + K_c/[\text{H}^+] + K_c K_{c_1}/[\text{H}^+]^2} \right).$$

The titration curve calculated for $K_1=10^{5.90}$, $K_c=10^{-3.85}$, $K_c=10^{-9.25}$ and $K_{c_1}=10^{-19.75}$ is also shown in Fig. 8.

The titration curve of the 2:1 glycylytyrosine-copper solution shows that 6 equivalents of alkali/copper atom are taken up between pH 3.7 and 11.5. This has been interpreted as due to the occurrence of complex-formation as in a 2:1 glycyglycine-copper solution, ML_2^{3-} being formed on the addition of 4 equivalents of alkali, followed by the separate ionizations of the two phenolic hydroxyl groups in this complex to give ML_2^{3-} and ML_2^{4-} .

$K_{c''}$ and $K_{c''}$, the stability and ionization constants of the complex ML_2^{3-} , respectively, may be evaluated as for glycyglycine using the titration figures obtained during the addition of the third equivalent of alkali to the 2:1 glycylytyrosine-copper solution. Values are chosen for

$$K_{c''} = [\text{ML}_2^{3-}][\text{H}^+]/[\text{ML}_2^{2-}]$$

and

$$K_{c_{iv}} = [\text{ML}_2^{4-}][\text{H}^+]/[\text{ML}_2^{3-}]$$

from inspection of the titration curve above pH 9.5. When $\bar{n} < 1$, i.e. below pH 5.5, the theoretical titration curve of the 2:1 glycylytyrosine-copper solution may be calculated as for glycyglycine, putting $K_1=10^{5.90}$ and $K_c=10^{-3.85}$. Above $\bar{n}=1$ it may be calculated using the eqns. 10, 11 and 12:

$$\frac{(2-\bar{n})^2}{(\bar{n}-1)} = \frac{\alpha}{K_2[\text{M}_0]} \frac{1}{1 + \frac{K_{c''}}{[\text{H}^+]} + \frac{K_{c''}K_{c''}}{[\text{H}^+]^2} + \frac{K_{c''}K_{c''}K_{c_{iv}}}{[\text{H}^+]^3}}, \quad (10)$$

$$\alpha = \frac{[\text{H}^+]^2}{K_a K_{c''}} + \frac{[\text{H}^+]}{K_a} + 1 + \frac{K_{a_1}}{[\text{H}^+]}$$

$$\alpha[\text{L}^-] = [\text{M}_0](2-\bar{n}), \quad (11)$$

$$\text{and } [\text{B}^+] = (\delta' + 2)[\text{M}_0] + [\text{OH}^-] - \beta'[\text{L}^-], \quad (12)$$

$$\text{where } \delta' = \frac{1 + \frac{2K_{c'}}{[H^+]} + \frac{3K_{c'}K_{c''}}{[H^+]^2} + \frac{4K_{c'}K_{c''}K_{c_{iv}}}{[H^+]^3}}{1 + \frac{K_{c'}}{[H^+]} + \frac{K_{c'}K_{c''}}{[H^+]^2} + \frac{K_{c'}K_{c''}K_{c_{iv}}}{[H^+]^3}},$$

and

$$\beta'' = (\delta + 1) \frac{[H^+]^2}{K_a K_{a'}} + \delta \frac{[H^+]}{K_a} + (\delta - 1) + (\delta - 2) \frac{K_{a_1}}{[H^+]}$$

The titration curve calculated with $K_1 = 10^{5.90}$, $K_2 = 10^{3.34}$, $K_c = 10^{-3.85}$, $K_{c'} = 10^{-9.60}$, $K_{c''} = 10^{-10.3}$ and $K_{c_{iv}} = 10^{-10.7}$ is shown in Fig. 8.

Carnosine

This dipeptide is of special interest because of its biological importance and because it contains a β -alanine group.

The values found for the ionization constants of the carboxyl, iminazole and amino groups of carnosine are $K_{a'} = 10^{-2.77}$, $K_{a_1} = 10^{-6.90}$ and $K_a = 10^{-9.70}$ at 20°. Those reported by Deutsch & Eggleton (1938) are $10^{-2.63}$, $10^{-6.88}$ and $10^{-9.54}$ at 22°.

The experimental titration curves of solutions containing CuCl_2 and carnosine in the molecular ratios of 1:1, 1:2 and 1:4 are shown in Fig. 9. Inspection of these curves reveals the following points. When CuCl_2 (0.005M) and carnosine are mixed in equimolar quantities, the pH of the solution is 5.12 and on addition of 1 equivalent of alkali/copper atom the pH is raised to 5.60. Another 0.8 equivalent of alkali brings the pH to 6.50, but thereafter there is little buffering in the solution, since only a further 0.3 equivalent is required to raise the pH to 11.00. There is no evidence of buffering by the carnosine iminazole group in the neighbourhood of pH 6.9, which implies that the nitrogen atom of this group is associated with the copper atom at an early stage in the reaction

Fig. 9. Titration curves of solutions containing A, 28.27 mg. carnosine (0.005M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 23.75 ml. B, 56.54 mg. carnosine (0.01M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 22.5 ml. C, 113.08 mg. carnosine (0.02M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 22.5 ml. —, observed; $\odot$, $\square$ and $\triangle$, calculated.

between metal and peptide. If the copper were thus to combine with the amino and iminazole nitrogen atoms of the carnosine molecule, then it is likely that it would also become linked to the peptide-bond nitrogen atom giving a tridentate dichelate complex from which the peptide-bond proton would be readily dissociated.

The titration curve of either of the solutions containing more than one molecule of carnosine for each cupric ion is to a first approximation the same as when the ratio is 1:1, provided allowance be made for the alkali required to titrate the ionizable groups of the extra carnosine molecules. This suggests that even when excess carnosine is present only one molecule is bound by each copper atom.

Rough calculations show that the pH of an aqueous solution containing 0.02M carnosine and 0.005M CuCl_2 , namely pH 6.64, is compatible with the suggestion that two protons are initially liberated for each 1:1 copper-peptide complex molecule formed, and that these protons combine reversibly with the free carnosine molecules present in the solution. In fact we have two protons distributed between three free carnosine molecules. These will combine with the iminazole groups of these molecules so that the pH of the solution will be

$$\text{pH} = \text{p}K_{a_1} - \log \frac{\text{combined iminazole groups}}{\text{free iminazole groups}} = 6.9 - \log_{10} 2 = 6.6.$$

This rough analysis of the situation supports the view that one copper atom combines with the amino group of one carnosine molecule, liberating a proton and also much reducing the pK of the iminazole group, presumably as a result of bonding between the metal and the basic nitrogen atom of that group. The copper is also presumably united to the nitrogen or oxygen atom of the peptide bond and this union promotes dissociation of the proton from the amide nitrogen of that bond in the pH region 5-6. The resulting chelate structure would consist of two six-membered rings. There is apparently no tendency for a complex containing two carnosine molecules to be formed.

The only constants to be evaluated here are therefore K_1 and K_c , the stability and ionization constants respectively, of the complex ML^+ . The values of these are calculated from the titration data as for glycylglycine with slight modification of eqn. 1 to take into account the extra ionization constant of carnosine itself. Eqn. 1 becomes

$$\beta''[\text{L}^-] = \gamma[\text{L}_0] - [\text{B}^+] - [\text{H}^+], \quad (13)$$

$$\text{where } \gamma = \frac{1 + 2K_{a'}/[\text{H}^+]}{1 + K_{a'}/[\text{H}^+]}, \text{ as before}$$

and

$$\beta'' = \frac{(\gamma + 2)[\text{H}^+]^3}{K_a K_{a_1} K_{a'}} + \frac{(\gamma + 1)[\text{H}^+]^2}{K_a K_{a_1}} + \frac{\gamma[\text{H}^+]}{K_a} + \gamma - 1.$$

The values found for K_1 and K_2 are $10^{8.65}$ and $10^{-5.55}$, respectively. The theoretical titration curves calculated using these figures are also shown in Fig. 9.

DISCUSSION

In dilute aqueous solutions of simple amino acids such as glycine and divalent metal ions such as copper, the equilibrium is described by two constants K_1 and K_2 , which refer to the reactions $M^{2+} + L^- = ML^+$ and $ML^+ + L^- = ML_2$ respectively. It now appears that when a simple dipeptide such as glycylglycine replaces the amino acid, the equilibrium can no longer be described by the two dissociation constants. However, with these dipeptides, the first reaction is still that represented by $M^{2+} + L^- = ML^+$, and so it is legitimate to compare the value of K_1 for the amino acids with the value of K_1 for polypeptides with the corresponding metal. The results of such a comparison are shown in Table 3, which confirms the conclusions of Monk (1951) and Perkins (1952) that peptide formation in general reduces the value of the first dissociation constant. However, it has to be remembered that the dissociation constants for the amino groups of the peptides are smaller than those of the acid so that at a pH of say 7.3, the relatively low stability of the complex of metal with peptide anion is to some extent compensated by the increase in the amount of anion present as compared with the amino acid under similar conditions. There must also be taken into account the effect of the equilibrium of the conversion of ML^+ into ML which promotes the tendency to complex-formation between peptide and metal. This point will be considered further in the discussion in the following paper.

The most novel result in this work as far as the dipeptides glycylglycine, glycyllucine and glycylytyrosine are concerned is the tendency of the 1:1 and of the 2:1 peptide-copper complex to react with an extra molecule of alkali in the neighbourhood of pH 4 and 10 respectively. The main problem is to decide the nature of the reaction involved, the two possibilities being (a) that the complex takes up a hydroxyl group and (b) that it gives up a hydrogen ion. As this phenomenon is not observed with simple amino acids but only with compounds containing the peptide bond, it is natural to consider that it may be dependent on some reaction between

the copper atom and the $-\text{CO.NH}-$ group. Whereas the simple uptake of a hydroxyl group by the metal could take place equally well with glycine, the loss of a hydrogen ion could only take place when a peptide bond or some similar structure was available. The conclusion that the loss of a hydrogen ion from a peptide bond is involved is borne out by the following roughly quantitative considerations.

The copper-glycylglycine complex CuG^+ has an apparent acid dissociation constant of value $10^{-4.25}$. If this is considered in terms of hydroxyl-ion uptake it means that a complex CuG.OH is formed with dissociation constant $K_b = 10^{-10}$ approximately. Since cupric chloride (0.005M) dissolved in water gives a solution of about pH 5.2, it would seem that the dissociation constant of CuCl.OH has the value $K_b = 10^{-6}$ approximately. This means that the complex CuG^+ has a much greater affinity for OH^- than have Cu^{2+} or CuCl^+ . It seems, however, unlikely that the effect of chelation of the copper by the large glycylglycine anion would be to increase the affinity of the copper for hydroxyl ions. In any case an increase of the order of 10^4 would be impossible.

The neutral complex formed from CuG^+ has an absorption spectrum with λ_{max} at 640 m μ . and $E_{\text{max}} = 84$. CuG^+ has λ_{max} at 700 m μ . with $E_{\text{max}} = 36$. This shift in wavelength and the considerable rise in E_{max} seems to favour the interpretation of CuG as a complex containing a chelate ring rather than the complex CuG^+ with an OH^- attached.

Further evidence has been obtained from a study of the copper complexes formed by diglycylglycine. This will be discussed in a later paper, but it may be referred to here as in our opinion it strongly supports the view that the simple uptake of a hydroxyl group does not adequately explain the observed facts.

Reference may also be made to the work of Rising *et al.* (1934) and Wenaas (1937), who isolated crystalline products of the combination of copper with triglycylglycine and tetraglycylglycine, respectively. Analyses of these substances supported the view that their formation involved the loss of protons from the peptide molecules.

In the present work, no attempt was made to examine the residues obtained by evaporation of solutions containing copper and glycylglycine since these residues were extremely hygroscopic.

The observations with carnosine would seem to support the view that in complex-formation between copper and a peptide the amide link of the peptide plays an important part. As we have seen above it is difficult to avoid the conclusion that union of a cupric ion with both the nitrogen of the free NH_2 group and the basic iminazole nitrogen of carnosine takes place with great ease, giving rise to

Table 3. Comparison of the value of K_1 for the combination of cupric ions with glycine or a dipeptide

Chelating agent	$\log K_1$
Glycine	8.48
Glycylglycine	5.88
Glycyllucine	6.10
Glycylytyrosine	5.90
Carnosine	8.65

a compound of very great stability ($\log K_1 = 8.65$). If the peptide link were not involved in the chelation system, this would imply the formation of a very stable 10-membered ring, which is unlikely. If, however, the peptide bond is associated with the metal through its nitrogen atom, then the co-ordination compound would have two six-membered rings. Such a system might well be expected to possess great stability. It has been recorded that the blue crystals of copper-carnosine possess the formula: $C_9H_{14}O_3N_4 \cdot CuO$ (Mauthner, 1913). This confirms that the stable complex is of the ML type. The actual formula is compatible with the uptake of a hydroxyl group by ML^+ or with the hydrated form of the complex minus one hydrogen ion. We have, in fact, the two situations represented by *A* and *B* in Fig. 10. If the hydrogen ion is attached to the nitrogen atom as in *A*, we have the uptake of a hydroxyl group by CuG^+ and if to the oxygen atom as in *B* then we have the uptake of a water molecule by the complex CuG .

In the light of the considerations mentioned above we tend to favour the second interpretation.

If we conclude from the above considerations that the most probable interpretation of the results is that a proton is liberated from the peptide bond as the result of chelation with the copper atom, the question arises as to whether the copper should be considered to be linked to the nitrogen or to the oxygen, i.e. are we to consider the copper as displacing the hydrogen from the peptide bond in the ketimic $[-CO.NH-]$ form or in the enimic $[-C(OH):N-]$ form. It appears most probable that the ionized amide group in these complexes exists as a resonance hybrid with the negative charge distributed between the oxygen and the nitrogen atoms so that the positively charged copper atom would be attracted to both. The relative importance of the two resonating forms will presumably be dependent on the precise structure of the peptides employed and it is to be expected that

with carnosine for example, the nitrogen atom will be mainly involved as the linking of copper with the oxygen atom would imply the formation of a seven-membered ring.

A point of considerable interest is the very high affinity of carnosine for cupric ions and the fact that it forms only a 1:1 complex even in the presence of excess carnosine. It is one of the few dipeptides which readily gives a crystalline compound with this metal.

SUMMARY

1. Complex-formation between cupric ions and glycylglycine has been examined using the methods of potentiometric titration, spectrophotometric examination, electrophoresis and copper-electrode potential measurements. The constants characterizing the reaction between cupric ions and the dipeptide have been evaluated from the potentiometric measurements. It has been shown that the expected titration figures for solutions containing cupric chloride and glycylglycine calculated using these values of the constants agree with the experimental figures. The other experimental observations are also consistent with the theory.

2. Potentiometric measurements have been made on solutions containing cupric ions along with either glycyl-L-leucine or glycyl-L-tyrosine in various molecular proportions. Stability constants have been evaluated for the complexes formed. These are similar in magnitude to those of the copper-glycylglycine complexes. The phenolic hydroxyl group of glycyltyrosine does not appear to take part in the reaction between this peptide and cupric ions.

3. It appears that one cupric ion can combine with either one or two anions of these peptides producing complexes, each of which either loses a proton or takes up a hydroxyl group.

4. Potentiometric observations on solutions containing cupric chloride and carnosine indicate that one cupric ion combines with one carnosine molecule at three points, namely the nitrogen atom of the free NH_2 group, the basic iminazole nitrogen atom and the nitrogen or oxygen atom of the peptide bond. This produces a dichelate complex in which the co-ordinated iminazole group has no tendency to take up a proton, i.e. it has lost its salt-forming capacity. Even when carnosine is present in excess a second molecule is not apparently taken up by the metal.

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REFERENCES

- Albert, A. (1950). *Biochem. J.* **47**, 531.
 Chouteau, J. (1951). *C.R. Acad. Sci., Paris*, **232**, 2314.
 Chouteau, J. & Lenormant, H. (1951). *C.R. Acad. Sci., Paris*, **232**, 1479.

Fig. 10. Possible structural formulae for the copper-carnosine complex ML.

- Deutsch, A. & Eggleton, P. (1938). *Biochem. J.* **32**, 209.
 Dobbie, H., Kermack, W. O. & Lees, H. (1955). *Biochem. J.* **59**, 240.
 Glasstone, S. & Hammel, E. F. (1941). *J. Amer. chem. Soc.* **63**, 243.
 Greenstein, J. P. (1932). *J. biol. Chem.* **95**, 465.
 Kober, P. A. & Haw, A. B. (1916). *J. Amer. chem. Soc.* **38**, 457.
 Kober, P. A. & Sugiura, K. (1912). *Amer. chem. J.* **48**, 383.
 Mauthner, M. (1913). *S.B. Akad. Wiss. Wien, Abt. IIb*, **122**, 269. [(1913). *Mh. chem.* **34**, 883.]
 Monk, C. B. (1951). *Trans. Faraday Soc.* **47**, 292.
 Perkins, D. J. (1952). *Biochem. J.* **51**, 487.
 Plekhan, M. I. (1951). *Zh. Obshch. Khim.* **21**, 312, 316.
 Plekhan, M. I. & Gavrilov, N. I. (1948). *Zh. Obshch. Khim.* **18**, 1843.
 Plekhan, M. I., Gavrilov, N. I. & Poddubnaya, N. A. (1942). *Chem. Zbl.* **11**, 385.
 Rising, M. M., Parker, F. M. & Gaston, D. R. (1934). *J. Amer. chem. Soc.* **56**, 1178.
 Wenaas, P. C. (1937). *J. Amer. chem. Soc.* **59**, 1353.

Complex-Formation between Polypeptides and Metals

3. THE REACTION BETWEEN CUPRIC IONS AND DIGLYCYLGLYCINE

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In the preceding paper (Dobbie & Kermack, 1955) a study of complex-formation between various dipeptides and cupric ions has been reported. It appeared desirable to extend this work to tripeptides, the obvious initial choice being diglycylglycine. It soon became clear that the situation was more complex than those dealt with in the preceding paper, since a greater variety of distinct molecular species were apparently formed and the potentiometric-titration data alone did not permit calculation of all the relevant constants. These were calculated from consideration of both potentiometric and spectrophotometric data. Because of the more elaborate and time-consuming calculations involved, only one tripeptide has so far been studied.

MATERIALS AND METHODS

Potentiometric titrations and spectrophotometric examinations of solutions were carried out as described previously. No copper-electrode potential measurements were made since the reliability of such readings is limited.

The diglycylglycine was obtained from Roche Products Ltd. and examined for purity on one-dimensional chromatograms run with (a) phenol-water (4:1, w/v), (b) phenol-*m*-cresol-water (2:2:1, w/v/v), or (c) *n*-butanol-acetic acid-water (4:1:5, by vol.) as solvent. Generally a single spot with R_F 0.55 was detected. One sample of the peptide, apparently contaminated with glycine, was recrystallized from 25% (v/v) aqueous ethanol until chromatographically pure.

RESULTS

The values found here for the ionization constants K_a and $K_{a'}$ of the amino and carboxyl group, respectively, of diglycylglycine are $10^{-8.20}$ and

$10^{-3.24}$ at 20°. Glasstone & Hammel (1941) report the values of $10^{-8.11}$ and $10^{-3.19}$ at 20°.

The potentiometric-titration curves of solutions containing CuCl_2 (0.005M) and diglycylglycine in the molar ratios of 1:1, 1:2, 1:4 and 1:6 are shown in Figs. 1-4, respectively. Inspection of the first of these curves reveals that three equivalents of alkali/copper atom are required to raise the pH of the solution to 10.9, one equivalent being taken up in each of the pH ranges 4.2-5.4, 5.4-6.4 and 6.4-*ca.* 8. Above about pH 8 the titration curve rises steeply. To explain these observations we assume that each cupric ion unites first with the amino nitrogen atom of a peptide anion and that the complex ML^+ thus formed reacts with two further molecules of alkali to give the complexes ML and ML^- (where L^- can represent the diglycylglycine anion).

When two molecules of diglycylglycine are present per copper atom, three equivalents of alkali/copper atom are taken up over pH ranges similar to those just mentioned and then a fourth between pH 7.5 and 11. It might appear that the process of complex-formation here is the same as in the 1:1 solution with the fourth equivalent of alkali being required to titrate the NH_3^+ group of the extra peptide molecule. As we shall see later, this interpretation does not fit in with the spectrophotometric observations made with the two solutions.

The titration curves of the solutions containing copper and diglycylglycine in the ratios of 1:4 and 1:6 correspond with the 1:2 copper-peptide curve except that two and four more equivalents of alkali/copper atom, respectively, are taken up in the neighbourhood of pH 8.2.