glycosyl bond is considerably smaller in the C_2'-*exo* and C_3'-*exo* conformation than in any other conformation which has been considered acceptable. Thus, intramolecular conversions from one conformation to another of the ribose ring will cause changes in the diameter of polynucleotides and nucleic acids which may or may not be followed by changes in specific rotation. A systematic survey of the stereo configuration of five-membered rings and their derivatives by p.m.r. and optical rotation studies is planned, as well as studies on the implications of the furanose conformation on nucleic acid structure and on the specificity of enzymes which attack these compounds.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF FLORIDA SCHOOL OF MEDICINE, AND THE BUREAU OF MEDICAL RESEARCH, EQUITABLE LIFE ASSURANCE SOCIETY OF THE UNITED STATES]

Coördination Complexes and Catalytic Properties of Proteins and Related Substances. IV. Reactions of Glycine-containing Dipeptides with Cupric Ions and with *p*-Nitrophenyl Acetate[1]

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Equilibria between Cu(II) ions and glycylglycine (GG), sarcosylglycine (GS), L-prolylglycine (PG), glycyl-L-valine (GV), L-valylglycine (VG), glycylsarcosine (GS) and glycyl-L-proline (GP) have been measured by potentiometric titration. All the peptides except the last two form complexes with Cu(II) in which the peptide hydrogen atom is displaced. The potentiometric results are correlated with spectral measurements. Analysis of the equilibria above *p*H 7 in Cu(II)–dipeptide mixtures is coupled with the demonstration that a certain basic complex catalyzes the hydrolysis of *p*-nitrophenyl acetate (NPA). The formation of a catalytically-inert olate complex is also explored. The rate of acetylation of the various dipeptides by NPA is correlated with their basicity. Lastly, a kinetic method is used to measure the formation of a mixed complex of Cu(II) with both glycylglycine and imidazole.

Introduction

The preceding paper of this series[5] dealt with the effect of zinc and cupric ions on the reaction of glycylglycine (GG) with *p*-nitrophenyl acetate (NPA). By a combination of kinetic and equilibrium measurements similar to previous studies[6,7] on systems containing imidazoles, it was possible to determine unambiguously the association constant for the formation of the complex $CuGG_2^-$, according to equation 3, and to confirm the picture of successive equilibria described by the following sequence of reactions[8]

$$Cu^{++} + GG^- \rightleftharpoons CuGG^+ \quad k_1^+ = \frac{(CuGG^+)}{(Cu^{++})(GG^-)} \quad (1)$$

$$CuGG^+ \rightleftharpoons CuGG + H^+ \quad k_{1,0} = \frac{(H^+)(CuGG)}{(CuGG^+)} \quad (2)$$

$$CuGG + GG^- \rightleftharpoons CuGG_2^- \quad k_{11,-} = \frac{(CuGG_2^-)}{(CuGG)(GG^-)} \quad (3)$$

In reaction 2 a hydrogen ion is displaced from the peptide linkage. The participation of the peptide linkage as well as the α-amino group in the formation of metal complexes means that a knowledge of the reactivity of only the first residue in a peptide sequence is insufficient to permit the prediction of the behavior of the N-terminus of a peptide or protein. For this reason the present study was undertaken to compare the reactivities of several dipeptides with Cu(II) ions and with NPA.

The dipeptides studied primarily are glycylglycine (GG), sarcosylglycine (SG), L-prolylglycine (PG), glycyl-L-valine (GV) and L-valylglycine (VG), all of which follow the reaction sequence 1–3 with Cu(II) ions. The evidence of Datta and Rabin[9] that glycylsarcosine (GS) and glycyl-L-proline (GP) follow equations 1 and 4 has been confirmed.

$$CuGS^+ + GS^- \rightleftharpoons CuGS_2 \quad k_{11,0} = \frac{(CuGS_2)}{(CuGS^+)(GS^-)} \quad (4)$$

The values for the separate formation constants for these various peptides are compared, and with their help the properties of the absorption spectra of the individual complexes are estimated.

Equilibria in equimolar mixtures of Cu(II) ion and dipeptide at higher *p*H values have been explored and the processes described in equations 5, 6 and 7 are shown to be possible sequels to that in equation 2.

$$CuGG \rightleftharpoons CuGGOH^- + H^+ \quad k_{1,-} = \frac{(H^+)(CuGGOH^-)}{(CuGG)} \quad (5)$$

$$CuGGOH^- + CuGG \rightleftharpoons (CuGG)_2OH^- \quad k_{21,-} = \frac{((CuGG)_2OH^-)}{(CuGGOH^-)(CuGG)} \quad (6)$$

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(2) A preliminary report presented at the 132nd National Meeting, American Chemical Society, New York, N. Y., Sept. 8–13, 1957, described work begun in the Department of Biochemistry, Washington University School of Medicine, St. Louis, Missouri.

(3) Senior Postdoctoral Fellow of the United States Public Health Service.

(4) John Simon Guggenheim Memorial Fellow and Helen Hay Whitney Fellow, Washington University, St. Louis, 1954–1955.

(5) W. L. Koltun and F. R. N. Gurd, THIS JOURNAL, **81**, 301 (1959).

(6) W. L. Koltun, R. N. Dexter, R. E. Clark and F. R. N. Gurd, *ibid.*, **80**, 4188 (1958).

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(8) The nomenclature of the association constants is defined in ref. 5; see especially footnote 17.

(9) S. P. Datta and B. R. Rabin, *Trans. Faraday Soc.*, **52**, 1117 (1956).

$$CuGGOH^- \rightleftharpoons CuGG(OH_2)^- + H^+$$

$$k_{I,-} = \frac{(H^+)(CuGG(OH)_2^-)}{(CuGGOH^-)} \quad (7)$$

The complex $CuGGOH^-$ is shown to catalyze the hydrolysis of NPA.

The various peptides are also compared with respect to their rate of reaction with NPA, and kinetic measurements on some of the Cu(II)-dipeptide systems are used to confirm the values of $k_{II,-}$ adduced from titration behavior. Lastly, kinetic measurements are used to show that CuGG may combine with imidazole in a reaction comparable to equation 3.

Materials and Methods

All peptides except glycylsarcosine (GS) and sarcosylglycine (SG) were supplied by Mann Research Laboratories, Inc., New York, N.Y. The commercial materials were titrated with HCl and NaOH under the same standard conditions as were used in the presence of Cu(II) ions and all gave association constants with hydrogen ions which showed no trends during the course of the titration. Glycylglycine (GG), glycyl-L-proline (GP) and L-prolylglycine (PG) were recrystallized from water at 50–60° by the addition of ethanol. SG was prepared according to Levene, *et al.*[10] The m.p. was 196–198°. GS was prepared by coupling carbobenzoxyglycine with sarcosyl ethyl ester by means of N,N'-dicyclohexylcarbodiimide.[11] The resulting ester was saponified and the product reduced with hydrogen in the presence of 5% Pd on charcoal. The GS was crystallized from water by the addition of ethanol. The m.p. was 201.5–202.5°. Acetylglcyine (AcG) and acetylglycylglycine (AcGG) were prepared according to the methods of Fischer[12] and Gordon, Martin and Synge.[13]

All stock solutions of peptides were tested for purity by paper electrophoresis in the Spinco Model R apparatus, using Spinco B2 buffer adjusted to *p*H 8.2 by the addition of about 150 ml. of 0.1 *M* HCl per liter, ionic strength 0.075. Schleicher and Schüll No. 2043A paper was used. The duration of the run was either 6 hr. at 2.5 mamp. or 3 hr. at 5 mamp. The colors were developed by ninhydrin spray.[5] All peptides behaved as single components. The initial colors on spraying were yellow for GG, GP, GS and GV; orange for PG; and purple for VG. Very little color was given by SG.

The sample of imidazole and the other reagents were as used previously.[5-7] Kinetic and *p*H measurements were made as previously described[5-7] at 25.0 ± 0.1°. Some of the earlier titrations were performed using the Beckman Model G *p*H Meter with external electrodes thermostated at 20.0 ± 0.1°.

Results

Association Constants for Hydrogen Ions.—The logarithms of the association constants for equilibria with hydrogen ions in the absence of metal ions are shown in Table I. The constants describing the association of a hydrogen ion with the carboxylate group are denoted by k_+' to show the charge type of the peptide formed in the reaction; likewise, the association constants for the reactions with the amino group are denoted by $k_\pm'$. In addition to the measurements at ionic strength 0.16 the titrations of GG were performed in the absence of added electrolyte (ionic strength 0.0005–0.0045) and in the presence of 1 *M* KNO_3 at 20°. The values of log k_+' were 3.29 and 3.57, respectively, and of log $k_\pm'$ 8.46 and 8.43, respectively. Comparison with the results of Dobbie and Kermack[14] (20°) and of Datta and Rabin[9] (25°) is limited to the region of low and variable ionic strengths. Datta and Rabin reported values for log k_+' of 3.20 and for log $k_\pm'$ of 8.23, in good agreement with the present values when allowance is made for the difference in temperature. Dobbie and Kermack reported values at 20° of 3.12 and 8.37 for log k_+' and log $k_\pm'$, respectively. The values of log $k_\pm'$ shown in Table I for GS, SG and GP are somewhat below the values of 8.77, 8.63 and 8.66, respectively, reported by Datta and Rabin.[9] These peptides probably follow the same pattern as GG in terms of variations of log $k_\pm'$ with ionic strength.[15]

TABLE I

VALUES OF LOG k_+' AND LOG $k_\pm'$ FOR THE VARIOUS PEPTIDES

Temp. 25.1°; ionic strength 0.16

Peptide	log k_+'	log $k_\pm'$
Glycylglycine	3.19	8.13
Glycylsarcosine	2.98	8.57
Sarcosylglycine	3.15	8.56
Glycyl-L-proline	2.97	8.48
L-Prolylglycine	3.19	8.97
Glycyl-L-valine	3.15	8.18
L-Valylglycine	3.23	8.00

Formation of 1:1 Cu(II)–Dipeptide Complexes at *p*H Values below 7.—The course of the titration of a mixture approximately 0.01 *M* with respect to both $CuCl_2$ and GG is shown in curve 3 of Fig. 1 The evaluation of $k_{I,+}$ and $k_{I,0}$ in reactions 1 and 2 is complicated by the fact that the two reactions occur in the same *p*H range, primarily between *p*H 4 and 6, and that significant quantities of the GG^+ form are present at the lower end of this *p*H range. On the other hand the reactions described in equations 3–7 may be neglected below *p*H 7 in the presence of equimolar mixtures of cupric salts and peptides of the type of GG, SG, PG, GV and VG. The description of reactions 3, 5, 6 and 7 for these peptides is reserved for a later section.

A method of successive approximation has been used for evaluating $k_{I,+}$ and $k_{I,0}$. Arbitrary values of $k_{I,0}$ are chosen and substituted into appropriate expressions until a value is found that leads to a value of $k_{I,+}$ which is independent of *p*H over the appropriate range. The following expressions apply to the conditions under discussion

$$(Cu_T) = (Cu^{++}) + (CuGG^+) + (CuGG) \quad (8)$$

$$(GG_T) = (GG^+) + (GG^\pm) + (GG^-) + (CuGG^+) + (CuGG) \quad (9)$$

$$(NaOH) = (CuGG^+) + 2(CuGG) + \Delta(GG^-) - \Delta(GG^+) - \Delta(H^+) \quad (10)$$

Here the total concentrations of Cu(II) and GG are denoted by the subscript T and (NaOH) represents the formal concentration of NaOH added. In equation 10, the symbol Δ indicates the difference in concentration of the substance in the presence of the Cu(II) salt and NaOH and in the absence of the Cu(II) salt and NaOH. In the absence of added Cu(II) salt and NaOH the concentrations of GG^-, GG^+ and H^+ are negligible. In the experimental

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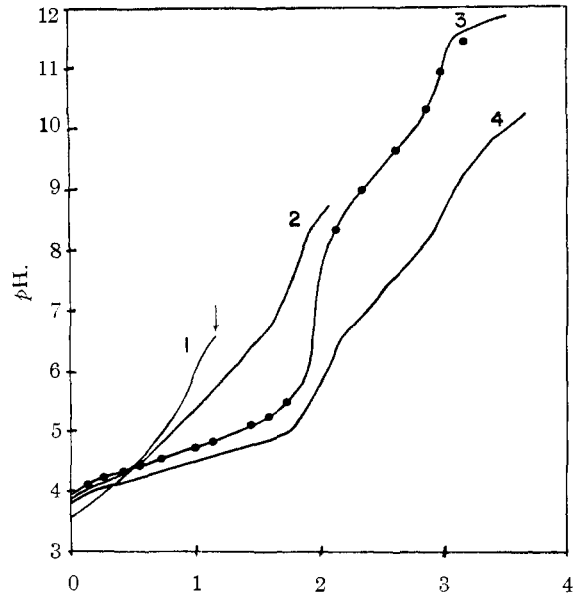

Fig. 1.—Titrations of mixtures of Cu(II) and dipeptides: curve 1, $CuCl_2$ 0.01 *M*, GS 0.01 *N*; curve 2, $CuCl_2$ 0.01 *M*, GS 0.02 *M*; curve 3, $CuCl_2$ 0.01 *M*, GG 0.01 *M*; curve 4, $CuCl_2$ 0.01 *M*, GG 0.02 *M*. The arrow on curve 1 indicates onset of precipitation. The points shown on curve 3 are computed as described in the text.

solutions containing Cu(II) the concentration of GG^- is also negligible. Accordingly, equation 10 reduces to

$$(NaOH) = (CuGG^+) + 2(CuGG) - (GG^+) - (H^+) \quad (11)$$

where (GG^+) and (H^+) now represent the concentrations present in the experimental solutions.

Introducing the expressions

$$\frac{(CuGG)}{(CuGG^+)} = \mathbf{k}_{I,0}(H^+) = a \quad (12)$$

and

$$\frac{(GG^\pm)}{(GG^+)} = \frac{1}{\mathbf{k'}_+(H^+)} = b \quad (13)$$

and

$$1 + \frac{(1 + 2a)(1 + b)}{1 + a} = c \quad (14)$$

into equations 8, 9 and 11, we obtain on substitution and rearrangement the following series of expressions

$$(Cu^{++}) = (Cu_T) - (GG_T) + \frac{1 + b}{c}\left[\left(\frac{1 + 2a}{1 + a}\right)(GG_T) - (NaOH) - (H^+)\right] \quad (15)$$

$$(GG^-) = \frac{b}{c(H^+)\mathbf{k}_\pm}\left[\left(\frac{1 + 2a}{1 + a}\right)(GG_T) - (NaOH) - (H^+)\right] \quad (16)$$

$$(CuGG^+) = \frac{(GG_T) - \frac{1 + b}{c}\left[\left(\frac{1 + 2a}{1 + a}\right)(GG_T) - (NaOH) - (H^+)\right]}{1 + a} \quad (17)$$

In a given solution at a given *p*H all parameters on the right side of equations 15–17 are known or

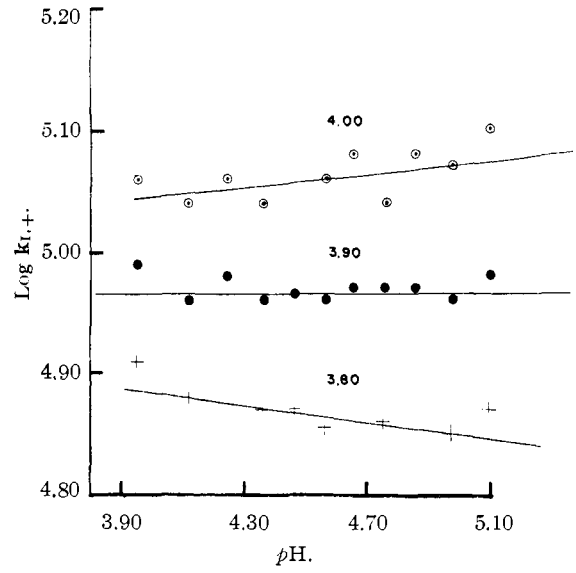

Fig. 2.—Dependence of log $\mathbf{k}_{I,+}$ on *p*H for values of $p\mathbf{k}_{I,0}$ of 3.80, 3.90 and 4.00, applied to the results of a titration of an equimolar mixture of $CuCl_2$ and GG of approximately 0.01 *M*.

fixed when *a* is fixed, and the results can be used to compute $\mathbf{k}_{I,+}$ according to equation 1. If the true value of $\mathbf{k}_{I,0}$ is converted to the appropriate *a* value for each experimentally observed *p*H and introduced into equations 15, 16, 17 and 1, the values of $\mathbf{k}_{I,+}$ so obtained will not vary with *p*H. Figure 2 shows the dependence of log $\mathbf{k}_{I,+}$ on *p*H for three different values of $p\mathbf{k}_{I,0}$ applied to the results of a titration in which the concentration of the equimolar mixture of Cu(II) and GG was about 0.01 *M*. Between *p*H 4.1 and 5.1, the values of log $\mathbf{k}_{I,+}$ for $p\mathbf{k}_{I,0}$ of 3.90 are very close to 4.96. The values corresponding to $p\mathbf{k}_{I,0}$ of 4.00 appear to increase gently with increasing *p*H, whereas those corresponding to 3.80 appear to decrease gently. The best value of $p\mathbf{k}_{I,0}$ appears to be close to 3.90. The results for a titration at a concentration of about 0.02 *M* were similar, although here a value of $p\mathbf{k}_{I,0}$ of 4.00 gave possibly the best fit.

Another test of the adequacy of the interpretation was made by expressing the various terms in equation 11 explicitly in terms of (H^+) and thereby computing values of the numbers of moles of NaOH reacted per mole of Cu(II) ion at a series of *p*H values. The value of log $\mathbf{k}_{I,+}$ was taken to be 4.96 and of $p\mathbf{k}_{I,0}$ 3.90. The results are plotted in Fig. 1 on the lower part of curve 3. The solid line represents the observed values.

The same method of analysis was applied to the results of titrations made on equimolar mixtures of Cu(II) and GG of about 0.005 *M* at 20° in the absence of added electrolyte. Under these conditions a value of 4.30 for $p\mathbf{k}_{I,0}$ yielded a value for log $\mathbf{k}_{I,+}$ of approximately 5.79 which was independent of *p*H between 4.0 and 5.0. These values confirm those of 4.25 and 5.88, respectively, computed by Dobbie and Kermack under nearly identical conditions.[14] Similar computations were applied to the results of titrations made under the same conditions but in the presence of approximately 1

M KNO_3. A value for $p\mathbf{k}_{I,0}$ of 4.60 was found to yield a value of $\log \mathbf{k}_{I,+}$ of 5.64 which was independent of pH over the range 4.0–5.0.

Equilibria of 1:1 Complexes at Higher pH Values.—Curve 3 of Fig. 1 shows that a 1:1 mixture of $CuCl_2$ and GG reacts with four moles of NaOH, two moles of which react above pH 7. The curve could not be fitted satisfactorily by assuming two constants $\mathbf{k}_{I,-}$ and $\mathbf{k}_{I,=}$ according to equations 5 and 7. A satisfactory fit of the curve could be made, however, by postulating the existence of the equilibrium described in equation 6, and by choosing an appropriate value of $\mathbf{k}_{21,-}$. The points shown above pH 8 on curve 3 in Fig. 1 are computed, using values of $p\mathbf{k}_{I,-}$, $\mathbf{k}_{2I,-}$ and $p\mathbf{k}_{I,=}$ of 9.37, 200 and 12.20, respectively. The same values were used to compute the form of the corresponding curve in a mixture containing twice as high initial concentrations of $CuCl_2$ and GG (0.02 M); the agreement with experiment was again very good. Similar results for 1:1 mixtures of $CuCl_2$ with SG, GV and VG are shown in Table II. For GV and VG, the values were again found to fit the titration curves obtained with 0.01 M and 0.02 M Cu(II)–dipeptide solutions.

The titration of a mixture of 0.02 M $CuCl_2$ and 0.02 M PG consumed only three equivalents of NaOH up to pH 12, so that the value of $p\mathbf{k}_{I,=}$ for this peptide must be considerably higher than for the other peptides, if indeed the process described in equation 7 occurs at all. Table II shows that the values of $p\mathbf{k}_{I,-}$ and $\log \mathbf{k}_{2I,-}$ for the Cu(II)–PG system fall in the same range as the other values listed for these constants.

TABLE II

EQUILIBRIUM CONSTANTS FOR FORMATION OF Cu(II)-DIPEPTIDE COMPLEXES

Temperature 25.0°; ionic strength 0.16

Peptide	$\log \mathbf{k}_{1,+}$	$p\mathbf{k}_{1,0}$	$\log \mathbf{k}_{11,-}$	$\log \mathbf{k}_{11,0}$	$p\mathbf{k}_{1,-}$	$\log \mathbf{k}_{21,-}$	$p\mathbf{k}_{1,=}$
Glycylglycine	4.96	3.90	3.07	[a]	9.37	2.30	12.2
Glycylsarcosine	6.13	[a]	[a]	4.62	[a]	[a]	[a]
Sarcosylglycine	4.39	3.45	2.42	[a]	9.19	1.48	11.9
Glycyl-L-proline	6.43	[a]	[a]	5.02	[a]	[a]	[a]
L-Prolylglycine	6.39	3.95	2.66	[a]	9.34	1.88	[a]
Glycyl-L-valine	5.62	4.75	2.94	[a]	9.30	2.18	12.0
L-Valylglycine	4.87	3.85	2.20	[a]	9.13	2.24	11.8

[a] Reaction not detected.

Catalytic Activity of $CuGGOH^-$.—Either or both the basic complexes, $CuGGOH^-$ and $(CuGG)_2OH^-$, may be expected to show a measurable rate of nucleophilic attack on NPA. The rate of splitting of NPA was measured in fifteen mixtures of the compositions described in Table III. The mixtures were prepared using a single stock solution of $CuCl_2$, GG and NaOH mixed in the molar proportions 1:1:2. It was found that dilutions of this mixture alone, in which the form CuGG is almost exclusively present, caused negligible increases in the rate of hydrolysis of NPA compared to control solutions of the same pH and ionic strength. Table III shows, however, that the rate of hydrolysis was increased markedly by addition of further NaOH. The first column lists various concentrations of total CuGG representing different dilutions of the stock solution. The second column shows total concentrations of NaOH added to the stock dilutions in the preparation of the mixtures for the kinetic experiments. Sufficient NaCl was also added to maintain a final ionic strength near 0.16. The observed pH values are listed in the third column. The initial concentration of NPA was 1×10^{-4} M, and the first-order rate constants were computed from the rate of formation of NP^- ion as measured at 400 mμ. The observed first-order rate constants were corrected for the rate of hydrolysis of NPA alone under comparable conditions of pH, temperature and ionic strength.[6] The corrected first-order rate constants are listed in the fourth column of Table III.

Computed values for the equilibrium concentrations of the three species CuGG, $CuGGOH^-$ and $(CuGG)_2OH^-$ are listed in columns 5, 6 and 7 of Table III. The computations were made by combining equations 5 and 6 with the expression for the sum of the concentrations of Cu(II)–GG complexes present and solving the resulting quadratic in $(CuGGOH^-)$. The values of $p\mathbf{k}_{I,-}$ and $\log \mathbf{k}_{2I,-}$ were taken from Table II and the pH values from column 3 of Table III. Since the pH values of the mixtures were between 7.20 and 10.03, the concentrations of the forms CuGG and $CuGG(OH)_2^-$ were computed to be at most 3×10^{-5} M, and these forms were neglected.

The eighth column of Table III shows the second-order rate constants k_2 computed on the assumption that the sole reactive species of the Cu(II)–GG system under these conditions is $CuGGOH^-$. The values of k_2 were obtained by dividing the values for the first-order rate constants (column 4) by the concentrations of $CuGGOH^-$ (column 6). The average value is 12.9 l. $mole^{-1}$ $min.^{-1}$ and the average deviation 1.6 l. $mole^{-1}$ $min.^{-1}$. By contrast, a similar computation to test the dependence of k_1 on $((CuGG)_2OH^-)$, in which the values in column 4 are divided by those in column 7, gives values which range from 0.6 to 56 l. $mole^{-1}$ $min.^{-1}$ and also show a strong inverse relation with the total concentration of the Cu(II)–GG system. The results of these tests lend support to the assumption that $CuGGOH^-$ is a much more reactive species than $(CuGG)_2OH^-$. The powerful basic catalysis by hydroxyl ion limits the usefulness of the present technique above pH 10, and no attempt was made to detect a kinetic effect due to the species $CuGG(OH)_2^=$.

Except in the most dilute mixtures studied in the experiments reported in Table III, the proportion of NaOH added in the final preparation of the experimental solutions (column 2) was much less than that which went into the preparation of the stock CuGG solution (column 1). It was for this reason that the single stock solution was used to minimize random errors. Since the stock solution was prepared by mixing standard solutions of $CuCl_2$, GG and NaOH, any imbalance in the ingredients is introduced into all experiments. An estimate of this imbalance is given by a comparison of the sum of the concentrations given in columns 6 and 7 of Table III with that in column 2. The imbalance may be seen to be the equivalent of an error of between 2 and 3% in the measurement of the NaOH used to prepare the stock solution.

TABLE III

DECOMPOSITION OF NPA IN BASIC Cu(II)-GG SOLUTIONS

Total CuGG, mole/l.	Added NaOH, mole/l.	pH	$k_1 \times 10^2$, min.$^{-1}$	Computed equilibrium concn., mole/l. CuGG $\times 10^3$	CuGGOH$^-$ $\times 10^3$	$(CuGG)_2OH^-$ $\times 10^3$	k_2, l. mole^{-1} min.$^{-1}$
0.1010	0.00434	7.20	0.670	82.1	0.555	9.16	12.1
.1010	.00651	7.32	0.691	78.4	.700	11.0	9.9
.1010	.00868	7.48	1.28	72.8	.938	13.6	13.7
.0505	.00217	7.47	0.459	41.4	.521	4.31	8.8
.0505	.00434	7.69	0.966	37.8	.789	5.96	12.2
.0505	.00651	7.98	1.46	32.3	1.31	8.46	11.1
.0505	.00868	8.16	2.32	28.5	1.76	10.1	13.2
.0202	.00217	7.99	0.92	15.5	0.647	2.01	14.1
.0202	.00434	8.56	2.02	11.0	1.70	3.77	11.9
.0202	.00651	8.88	3.95	7.66	2.48	5.03	16.0
.0202	.00868	9.26	5.84	5.70	4.42	5.04	13.2
.0101	.00217	8.66	1.43	6.05	1.18	0.935	12.1
.0101	.00434	9.17	3.60	3.88	2.44	1.89	14.8
.0101	.00651	9.69	7.05	2.09	4.36	1.83	16.2
.0101	.00868	10.03	8.35	1.28	5.84	1.49	14.3
						Av. k_2	12.9

That CuGGOH$^-$ acts primarily as a catalyst for the hydrolysis of NPA instead of being consumed in the reaction has been shown by measuring the rate of the reaction during the consumption of a clear excess of NPA. Because of limitations of solubility the reaction was carried out in 40% ethanol by volume and in four stages. In the first stage the reaction was begun by mixing 2.0 ml. of a solution of 0.02 *M* NPA in absolute ethanol with 3.0 ml. of a solution made up from $CuCl_2$, GG, NaOH and NaCl. The *p*H of the latter solution before the addition of the NPA solution was 8.42. At the moment of mixing the composition of the solution was as follows: $CuCl_2$ 0.0200 *M*, GG 0.0200 *M*, NaOH 0.04445 *M* and NPA 0.0080 *M*. The ionic strength at all stages was maintained at about 0.16 by the inclusion of NaCl in the reagents. The temperature was maintained at 25.0 ± 0.1° and the titration vessel was blanketed with a stream of N_2 bubbled through distilled water.

Within one minute after mixing the *p*H reading reached 8.48 ± 0.01 and was maintained thereafter at that value by addition by hand from a microburet of a solution 0.1011 *N* with respect to NaOH and 0.06 *M* with respect to NaCl. Buret readings were recorded at intervals until most of the reaction had occurred. The reaction mixture was then removed from the vessel and stored tightly stoppered at 25° overnight.

On the succeeding day 3.0 ml. of the reaction mixture was withdrawn and returned to the titration vessel. The *p*H was adjusted from 7.50 to 8.42 with the NaOH solution before the addition of 2.0 ml. of 0.020 *M* NPA in 40% ethanol containing 0.16 *M* NaCl. The *p*H of the new reaction mixture was again maintained at 8.48 and the buret readings recorded. The same procedure was followed through two more stages in an identical fashion, with the total concentration of Cu(II) and GG in each stage somewhat less than 60% of that in the preceding stage. Each stage was matched by a control experiment in which Cu(II) and GG were omitted but NPA and NP$^-$ were added in the concentrations present in the experimental solutions. The buret readings in the control experiments were subtracted from the values obtained with the Cu(II)–GG system to yield the values ascribable to the CuGGOH$^-$ catalysis proper. This procedure allowed for a possible small degree of catalysis due to the accumulation of NP$^-$ or any effects due to the absorption of traces of CO_2. The corrections were of the same order of magnitude in each stage; by the fourth stage they amounted to about 50% of the total experimental readings.

TABLE IV

CATALYSIS OF HYDROLYSIS OF NPA BY CuGGOH$^-$

Ethanol 40%, apparent *p*H 8.48, ionic strength 0.16, temp. 25.0°

Stage	Concn. of Total CuGG	Concn. of CuGGOH$^-$ $\times 10^3$	k_2(NPA)(CuGGOH$^-$), mole l.$^{-1}$ min.$^{-1}$ $\times 10^4$	k_2, l. mole^{-1} min.$^{-1}$
1	0.0200	0.800	0.340	5.3
2	.0109	.492	.296	7.5
3	.00595	.300	.220	9.2
4	.00324	.176	.100	7.1
			Av. k_2	7.3

Since the total consumption of NPA in the four stages that was attributable to the action of the Cu(II)–GG system was greater than the total quantity of the latter system present, it was concluded that the role of CuGGOH$^-$ was largely catalytic. A quantitative estimate of the catalytic activity was made from the initial slopes of the plots of the rate of NaOH consumption against time in minutes. The number of moles of NaOH consumed per mole of NPA hydrolyzed at *p*H 8.48 was taken as 1.92, where 1.00 mole is ascribed to neutralization of acetic acid and 0.92 mole to neutralization of NP which was found to have a pK' of 7.44 in the solvent containing 40% ethanol and 0.16 *M* NaCl at 25°. From this value the initial rate of hydrolysis of NPA was computed and set equal to k_2(NPA)(CuGGOH$^-$). In each case the initial value of NPA was taken as 0.0080 *M* and (CuGGOH$^-$) was computed using values for $p\mathbf{k}_{I,-}$ of 9.66 and for $\mathbf{k}_{21,-}$ of 400 which had been derived from titration data obtained in the same solvent.

The results are shown in Table IV. The average value of k_2, 7.3 l. mole^{-1} min.$^{-1}$, agrees with the

value of 7.5 l. mole^{-1} min.$^{-1}$ measured separately in the same solvent by the procedure described above for the measurements reported in Table III. Although the pH values obtained in 40% ethanol cannot be compared directly with those obtained in 0.2% ethanol, the computations of k_2 made in this solvent are internally consistent and are not dependent on such a comparison. The lower value of k_2 in the ethanol–water mixture has a parallel in the imidazole system.[6]

Effects of Substituents on Complex Formation.—The evaluation of $\mathbf{k}_{II,-}$ according to equation 3 from titration of 2:1 mixtures of dipeptide and Cu(II) ion (Fig. 1, curve 4) has been described.[5] The value of log $\mathbf{k}_{II,-}$ for GG given in Table II is taken from the preceding publication of this series and is derived from kinetic measurements as well as titration.[5] Other values in Table II are derived from titrations; in the case of PG, kinetic measurements were also made as described below and confirm the titration values.

In these 2:1 complexes the second peptide molecule appears to retain its peptide hydrogen atom, at least below pH 9. The values for log $\mathbf{k}_{II,-}$ in Table II suggest that substituents on the α-carbon atom of the N-terminal residue of the peptide may hinder the formation of the 2:1 complex. For example, log $\mathbf{k}_{II,-}$ for GV almost equals that for GG, whereas the value for VG is distinctly lower. At the same time all three peptides have almost the same value of $\mathbf{k}_{\pm}'$ and VG and GG have very similar values for log $\mathbf{k}_{I,+}$ and $p\mathbf{k}_{I,0}$. A similar type of effect may be apparent in the low values of log $\mathbf{k}_{II,-}$ for SG and PG compared to GG. Here the hindrance may be due to the presence of substituents on the terminal nitrogen atom.

The lower values of log $\mathbf{k}_{II,-}$ for VG and SG relative to GG may represent the partial hindrance of reactivity by substitution. Much more marked effects are shown by GS and GP in which a hydrogen ion cannot be displaced from the peptide bond to yield a structure of the form CuGG. The use of such substituents to establish unequivocally the nature of reaction 2 was initiated by Datta and Rabin[9] and, independently, by two of us.[16] These peptides do not follow reactions 2 and 3, but reaction 1 is succeeded directly by reaction 4. The course of the titrations of GS in the total molar proportions relative to Cu(II) ion of 1:1 and 2:1 is shown in Fig. 1, curves 1 and 2. The reaction occurs in only two steps. The successive constants, $\mathbf{k}_{I,+}$ and $\mathbf{k}_{II,0}$, were computed from the titrations according to the procedure described for the Zn(II)–GG system[5] or by a variant of the procedure of successive approximations used for the Cu(II)-GG system in the present study. The values of log $\mathbf{k}_{I,+}$ and log $\mathbf{k}_{II,0}$ in Table II for GS and GP agree fairly well with the respective values found at lower ionic strengths by Datta and Rabin[9]: 6.50 and 5.24 for GS; 6.66 and 5.24 for GP.

Acetylation of the α-amino group to form acetylglycylglycine (AcGG) markedly reduced the affinity for Cu(II) ions. At 20° and ionic strength about 0.015, the value of log $\mathbf{k}_{I,}^{+}$ for acetylglycylglycinate ($AcGG^-$) and Cu(II) ions was found to be 2.07; at ionic strength 1.0, the value was 1.41. The corresponding values for acetylglycinate (AcG^-) and Cu(II) ions are 2.14 and 1.71. Both acetyl derivatives, therefore, show much the same affinity for Cu(II) ions as acetate, and the amide groups do not appear to contribute to the stability of these complexes. In the case of $AcGG^-$ or AcG^- reaction 4 was not detected, presumably because the concentrations of ions employed, in the range of 0.01 M, were too low. The respective values of the association constants at 20° of $AcGG^-$ and AcG^- for hydrogen ions were found to be 3.66 and 3.84 at ionic strength near 0.015 and 3.50 and 3.68 at ionic strength near 1.0.

For comparison with the substituted peptides, the complex formation of sarcosinate with Cu(II) ions was measured at 20°. The values of log $\mathbf{k}_{I,+}$ and log $\mathbf{k}_{II,0}$ were found to be 8.08 and 6.70 at ionic strength 0.015 and 7.84 and 6.50 at ionic strength 1.0. The values of log $\mathbf{k}_{+}'$ and log $\mathbf{k}_{\pm}'$ for sarcosine at ionic strength near 0.015 were 2.63 and 10.31, respectively, and at ionic strength 1.0, 2.64 and 10.28, respectively. For the Cu(II)–glycinate system Flood and Lorås[17] reported values of 8.22 and 6.97 for log $\mathbf{k}_{I,+}$ and log $\mathbf{k}_{II,0}$ at 20° and ionic strength 0.5.

Visible Spectral Characteristics.—The absorption spectra of mixtures of dipeptides with $CuCl_2$ and varying proportions of NaOH were measured in the Beckman Model DU Spectrophotometer under the same conditions of temperature and ionic strength as were used to measure complex formation. The spectra of the CuGG form of complex were obtained directly from measurements on solutions of the appropriate dipeptides mixed with Cu(II) ions and NaOH in the molar proportions 1:1:2. The known values of $\mathbf{k}_{I,+}$ and $\mathbf{k}_{I,0}$ and the observed pH values were used to compute the concentrations of the $CuGG^+$ and CuGG forms of complexes in equimolar mixtures of peptide and Cu(II) ions containing less than two equivalents of NaOH; from these computations the spectra of the $CuGG^+$ forms were obtained. The spectra of the $CuGG_2^-$ complexes were measured by preparing solutions containing molar proportions of Cu(II) ions, dipeptide and NaOH of 1:2:3, respectively. The approximate wave lengths of maximum absorption λ_{max}, and corresponding molar extinction coefficients ϵ_{max}, are listed in Table V.

TABLE V

THE ABSORPTION CHARACTERISTICS IN THE VISIBLE REGION OF INDIVIDUAL Cu(II)–DIPEPTIDE COMPLEXES

	$CuGG^+$		CuGG		$CuGG_2^-$	
Peptide	λ_{max}	ϵ_{max}, l. mole^{-1} cm.$^{-1}$	λ_{max}	ϵ_{max}, l. mole^{-1} cm.$^{-1}$	λ_{max}	ϵ_{max}, l. mole^{-1} cm.$^{-1}$
GG	735	65	635	84	625	82
PG	735	34	635	100	645	105
GV	760	27	640	95	625	87
VG	715	100	635	92	620	91

The results in Table V show general similarities in the values of λ_{max} for the individual types of

(16) M. Fried and F. R. N. Gurd, Abst. 132nd National Meeting, American Chemical Society, New York, N. Y., Sept. 8–13, 1957, p. 29C.

(17) H. Flood and V. Lorås, *Tidsskr. Kjemi Bergvesen Met.*, **5**, 83 (1945).

complexes. The values of both λ_{max} and ϵ_{max} for the type $CuGG^+$ are the most difficult to define precisely because solutions containing a preponderance of this form of complex cannot be prepared. The values listed for the Cu(II)–GG complexes agree in a general way with those reported previously by Dobbie and Kermack.[14] These authors showed also that the addition of more NaOH to solutions of CuGG did not cause large changes in the absorption spectra of the solutions.

Similar measurements were made on the systems Cu(II)–GS and Cu(II)–GP. For $CuGS^+$ λ_{max} was found to be 720 mμ and ϵ_{max} 32; for $CuGP^+$ the corresponding values were 745 mμ and 32. For $CuGS_2$ λ_{max} was found to be 675 mμ and ϵ_{max} 45; for $CuGP_2$ the corresponding values were 665 and 50.

The system Cu(II)–AcGG formed faintly colored solutions. A weak maximum was observed near 760 mμ.

Various mixtures of complexes in the Cu(II)–GG and Cu(II)–GS systems were studied for their absorption characteristics in the ultraviolet region down to 210 mμ. In each case an increase in absorption set in at about 320 mμ and continued to the lower limit of wave lengths measured. Measured separately, the $CuCl_2$ and dipeptide solutions both exhibited end absorption below 240 mμ. The sum of the separate absorptions, however, was less than that of comparable solutions containing complexes.

Reaction of Dipeptides with NPA.—It has been shown that GG^- may be acetylated by NPA.[5] In the presence of a large excess of peptide the reaction may be studied under pseudo first-order conditions, and the measurement of the rate of release of *p*-nitrophenolate gives a measure of the concentration of GG^- present. This procedure has been applied[5] already to the measurement of the equilibrium described in equation 3 for GG.

The second-order rate constants k_2 for the reaction of NPA with PG, GP, GG, GV and VG have been measured at 25.1° and ionic strength 0.16. Similar measurements for glycinate, G^-, and glycine ethyl ester, GEE, have been made for comparison. That k_2 is related in general to the basicity of the peptide is indicated in Fig. 3, in which log $\mathbf{k}_{\pm}'$ is plotted against log k_2 in the usual Brönsted plot. All the compounds except VG adhere quite closely to the relation $\log k_2 = 0.84 \log \mathbf{k}_{\pm}' - 5.87$. The value of k_2 for VG is, however, about one-tenth of that predicted from this relation. The low rate of reaction between VG^- and NPA has a parallel in the great resistance of D,L-valylglycine to hydrolysis in acid or alkali.[18]

Measurements of the kinetics of the reaction of PG^- with NPA were made in the presence of Cu(II) ions. The results of measurements on six mixtures were applied to the computation of $\mathbf{k}_{II,-}$ as described previously.[5] The average value of log $\mathbf{k}_{II,-}$ was 2.75, with an average deviation of 0.22. This value agrees well with the value of 2.71 obtained using the *p*H values which were measured as part of the kinetic studies rather than the kinetic measurements themselves. It is in fair agreement with the value of 2.66 obtained from the stepwise titration curve (Table II).

(18) R. L. M. Synge, *Biochem. J.*, **39**, 351 (1945).

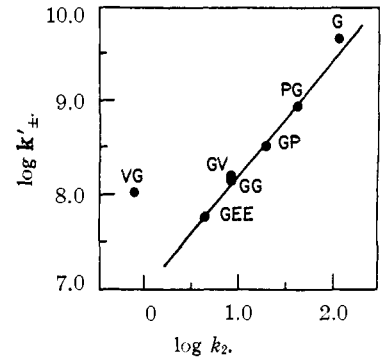

Fig. 3.—Relation between log k_2 and log $k'_{\pm}$: VG, L-valylglycine; GEE, glycine ethyl ester; GG, glycylglycine; GV, glycyl-L-valine; GP, glycyl-L-proline; PG, L-prolylglycine; G, glycine.

The agreement between the kinetic and equilibrium methods of measuring the concentration of PG^- and $\mathbf{k}_{II}^-$, extends the demonstration of the compatibility of the two methods to an α-imino peptide.

Mixed Complex with Imidazole.—A counterpart to reaction 3 was studied in the reaction between CuGG and imidazole, Im

$$CuGG + Im \rightleftharpoons CuGGIm;$$

$$\mathbf{k}_{CuGGIm} = \frac{(CuGGIm)}{(CuGG)(Im)} \quad (18)$$

Mixtures of $CuCl_2$, GG, NaOH and Im were prepared to yield the concentrations listed in Table VI after admixture with NPA. Sufficient NaCl was included to maintain an ionic strength of 0.16. The solutions were mixed with NPA solution and the rate of release of NP^- measured spectrophotometrically. The range of final *p*H was 5.89 to 7.08, in which the forms $CuGG^+$ and $CuGGOH^-$ are relatively negligible for 1:1 Cu(II)–GG mixtures (Fig. 1, curve 3; Table II).

The concentration of free basic imidazole, (Im), was computed from the kinetic measurements using the value of 31.3 l. $mole^{-1}$ $min.^{-1}$ for the specific second-order rate constant for imidazole under these conditions.[6] It was assumed that CuGG and CuGGIm do not react with NPA; the lack of participation of CuGG was confirmed experimentally. The results of the kinetic assay for (Im) were used to compute $\mathbf{k}_{CuGGIm}$ according to equation 18. In this computation the value of (Im) was combined with the *p*H value and that of pK' (7.08) to yield (HIm^+). The value of (CuGGIm) was then taken as the difference between the total concentration of imidazole and the sum of (Im) and (HIm^+). The value of (CuGG) in turn was obtained by difference. The results of these computations are shown in Table VI. The average value of log $\mathbf{k}_{CuGGIm}$ is 3.85 with an average deviation of 0.10. The constancy of this value confirms the assumption that CuGGIm itself does not react with NPA at a comparable rate.

The affinity of CuGG for Im is so great that the lower limit of reliability of the kinetic measurement of (Im) was reached with nearly one-third of the

TABLE VI

EQUILIBRIUM BETWEEN CuGG AND IMIDAZOLE

Total $CuCl_2$ 0.0100 *M*; total GG$^{=}$ 0.0100 *M*; temperature 25.1°; ionic strength 0.16

Initial total molar concn. of Im	NaOH	*p*H	$k_1 \times 10^2$, min.$^{-1}$	(Im) $\times 10^3$	(HIm^-) $\times 10^3$	(CuGGIm) $\times 10^3$	(CuGG) $\times 10^3$	log k_{CuGGIm}
0.00504	0.0190	6.40	0.382	0.122	0.584	4.33	5.67	3.80
.01008	.0150	5.89	0.743	.237	3.67	6.17	3.83	3.83
.01008	.0170	6.32	1.45	.463	2.67	6.95	3.05	3.69
.01008	.0180	6.50	1.54	.492	1.87	7.72	2.28	3.84
.01008	.0180	6.62	1.69	.540	1.56	7.98	2.02	3.86
.01008	.0190	7.08	2.81	.897	1.79	8.29	1.71	3.73
.02016	.0100	6.28	4.53	1.45	9.14	9.57	0.43	4.19
							Av. log k_{CuGGIm}	3.85

total CuGG in the form of CuGGIm. Measurements at higher values of (Im) failed to reveal any evidence for the formation of a complex containing two imidazole molecules, *e.g.*, $CuGG(Im)_2$.

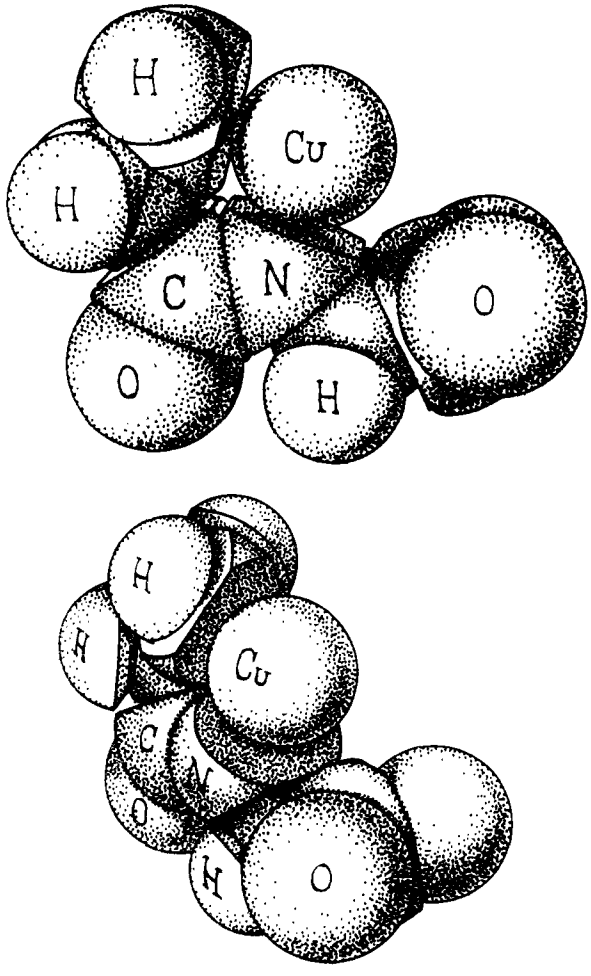

Fig. 4.—Molecular model of CuGG.

The large affinity of CuGG for Im made possible the preparation of solutions in which essentially all the Cu(II) was in the form CuGGIm. From measurements of the absorption spectra of such solutions, it was computed that the value of λ_{max} for the complex was 610 mμ and of ϵ_{max} 94.

Discussion

The results of the analysis of the titration curves below *p*H 7 of equimolar mixtures of $CuCl_2$ and dipeptides confirm the occurrence of the reactions shown in equations 1 and 2. Rabin[19] has pointed out that the values of log $\mathbf{k}_{I,+}$ for Cu(II)-complexes with several compounds containing N-terminal glycine are linearly related to log $\mathbf{k}_{\pm}'$. Datta, Leberman and Rabin[20] have extended these observations to series of compounds containing N-terminal leucyl and sarcosyl residues. For each family of compounds with the same N-terminal residue the linear relation between log $\mathbf{k}_{I,+}$ and log $\mathbf{k}_{\pm}'$ holds, but results for the different families of compounds are not superimposable. In the present series the same conclusions appear to hold: a plot of log $\mathbf{k}_{I,+}$ *vs.* log $\mathbf{k}_{\pm}'$ for GG, GS, GP and GV is linear, whereas the values for SG and PG do not obey the same relation. The values for VG appear to fit the N-terminal glycine series.

The Cu(II) atom in complexes of the type $CuGG^+$ probably is associated with the α-amino N atom and the carbonyl O atom of the peptide bond. It has been shown that the relation between log $\mathbf{k}_{I,+}$ and log $\mathbf{k}_{\pm}'$ for peptides containing N-terminal glycine can be extended to predict the observed value of log $\mathbf{k}_{I,+}$ for glycine itself.[19] Similar extensions to leucine and sarcosine have been reported.[20] The successful inclusion of the compounds GS and GP in the N-terminal glycine series gives further support to the suggestion that the Cu(II) ion is bonded to the carbonyl O atom of the peptide bond in complexes of the type $CuGG^+$, particularly in view of the present demonstration of the similarity of the absorption spectra of the forms $CuGG^+$, $CuGS^+$ and $CuGP^+$. This similarity in absorption spectra implies a common structure for both $CuGG^+$ and the complexes in which the peptide N is substituted.

By contrast, the failure of the Cu(II)–GS and Cu(II)–GP systems to form a complex of the type CuGG is strong evidence for the involvement of the peptide N in bonding to Cu(II) in the latter type of complex. A molecular model of CuGG has been constructed to a scale corresponding to a distance of 2.0 Å.[21] The N–Cu–N angle is close to 90° without distortion and the ring formed of Cu, α-amino N, methylene C, peptide carbonyl C and peptide N is coplanar. Drawings of the model are shown in Fig. 4. A structure of this sort preserves resonance in the peptide bond.[19] The stability

(19) B. R. Rabin, *Trans. Faraday Soc.*, **52**, 1131 (1956).

(20) S. P. Datta, R. Leberman and B. R. Rabin, *Nature*, **183**, 745 (1959).

(21) A. M. Mathieson and H. K. Welsh, *Acta Cryst.*, **5**, 599 (1952); H. Scouloudi, *ibid.*, **6**, 651 (1953).

of the complex is remarkable, considering that the dissociation of a proton from the peptide bond is usually taken to be negligible at *p*H values below 13 or 14.

The present method of computing $\mathbf{k}_{I,+}$ and $\mathbf{k}_{I,0}$ is equivalent to those employed by Dobbie and Kermack[14] and by Datta and Rabin.[9] The values computed for Cu(II)–GG at low ionic strength are in essential agreement with those of both these groups of authors. The present method of computation subjects the data to a rather severe and readily visualized test of internal consistency as illustrated in Fig. 2. The certainty with which the data of a given titration may be analyzed mathematically by this method is probably within ± 0.05 unit in log $\mathbf{k}_{I,0}$. The over-all uncertainty is probably at least ± 0.10 unit. In making these computations it is assumed that forms such as $CuGG^{+++}$ and $CuGG^{++}$ are not present in significant concentrations; these assumptions appear reasonable in view of the low values of $\mathbf{k}_{I,+}$ found for Cu(II)–AcGG and the probable increase in acidity of the carboxyl group of GG when the complex $CuGG^{+}$ is formed.

The complex $CuGGOH^{-}$ attacks NPA to catalyze its hydrolysis. Numerically the rate of the hydrolysis is very close to the rate of the reaction in which copper-free GG^{-} undergoes acetylation by NPA. Because of the great stability of the complexes in equimolar mixtures of Cu(II) and GG, very little of the latter reaction appears to occur in the presence of the Cu(II). The complex $CuGGOH^{-}$ may be written in several canonical forms, and it will be interesting to compare its catalytic properties with those of other Cu(II)–N chelates in the hydroxo form. The possible catalytic activities of other Cu(II)–dipeptide complexes of the $CuGGOH^{-}$ form have not yet been measured. Certain similarities between the system studied here and those described by Courtney, *et al.*, should be noted.[22]

The nucleophilic properties of $CuGGOH^{-}$ find expression as well in the combination with CuGG described in equation 6. Presumably the two CuGG moieties are joined to a common hydroxide ion to form an olate complex. The preference of the dipeptide complexes to form a monolate complex instead of a diolate complex may reflect the tendency of the carboxylate group of the dipeptide to act as a ligand to the Cu(II) ion. The possibility that the GG in CuGG should be looked upon as a tridentate ligand may explain the reluctance of a second GG to give up its peptide hydrogen atom when it combines with CuGG.

The studies reported here may serve to some degree as models for the behavior of the N-terminus of longer peptide chains such as are present in proteins. It has been shown that the N-terminus may react with NPA either to form the N-acetyl derivative or, in the presence of Cu(II) ion, to catalyze the hydrolysis of the NPA. Under appropriate conditions the N-terminus may be bridged by Cu(II) ion to another N-terminus or to a side-chain imidazole group. Some of these properties have been shown to vary materially with the nature of the dipeptide model and it may be expected that N-termini in proteins may exhibit a similar range of behavior. Whether or not longer peptide sequences will differ markedly from dipeptides in these properties is under study.

The high stability of complexes of the form CuGG and their apparent tendency to combine preferentially with one additional ligand point to their probable usefulness as reagents which might form ternary complexes with proteins. The present model studies suggest that such complexes would bind both to N-terminal α-amino groups and to imidazole groups and would probably show a preference for the latter.

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