- 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.

Fig. 1. Titration curve of solution containing 47.28 mg. diglycylglycine (0.005M) + 42.63 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 46 ml. —, observed; $\odot$, calculated.

Fig. 3. Titration curve of solution containing 94.56 mg. diglycylglycine (0.02M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 21 ml. —, observed; $\odot$, calculated.

Fig. 2. Titration curve of solution containing 94.56 mg. diglycylglycine (0.01M) + 42.63 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 45 ml. —, observed; $\odot$, calculated.

Fig. 4. Titration curve of solution containing 141.84 mg. diglycylglycine (0.03M) + 21.31 mg. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.005M) in 20 ml. —, observed; $\odot$, calculated.

To account for these observations the following scheme was postulated:

where the prefixes s and a denote respectively the symmetrical and asymmetrical forms of the complex ML_2^{2-} . These are shown in Fig. 5. As in the case of combination between cupric ions and glycylglycine, K_1 may be calculated from the titration figures obtained during the addition of the first two equivalents of alkali (BOH) to a solution in which the initial concentrations of peptide $[\text{L}_0]$ and of metal $[\text{M}_0]$ are equal. At any pH in this range, $[\text{L}^-]$ the concentration of free peptide anions is given by

$$\beta'[\text{L}^-] = \gamma[\text{L}_0] - [\text{B}^+] - [\text{H}^+], \quad (7)$$

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

$$\text{and } \beta' = (\gamma + 1) \frac{[\text{H}^+]^2}{K_a K_{c'}} + \frac{\gamma[\text{H}^+]}{K_a} + \gamma - 1,$$

and the corresponding value of $\bar{n}$ (the average number of peptide anions bound/metal ion at this pH) is given by

$$\bar{n} = 1 - \alpha[\text{L}^-]/[\text{L}_0], \quad (8)$$

$$\text{where } \alpha = [\text{H}^+]^2/K_a K_{c'} + [\text{H}^+]/K_a + 1.$$

Hence K_1 is obtained from

$$K_1 = \frac{\bar{n}}{(1 - \bar{n})[\text{L}^-]} \frac{1}{(1 + K_c/[\text{H}^+])}. \quad (9)$$

Values of K_c , consistent with the titration data, were tried in eqns. 7, 8 and 9 until that value was found which provided the most constant set of values for K_1 when calculated at many points on this part of the titration curve. The values thus found are $K_1 = 10^{5.30}$ and $K_c = 10^{-5.20}$.

From consideration of the titration figures obtained during the addition of the third equivalent of alkali to a solution in which $[\text{L}_0] = [\text{M}_0]$, $K_{c'}$ was given the value of $10^{-7.0}$.

Some difficulty was encountered when a procedure similar to that used for K_2 with glycylglycine was adopted to evaluate the stability constants K_2

and K_3 in the diglycylglycine-copper system. This difficulty arose from the fact that the value of $[\text{L}^-]$ above pH 6 in a solution where $[\text{L}_0]/[\text{M}_0] (= X) > 1$ cannot be calculated without making use of either K_2 or K_3 . $[\text{L}^-]$ may be found from either

$$\left(\frac{\alpha \epsilon K_3}{1 + K_{c''}/[\text{H}^+]} \right) [\text{L}^-]^2 - \left(\frac{\epsilon K_3 [\text{M}_0] (X - 2)}{1 + K_{c''}/[\text{H}^+]} + \beta'' \right) [\text{L}^-] + (2 + \epsilon_1(X - 2) + \delta(X - 1)) [\text{M}_0] = [\text{B}^+], \quad (10)$$

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

$$\epsilon_1 = \frac{1 - K_c K_{c'}/[\text{H}^+]^2}{1 + K_c/[\text{H}^+] + K_c K_{c'}/[\text{H}^+]^2}$$

$$\delta = \frac{1 + 2K_{c''}/[\text{H}^+]}{1 + K_{c''}/[\text{H}^+]}$$

and

$$\beta'' = (\delta + \epsilon_1 + 1) \frac{[\text{H}^+]^2}{K_a K_{c'}} + (\delta + \epsilon_1) \frac{[\text{H}^+]}{K_a} + \delta + \epsilon_1 - 1$$

or a similar equation in K_2 , and thereafter K_2 or K_3 may be found from

$$K_2 = \frac{[\text{M}_0] (X - 1) - \alpha[\text{L}^-]}{(\alpha[\text{L}^-] - [\text{M}_0] (X - 2)) \epsilon_{11}[\text{L}^-]} - \left(\frac{K_{c'}}{1 + \frac{K_{c'}}{[\text{H}^+]}} \right) K_3, \quad (11)$$

$$\text{where } \epsilon_{11} = \frac{\left(1 + \frac{K_{c''}}{[\text{H}^+]} \right) \frac{K_c}{[\text{H}^+]}}{1 + \frac{K_c}{[\text{H}^+]} + \frac{K_c K_{c''}}{[\text{H}^+]^2}}.$$

It was found to be impossible to decide upon the most likely values of K_2 , K_3 and $K_{c''}$ by using these equations and the usual method of successive approximations.

So it was decided to estimate K_2 from the titration data for a 2:1 diglycylglycine-copper solution in the pH range 6-7, where comparatively little of the species $a\text{ML}_2^{2-}$ would be expected to be present, putting $K_3 = 0$ in eqns. 10 and 11 and giving $K_{c''}$ various values in the neighbourhood of 10^{-8} . For values of $K_{c''}$ about $10^{-8.5}$ a relatively constant value of $10^{3.7}$ was thus obtained for K_2 in the pH range 6-7. With these values for K_2 and $K_{c''}$, eqns. 10 and 11 were applied to the titration data obtained above pH 7.5 for the 2:1 and 4:1 diglycylglycine-copper mixtures. The values thus found for K_3 were of the order 10^2 .

In order to establish the accuracy of these values calculated for K_2 , K_3 and $K_{c''}$ it was necessary to consider also the spectrophotometric observations made on the copper-peptide solutions. During the addition of a third equivalent of alkali to a solution containing equimolar quantities of diglycylglycine

Table 1. *Copper-diglycylglycine complexes*

A: distribution of copper in form of cupric ions and copper-diglycylglycine complexes at various pH values in solutions containing 0.005 M-CuCl₂, 2H₂O and different concentrations of diglycylglycine. B: comparison of observed and calculated optical densities of the solutions at various pH values. The figures are $\log I_0/I \times 10^3$ and have been adjusted to refer to a solution in which [Cu₀] = 0.005 M at each pH. G⁻ represents the diglycylglycine anion.

pH	A. Distribution of metal as						B. Optical densities at (m μ .)											
	Cu ²⁺ (%)		CuG ⁺ (%)	CuG (%)	CuG ⁻ (%)	CuG ₂ ⁻ (%)	550		600		650		700		750			
	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG (%)	CuG (%)	CuG (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.		
(a) Diglycylglycine concn., 0.005 M (composition of solution as in Fig. 1)																		
5.06	58.0	24.2	17.5	0.2	0.1	—	—	—	59	60	102	100	113	116	107	110		
5.93	15.1	12.3	65.9	5.6	1.1	—	118	113	218	213	288	282	257	255	174	176		
6.95	1.3	0.9	51.3	45.8	0.7	—	415	426	413	417	328	331	224	223	131	127		
(b) Diglycylglycine concn., 0.01 M (composition of solution as in Fig. 2)																		
A. Distribution of metal as						500		550		600		650		700		750		
pH	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG (%)	CuG (%)	CuG (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.		
	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG (%)	CuG (%)	CuG (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.		
4.83	52.5	33.1	14.1	0.1	0.2	—	—	—	—	—	—	—	94	95	115	114		
5.78	1.9	17.9	68.1	4.1	5.4	—	—	—	115	111	227	225	306	303	276	275		
6.86	—	0.9	41.7	30.2	26.3	0.9	171	167	342	338	380	376	327	329	228	228		
7.88	—	—	7.1	53.9	28.3	10.7	294	294	527	529	463	464	280	288	143	143		
10.36	—	—	—	61.7	0.3	38.0	272	273	616	617	476	475	236	234	86	87		
(c) Diglycylglycine concn., 0.02 M (composition of solution as in Fig. 3)																		
A. Distribution of metal as						500		550		600		650		700		750		
pH	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG (%)	CuG (%)	CuG (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.		
	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG (%)	CuG (%)	CuG (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.		
4.53	53.2	38.5	8.2	0.1	—	—	—	—	—	—	—	—	83	78	114	104		
5.82	1.3	15.4	64.1	4.2	15.0	—	—	—	124	125	236	243	306	315	271	276		
6.82	—	0.6	27.0	17.9	52.7	1.8	116	120	264	273	337	345	313	320	218	220		
7.97	—	—	2.8	26.5	48.2	22.5	194	198	369	373	371	372	264	268	148	149		
9.22	—	—	0.2	29.9	7.5	62.4	276	277	445	445	360	358	193	196	83	86		
11.31	—	—	—	30.4	0.1	69.5	286	290	454	456	361	356	186	184	73	76		
(d) Diglycylglycine concn., 0.03 M (composition of solution as in Fig. 4)																		
A. Distribution of metal as						500		550		600		650		700		750		
pH	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG (%)	CuG (%)	CuG (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.		
	Cu ²⁺ (%)	CuG ⁺ (%)	CuG (%)	CuG (%)	CuG (%)	CuG (%)	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.		
4.65	34.9	50.4	14.2	0.1	0.4	—	—	—	—	—	62	64	112	111	144	137		
5.53	2.5	26.7	57.0	1.9	11.9	—	—	—	92	94	194	207	268	283	253	261		
6.87	—	0.4	17.5	13.0	66.6	2.5	103	104	246	251	329	334	308	314	215	211		
8.18	—	—	1.1	17.3	46.4	35.2	179	180	327	331	334	336	246	246	139	139		
9.44	—	—	—	19.0	5.4	75.6	252	252	390	391	315	316	170	175	79	79		
11.28	—	—	—	19.3	0.1	80.6	261	261	397	399	316	313	166	166	71	72		

and CuCl_2 , the colour of the solution changes from deep blue through violet to purple. The actual change in absorption spectrum is shown in Table 1B. It will be seen that in the neighbourhood of pH 7 the solution has a high absorption peak at about $550 \text{ m}\mu$. This absorption is quite different in position and intensity from any we have observed with dipeptides or amino acids, an observation which agrees with the view that here a new type of complex, namely a tridentate dichelate structure is present in considerable concentration. We have thus ascribed an absorption spectrum with λ_{max} in the neighbourhood of $550 \text{ m}\mu$ to complexes with this structure, i.e. to ML^- and $a\text{ML}_2^{2-}$.

From Table 1B we see also that the 2:1, 4:1 and 6:1 peptide-copper solutions in the pH range 7-10 have each an absorption maximum in the neighbourhood of $550\text{--}560 \text{ m}\mu$. However, with increase in peptide concentration the intensity of absorption at this maximum is lowered. This implies that in the presence of a large concentration of tripeptide anions the absorption of the species ML^- is affected, and this can only arise if these anions attach themselves to the complex. It is for this reason that we associate the uptake of the fourth equivalent of alkali/copper atom in the solution containing peptide and copper in molar ratio of 2:1 with the formation of $a\text{ML}_2^{2-}$ shown in Fig. 5. To account for the change in intensity of absorption, the complex $a\text{ML}_2^{2-}$ must have an absorption spectrum with ϵ_{max} much less than that of ML^- . Since $s\text{ML}_2^{2-}$ and $a\text{ML}_2^{2-}$ are isomers which exist in any solution in an equilibrium unaffected by pH or proportion of peptide to metal, their individual absorption spectra cannot be found from the data given here. Instead an absorption curve has been assigned to

ML_2^{2-} which denotes the two complexes taken together in their equilibrium ratio

$$s\text{ML}_2^{2-}/a\text{ML}_2^{2-} = K_c \cdot K_2/K_c \cdot K_3.$$

In order to enable definite arithmetical calculations to be made it is necessary to give this fraction a value. The value taken here is unity, although the actual value might be substantially less than this since the wavelength of maximum absorption of a solution in which these complexes are present in considerable concentration is about $550 \text{ m}\mu$. By analogy with ML^- we expect $a\text{ML}_2^{2-}$ to have λ_{max} in this neighbourhood, while $s\text{ML}_2^{2-}$ might be expected to have an absorption spectrum similar to that of the corresponding complex of glycylglycine, i.e. with λ_{max} about $620 \text{ m}\mu$. If the value is less than 1, then an appropriate modification would have to be made in the values given here for K_3 and also K_c in order to satisfy both the potentiometric and spectrophotometric data.

Putting $K_c = 10^{-7.0}$, $K_c = 10^{-8.4}$, $K_2 = 10^{3.7}$ and making the above fraction = 1, we obtain $K_3 = 10^{2.3}$. Using these values along with $K_1 = 10^{5.3}$ and $K_c = 10^{-5.2}$, tentative absorption curves for the

Fig. 5. Proposed structural formulae of A, the symmetrical copper-diglycylglycine complex $s\text{CuG}_2^{2-}$ and B, the asymmetrical copper-diglycylglycine complex $a\text{CuG}_2^{2-}$. (G^- represents the diglycylglycine anion.)

Fig. 6. Computed absorption curves of copper-diglycylglycine complexes. Curve A, CuG^+ ; curve B, CuG ; curve C, CuG^- ; curve D, CuG_2^- and curve E, CuG_2^{2-} . (G^- represents the diglycylglycine anion.)

various molecular species were derived as described for glycylglycine. However, when the composite absorption curves were recalculated and compared with the observed values, although there was broad general agreement there were a number of divergencies. This suggested that the values of the constants required some adjustment. Suitable modifications were therefore made on an empirical basis taking care that the theoretical titration curves of all four solutions calculated using these modified constants in eqns. 7-11 remained consistent with those observed.

It was found that the best agreement between all of the observed and calculated figures was obtained when the following values were given to the reaction constants: $K_1 = 10^{5.3}$, $K_2 = 10^{3.6}$, $K_3 = 10^{2.0}$, $K_4 = 10^{-6.2}$, $K_{4'} = 10^{-7.0}$ and $K_{4''} = 10^{-8.6}$.

The potentiometric-titration curves calculated using these figures are shown in Figs. 1-4, and the absorption curves derived for the copper-diglycylglycine complexes in Fig. 6. A selection of the spectrophotometric figures calculated by combination of the latter set of curves with the figures showing the distribution of the copper among the various complexes formed at any pH (Table 1A) are compared in Table 1B with those observed.

DISCUSSION

If we compare the experimental observations for copper and glycylglycine with those reported here we find that there are three main differences. The first comes to light on considering the potentiometric-titration curves of solutions containing one molecule of glycylglycine or diglycylglycine for each cupric ion. In the first case two equivalents of KOH/copper atom are taken up over the pH range 4-8, whereas with diglycylglycine three equivalents of KOH are required in this range.

Secondly, during the addition of alkali to the glycylglycine-copper solution the colour of the solution deepens from pale greenish blue to very dark blue. With diglycylglycine a similar deepening of colour is observed during the addition of the first two equivalents of alkali/copper atom, but thereafter the colour of the solution changes through violet to deep purple, while the third equivalent of alkali is being added. This corresponds to an actual shift in the wavelength of maximum absorption of the solution from about 650 $m\mu$. to about 550 $m\mu$.

Thirdly, on considering solutions containing two or more molecules of diglycylglycine/copper atom, we find that the intensity of absorption in the pH range 9-11 decreases substantially with increase in peptide concentration, whereas with glycylglycine only a very slight falling off in intensity is observed.

These three distinctions between the behaviour of glycylglycine and diglycylglycine in the presence of

cupric ions can be explained by the existence, in the case of the tripeptide, of a dichelate complex ML^- . The formation of such a complex was postulated initially to account for the uptake between pH 6.5 and 8.5 of a third equivalent of alkali by the copper in a solution containing equimolar quantities of $CuCl_2$ and diglycylglycine. To this complex we may assign a characteristic absorption spectrum with maximum absorption at a much lower wavelength than that of a simple monochelate complex. In the presence of excess peptide the concentration of ML^- is reduced as it becomes replaced by a 2:1 peptide-metal complex with much lower absorption.

These results have a bearing on the question as to how far the uptake of alkali during the potentiometric titrations of copper-peptide mixtures is to be attributed to the direct attachment of OH groups to the metal or to the release of protons from the peptide. If we consider complexes of the type ML formed by glycylglycine and by diglycylglycine, there would appear to be no reason why the first should not take up a hydroxyl group as easily as the second. On the other hand, the first could not lose a proton, as it has no free amide link, while the second might be expected to lose a proton with considerable ease. The fact that the 1:1 diglycylglycine-copper solution readily takes up almost three equivalents of alkali below pH 8, while a similar glycylglycine solution only takes up a third equivalent of alkali in the pH range 9-11 after a very steep rise in the titration curve suggests that the two complexes of type ML^- thus formed after the addition of three equivalents of alkali are quite different in structure. In the case of glycylglycine, it is difficult to assign to this complex a structure other than $ML.OH^-$, so that for the diglycylglycine complex to be different it must be formed by the loss of a proton from the second amide link of this peptide. Actually there was some slight experimental suggestion that a fourth equivalent of alkali was taken up by the 1:1 diglycylglycine-copper solution in a high pH region. However, some turbidity developed in the solution at this point with consequent disturbance of the equilibrium between the various molecular species, so that it was not possible to obtain reliable potentiometric-titration readings.

These results support the view that copper-peptide complexes may lose the protons of their peptide links.

We may refer here to the possibility mentioned in the preceding paper that a proton might be lost not from a peptide bond but from a hydrated form of the copper-peptide complex. This is equivalent to the uptake of a hydroxyl group by the copper atom. However, in the tridentate copper complex which we now postulate with diglycylglycine a difficulty would arise in this interpretation. We

would have attached to one copper atom three nitrogen atoms and two oxygen atoms, these latter being the oxygens of the two water molecules. Such a complex containing 5-co-ordinated copper would seem to be unlikely. We are therefore inclined to favour the view that the uptake of alkali is the result of the direct loss of protons from the amide groups of the copper-peptide complexes.

We have already referred to the fact that the uptake of the fourth equivalent of alkali by the solution containing two molecules of peptide/copper atom takes place at a pH value which corresponds roughly to the pK_a of the $-\text{NH}_3^+$ group of the peptide. This means that only when considerable concentration of chelating anion, i.e. the peptide anion L^- , has been produced does any appreciable amount of complex-formation take place. In accordance with this the value of K_3 is small, namely 10^2 , which may be compared with $K_1 = 10^6$. When complex formation takes place with a K of the latter magnitude or greater, there is considerable complex-formation at pH values much below pK_a , that is in the presence of even very low concentration of the chelating anion L^- . The consequent reduction in the concentration of L^- results in further dissociation of the $-\text{NH}_3^+$ groups of the zwitterion. Thus, though we tend to regard the liberation of protons as due to the combination of the zwitterion with the metal, yet the immediate source of the protons is the dissociation of the $-\text{NH}_3^+$ groups of the zwitterions into NH_2 and H^+ . When the value of K is smaller, there is no essential change in the process of complex-formation and proton-liberation, but as remarked above, little complex-formation now takes place until dissociation of the $-\text{NH}_3^+$ groups is considerable. The uptake of the resulting anions by the metal still promotes further dissociation, but the importance of this effect is now relatively slight, as the pH is so high that dissociation of the zwitterion into proton and anion is already considerable. Thus it is not altogether incorrect to say that in the potentiometric titration the uptake of the fourth equivalent of alkali is due to the pK_a of the peptide, but the equilibrium is modified somewhat by the simultaneous association of the anion with the metal. This description of the process remains true even

when the value of K is high and the equivalent of alkali is taken up at a much lower pH, only here the modifying effect of chelation on the equilibrium becomes predominant.

The situation with regard to the uptake of the third equivalent of alkali is especially interesting from this point of view. Here we are concerned with the conversion of ML into ML_2^- with $K_2 = 10^{3.6}$, the third proton being given up in the neighbourhood of pH 6–7. This value of K_2 is sufficiently high to displace the uptake substantially below the pH range corresponding to the pK_a of the $-\text{NH}_3^+$ group. Of course the situation is further complicated by the other equilibria in which ML and ML_2^- are involved, but the main effect is that indicated above.

It is of interest to compare the ability of diglycylglycine and the peptides previously studied to bind cupric ions at the physiologically interesting pH of 7.3 since this gives some indication of how these substances would compete with each other for free cupric ions.

In Table 2 is shown the distribution of the metal in the form of free ions and complexes in each of a series of solutions containing cupric chloride (0.005M) and either glycine or one of the peptides studied (0.005M).

It appears that under these conditions of concentration and pH the order of cupric-ion binding by these reagents is

Though the value of the stability constant K_1 is higher for glycine ($10^{8.48}$) than for any of these peptides except carnosine, the uptake of cupric ions by any one of the peptides under these conditions would be greater than that by glycine. This is partly to be attributed to the fact that the value of pK_a for any one of these peptides (except carnosine) is significantly lower than for the simple amino acid so that at pH 7.3 a greater proportion of each peptide exists as the free chelating anion. There is the further factor that the conversion of the copper-peptide complex ML^+ into ML by the intramolecular loss of a proton results in a shift of equilibrium which causes more copper to be taken up by the

Table 2. *Distribution of copper in form of free ions and complexes in solutions each containing CuCl_2 (0.005M) and either glycine or a peptide (0.005M) at pH 7.3*

Chelating agent	Free ligand concn. $[\text{L}^-]$	Distribution of metal (%)						
		Cu^{2+}	CuL^+	CuL	CuL^-	CuL_2	CuL_2^-	CuL_2^{2-}
Glycine	$10^{-7.74}$	13.3	73.4	—	—	13.3	—	—
Glycylglycine	$10^{-6.29}$	0.23	0.09	99.15	0.44	—	0.09	—
Glycylleucine	$10^{-6.13}$	0.34	0.31	98.76	0.44	—	0.15	—
Glycyltyrosine	$10^{-6.52}$	0.15	0.03	98.64	1.11	—	0.07	—
Carnosine	$10^{-7.63}$	0.17	1.73	98.10	—	—	—	—
Diglycylglycine	$10^{-5.69}$	0.64	0.26	32.94	65.87	—	0.26	0.03

Table 3. *Free cupric-ion concentration in solutions each containing CuCl₂ (0.005M) and either glycine or a peptide (0.025M) at pH 7.3*

Chelating agent	Free cupric-ion concentration (M)
Glycine	10 ^{-9.01}
Glycylglycine	10 ^{-8.86}
Glycylleucine	10 ^{-8.56}
Glycyltyrosine	10 ^{-9.33}
Carnosine	10 ^{-8.46}
Diglycylglycine	10 ^{-8.96}

peptide. It is true that the copper-glycine complex of the type ML^+ readily takes up a second glycine anion. However, under the conditions envisaged here, where the copper:ligand ratio is 1:1, the extent to which this reaction can take place is limited and therefore its effect on copper binding is less marked than that of proton loss by the copper-peptide complex ML^+ .

On inspection of Table 3, which shows the free cupric-ion concentration at pH 7.3 in a series of solutions each containing cupric chloride (0.005M) and one of the chelating agents in the molar ratio of 1:5 we find that the order of metal binding under these conditions would be

glycyltyrosine > glycine > diglycylglycine >
glycylglycine > glycylleucine > carnosine.

In these solutions containing excess chelating agent the order of copper binding is now markedly influenced by the value of K_2 . Since the value of K_2 for glycine ($10^{7.0}$) is very much higher than for any of the peptides, the amino acid is now second in the list, glycyltyrosine with its low pK , still remaining at the top. Furthermore, the inability of the copper-carnosine complex ML to take up a second carnosine

anion results in this peptide being displaced to the foot of the list.

It is clear that the competing powers of various ligands for copper depends to a considerable extent on the ratio of copper atoms to ligand molecules.

SUMMARY

1. Potentiometric titrations and spectrophotometric observations have been made with solutions containing cupric chloride (0.005M) and diglycylglycine in the ratio of 1:1, 1:2, 1:4 and 1:6.

2. The observations have been interpreted on the assumption of the existence in these solutions of the copper-diglycylglycine complexes ML^+ , ML , ML^- , ML_2^- , symmetrical ML_2^{2-} and asymmetrical ML_2^{2-} . Values have been calculated for the stability and dissociation constants of these complexes, and it is shown that these values are consistent with the experimental observations. A characteristic absorption spectrum has been assigned to each of these complexes.

3. The results support the view that in the process of formation of such complexes as ML , a link is established between the amide group and the copper atom, with the loss of a hydrogen ion.

4. The relative avidities of various glycine-containing peptides for cupric ions are compared with each other and with that of glycine itself, at the approximate physiological pH of 7.3.

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REFERENCES

- Dobbie, H. & Kermack, W. O. (1955). *Biochem. J.* **59**, 246.
Glasstone, S. & Hammel, E. F. (1941). *J. Amer. chem. Soc.* **63**, 243.

The Incorporation of ⁶⁵Zn in Mammary Tumours and Some Other Tissues of Mice after Injection of the Isotope

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The zinc content of normal tissues has been widely studied (Rost & Weitzel, 1919; Delezenne, 1919; Rost, 1921; Lutz, 1926; Dutoit & Zbinden, 1930; Simakov, 1940; Eggleton, 1940; Gettler & Bastian, 1947). Pathological material has been studied less extensively, and the zinc content of neoplastic tissue has been compared with that of normal tissues by only a few groups of workers. Some

investigators (Cristol, 1923; Labbé & Nepveux, 1927; Sugai, 1939, 1940; Addink, 1950) found that the concentration of zinc in some tumours was higher than that in normal tissues. On the other hand, Carruthers & Suntzeff (1945, 1946) and Cowdry, Carruthers & Suntzeff (1948) observed a decrease in the zinc content of epidermis associated with methylcholanthrene-induced tumours in this