[CONTRIBUTION FROM THE MALLINCKRODT LABORATORIES OF HARVARD UNIVERSITY AND FROM THE GEORGE HERBERT JONES LABORATORY OF THE UNIVERSITY OF CHICAGO]

Metal Ion Promoted Hydrolysis of Glycine Amide and of Phenylalanylglycine Amide

BY LEWIS MERIWETHER AND F. H. WESTHEIMER

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Cupric, nickelous and cobaltous ions promote the hydrolysis of glycine amide in slightly alkaline solution. In the absence of metal ions, phenylalanylglycine amide undergoes ring closure to 3-benzyl-2,5-diketopiperazine; in the presence of cupric ion at *p*H 5, hydrolysis at both the amide and peptide bonds is competitive with ring closure.

The action of many of the exopeptidases is promoted[1] by metal ions, and the function of metal ions in these enzymatic hydrolytic processes has been the subject of considerable speculation.[1,2] However, experimental data on model systems have been sparse. Kroll[3] discovered that cupric ion promoted the hydrolysis of glycine ethyl ester, but it is doubtful if the mechanism for this process is thoroughly understood.[4] The metal ion promoted hydrolysis of glycine amide has been partially investigated by Newton and Dauben[5]; further data on this system are discussed in this paper. But existing information on the cleavage of more complex systems by metal ions is at best fragmentary.

The present paper describes the hydrolyses of glycine amide and of phenylalanylglycine amide. A strong metal-ion catalysis of these reactions has now been discovered.

Experimental

Materials.—A chloroform solution of glycine amide was prepared from its hydrochloride[6] immediately before use. Phthaloylglycine[7] was prepared by refluxing glycine and phthalic anhydride in glacial acetic acid for 30 minutes[8]; m.p. 193.5–194.5°. Phthaloylglycylglycine was prepared in 71% yield by refluxing a mixture of equivalent quantities of phthalic anhydride, sodium acetate and glycylglycine hydrochloride in 1 *M* solutions in glacial acetic acid for 30 minutes. The product, which separated from the solvent on cooling, was recrystallized from water and melted[9] at 231–232°.

Phthaloyl-D,L-phenylalanylglycine amide was prepared by a procedure patterned after Sheehan's[10] method for the corresponding optically active anilide. A solution of 11.0 g. of phthaloyl-D,L-phenylalanyl chloride[11] in 450 ml. of chloroform was added at 0–2° over a period of 6 hours to a stirred solution of glycine amide (prepared from 7.43 g. of its hydrochloride) and 12.5 ml. of triethylamine in 500 ml. of chloroform. When the solution was chilled overnight, 5.75 g. of phthaloyl-D,L-phenylalanylglycine amide separated. An additional 2.9 g. could be obtained from the mother liquors. After recrystallization from water, it melted at 176–177.5°.

Anal. Calcd. for $C_{19}H_{18}O_4N_3$: C, 64.95; H, 4.88; N, 11.97. Found: C, 64.85; H, 4.96; N, 11.86.

D,L-Phenylalanylglycine Amide Hydrochloride.—A mixture of 6.93 g. of phthaloyl-D,L-phenylalanylglycine amide and 1.98 ml. of hydrazine hydrate was refluxed for an hour in 300 ml. of ethanol. The solution was taken to dryness in vacuum. The residue was dissolved in 60 ml. of water and acidified to *p*H 5. The mixture was chilled for 3 hr., the phthalhydrazide removed by filtration and the filtrate freeze-dried. The residue, after recrystallization from an absolute alcohol–ether mixture, weighed 3.39 g. and melted at 209–212°. A sample, prepared by recrystallization from absolute alcohol, was dried for analysis in a Fischer pistol at 56° under 10 cm. pressure of hydrogen chloride.

Anal. Calcd. for $C_{11}H_{16}O_2N_3Cl$: C, 51.26; H, 6.26; N, 16.31. Found: C, 51.19; H, 6.32; N, 16.03.

D,L-Phenylalanylglycine amide sulfate was prepared by the metathesis of the chloride salt with silver sulfate in aqueous solution. The suspension was shaken in the dark, the silver chloride removed by filtration and the filtrate freeze-dried. The residue was recrystallized from methanol-ether. The yield for the metathesis was about 65%; the compound melted at about 220°.

Anal. Calcd. for $C_{22}H_{32}O_8N_6S$: C, 48.88; H, 5.97; N, 15.55. Found: C, 49.01; H, 6.05; N, 15.92.

Phthaloyl-D,L-phenylalanylglycine ethyl ester was prepared according to the procedure of Sheehan[10] for the L-isomer. A sample, recrystallized from ethanol, melted at 128–131°.

Anal. Calcd. for $C_{21}H_{20}O_6N_2$: C, 66.60; H, 5.30; N, 7.37. Found: C, 66.41; H, 5.57; N, 7.30.

Phthaloyl-D,L-phenylalanylglycine, prepared according to Sheehan's[10] directions for the L-isomer, melted at 168–170°.

Anal. Calcd. for $C_{19}H_{15}O_6N_2$: C, 64.77; H, 4.58; N, 7.95. Found: C, 64.67; H, 4.60; N, 7.91.

D,L-Phenylalanylglycine was prepared following Sheehan's[10] procedure for the monohydrate of the L-isomer. The product was chromatographically pure and melted[12] at 274–276°.

D,L-3-Benzyl-2,5-diketopiperazine was prepared by refluxing 3.8 g. of phthaloyl-D,L-phenylalanylglycine ethyl ester and 1.5 ml. of hydrazine hydrate in 100 ml. of ethanol for an hour. The solution was evaporated to dryness, acidified to *p*H 5 and chilled. The solid product was triturated with sodium carbonate solution and filtered again. About 0.50 g. of diketopiperazine remained insoluble in the carbonate solution. An additional 0.63 g. could be obtained from the filtrate by warming it to 75° for 24 hr. at *p*H 5. The compound melts[13] at 277–279°; its solubility in water is 0.018 *M* at 75° and 0.00110 *M* at 2°.

Phthaloylglycyl-D,L-phenylalanine ethyl ester was prepared according to the procedure developed by Boissonnas[14] for phthaloylglycylglycine ethyl ester. A solution of 1.68 g. of phthaloylglycine and 1.95 ml. of tributylamine in 5 ml. of chloroform was treated at 0° with 0.79 ml. of ethyl chloroformate. After 10 minutes, a solution of 1.88 g. of D,L-phenylalanine ethyl ester hydrochloride and 1.95 ml. of tributylamine in 5 ml. of chloroform was added. The resulting suspension was washed successively with water, acid, bicarbonate and water. The chloroform solution was evaporated to a small volume and the ester crystallized from ether. The crude ester (m.p. 147–148°) was not purified but immediately used in the preparation of phthaloylglycylphenylalanine. This compound and glycylphenylalanine were prepared by the method of Sheehan and Frank.[11]

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(4) M. Bender, private communication.

(5) R. A. Newton, Dissertation, University of Washington, 1953.

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N-(2,4-Dinitrophenyl)-D,L-phenylalanine was prepared according to Sanger's[15] method for the L-compound. It melted at 214–215.5° (reported[16] 204–206°).

Anal. Calcd. for $C_{15}H_{13}O_6N_3$: N, 12.74. Found: N, 12.69.

N-(2,4-Dinitrophenyl)-glycine, similarly prepared, melted at 197–198° (reported[17] 205°).

Anal. Calcd. for $C_8H_7O_6N_3$: N, 17.43. Found: N, 17.38.

N-(2,4-Dinitrophenyl)-glycine amide was prepared by shaking 0.22 g. of glycine amide hydrochloride, 0.67 g. of sodium bicarbonate and 0.75 g. of 2,4-dinitrofluorobenzene in 30 ml. of 67% ethanol for 2 hr. at room temperature. The product, recrystallized from methanol–water, melted at 223–224°.

Anal. Calcd. for $C_8H_8O_5N_4$: C, 40.00; H, 3.36; N, 23.33. Found: C, 40.03; H, 3.52; N, 23.43.

N-(2,4-Dinitrophenyl)-D,L-phenylalanylglycine, prepared from D,L-phenylalanylglycine by a method paralleling that given above, melted at 173–174° after recrystallization from acetone–ligroin.

Anal. Calcd. for $C_{17}H_{16}O_7N_4$: C, 52.58; H, 4.15; N, 14.43. Found: C, 52.91; H, 4.30; N, 14.60.

N-(2,4-Dinitrophenyl)-D,L-phenylalanylglycine amide similarly prepared from the dipeptide amide hydrochloride melted at 204–205° after recrystallization from methanol–water.

Anal. Calcd. for $C_{17}H_{17}O_6N_5$: C, 52.71; H, 4.42; N, 18.11. Found: C, 53.10; H, 4.59; N, 18.11.

Buffers.—Sodium carbonate–bicarbonate buffers with a total carbonate content of 1 *M* were used in the *p*H range from 9.35 to 10.25, a veronal buffer (with total concentration of both species of 0.1 *M*) at *p*H 8.0, a 2,6-lutidine buffer (with total concentration of both species of 1 *M*) at *p*H 7.25 and acetate buffers (with total acetic acid plus acetate ion content of 1 *M*) in the *p*H range from 4.20 to 5.20. The *p*H determinations for these buffers were made at 25° and the values are certainly different at 75° where the rate measurements were made. However, the relative values were unaffected by the presence of cupric ion and, within any one buffer system, the relative acidities are probably largely independent of temperature. The *p*H, in these heavily buffered solutions, did not drift during any one experiment (with or without metal ion) by more than 0.1 *p*H unit; the usual drift was only a few hundredths of a *p*H unit.

Spectrophotometric Method.—The rate of hydrolysis of glycine amide was estimated by taking advantage of the difference between the absorption spectrum of the copper–glycine amide complex (λ_{max} 545 mμ) and those of copper–glycine and of copper–ammonia (λ_{max} 670 and 605 mμ). The composition of a solution containing the copper complexes of glycine, glycine amide and ammonia could be determined by comparison with an empirical standardization curve (Fig. 1). The ammonia made very little contribution to the absorption at 545 mμ, the losses of ammonia during transfer could be ignored. In a kinetic experiment, an aliquot of the solution was cooled to room temperature and then analyzed. If the hydrolysis mixture contained copper, the composition could be read directly from a comparison of the optical density with the data of Fig. 1. If it did not, an appropriate amount of copper sulfate solution was added to the cooled aliquot prior to the spectrophotometric reading.

Chromatography. (a) Ninhydrin Method.—Qualitative and semi-quantitative analyses of the products of hydrolysis were obtained by paper partition chromatography.[18] The descending chromatograms were obtained with Whatman No. 1 filter paper, using chromatography jars or, in later work, a Research Equipment Corp. "Chromatocab." *n*-Butyl alcohol saturated with 2 *N* aqueous ammonia was used as the solvent for the majority of the chromatograms. Regrettably, chromatograms made with this solvent system are difficult to reproduce, presumably due to the effects of variation in the solubility of butanol in water with small changes in temperature and of loss of ammonia by volatilization. After chromatography, the sheets were thoroughly dried in a stream of warm air, sprayed with 0.1% ninhydrin reagent and developed for 2–3 minutes at 110°. The papers were usually photographed, to preserve the colors, since some of the compounds (glycine amide, glycylphenylalanine) gave ninhydrin spots which were initially yellow but changed to purple during the next hour at room temperature. The approximate R_f values, in butanol–ammonia were[19]: Gly, 0.05; Gly–Am, 0.20; Phe–Gly, 0.39; Phe, 0.47; Phe–Gly–Am, 0.70. In order to carry out chromatography, metal ions had to be carefully removed from the solutions. This was best accomplished by precipitating the metal with H_2S and then centrifuging to remove the metal sulfide. No adverse effects were obtained from excess H_2S. Successive 5-μl. portions of the solutions were placed upon the paper and dried; the number of applications (1 to 10) was chosen in such a way as to get spots of the desired color intensity.

Chromatography. (b) Dinitrophenyl Derivatives.—The dinitrophenyl derivatives of the compounds present in a hydrolysis mixture were prepared by Sanger's method.[15] The reactions were carried out at room temperature and at *p*H 8 (bicarbonate solution) at high dilution[20] in 50% ethanol; the mixture remained homogeneous throughout. When the dinitrophenylation reaction was complete (about 2 hr.) the solution was evaporated to dryness in a stream of air. The residue was dissolved in 3:1 dioxane–water, and 10–50 μl. was transferred to the paper for chromatography. The mixture of dinitrophenyl derivatives was stored only as dry solid or as dry paper chromatograms; some of the derivatives slowly decomposed in solution.

The chromatography was carried out with *t*-amyl alcohol saturated with a *p*H 6 phthalate buffer.[21] The R_f values for the DNP derivatives are very sensitive to temperature in this system, probably because the saturation concentration of water in *t*-amyl alcohol is a sharp function of temperature. At 25°, the approximate values of the R_f numbers are: DNP–Gly, 0.25; 2,4-dinitrophenol, 0.46; DNP–PheGly, 0.54; DNP–Phe, 0.58; DNP–GlyAm, 0.78; DNP–Phe-GlyAm, 0.91; 2,4-dinitroaniline, 0.91; and 2,4-dinitrofluorobenzene, 0.95. (All of these compounds might be present in the dinitrophenylation mixture from the hydrolysate of phenylalanylglycine amide.) Under the conditions of the experiment, all of the dinitrofluorobenzene was converted to dinitrophenol, and the amount of dinitroaniline formed was very small. Therefore, the amounts of glycine, of glycine amide, of phenylalanylglycine amide and the sum of the phenylalanylglycine and phenylalanine could be determined.

After chromatography, the papers were dried, and the yellow spots cut out. The pieces of paper were then placed in 5 ml. of methanol in tightly stoppered test-tubes for an hour. The solutions were transferred to stoppered Corex cells and their optical densities determined at the wave length of maximum absorption with a model DU Beckman spectrophotometer. The method was tested by carrying out a series of controls of both the dinitrophenylation reaction and of the chromatography. Spots on the paper containing 1, 2, 5, 10, 15, 20, 25 and 30 μl. of a 0.01 *M* solution of various DNP derivatives were chromatographed, and the yields determined. These yields were remarkably constant but (probably because of the usual paper absorption factor) fell short of being quantitative. The paper absorption factors were: DNP–Gly, 1.23 ± 0.06; DNP–GlyAm, 1.25 ± 0.04; DNP–Phe, 1.60 ± 0.04; DNP–PheGly, 1.09 ± 0.04 and DNP–PheGlyAm, 1.06 ± 0.05.

In order to determine the yield on the dinitrophenylation reaction, a series of solutions was made up, representing various stages in the hydrolysis, and the entire analytical procedure carried to completion. The yield for DNP–PheGlyAm, for example, was 75 ± 7%; comparable yields were obtained for the other reactants. The uncertainty in the dinitrophenylation yields together with the uncertainties

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(19) The abbreviations are those of F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

(20) W. A. Schroeder and J. LeGette, THIS JOURNAL, **75**, 4612 (1953).

(21) S. Blackburn and A. G. Lowther, *Biochem. J.*, **48**, 126 (1951).

in the paper absorption factor reduced the accuracy of individual determinations to about ±10%.

Procedures.—The hydrolysis of phenylalanylglycine amide sulfate was carried out in an acetate–acetic acid buffer at 75°. A sealed ampoule was used for each point in the curve. When the sample was removed from the thermostat, it was cooled and stored at 0–2° for several days to allow the mixed diketopiperazine to crystallize. The solution was then carefully removed from the crystals and the latter washed with cold water; the amount of the mixed diketopiperazine was estimated both gravimetrically and spectrophotometrically. For the latter procedure, 5 ml. of glacial acetic acid was added to each tube, and the optical density of the resulting solution was measured at 264 mμ, where the extinction coefficient of the diketopiperazine is 127. The solution which was removed from the crystals of diketopiperazine was then analyzed by the dinitrophenylation and chromatographic procedures outlined above. The solutions which contained metal ions were treated, prior to the dinitrophenylation, with H_2S, filtered, and the filtrates freed of H_2S with a stream of nitrogen.

Results

Glycine Amide.—The data in Table I were obtained for the hydrolysis of glycine amide hydrochloride in sodium carbonate–sodium bicarbonate buffers, according to equation 1.

$$H_2NCH_2CONH_2 + H_2O \longrightarrow H_3\overset{+}{N}CH_2CO_2^- + NH_3 \quad (1)$$

TABLE I

RATES OF THE CUPRIC ION CATALYZED HYDROLYSIS OF 0.04 *M* GLYCINE AMIDE HYDROCHLORIDE

$CuSO_4$, m./l.	*T*, °C.	*p*H[a]	$10^5\ k_{obs}$, sec.$^{-1}$
0.02	65.5	9.35	5.19
.01	65.5	9.35	2.45
.004	65.5	9.35	1.47
...	65.5	9.35	0.19
.02	76	9.75	20.4
.02	76	9.75	20.6
...	76	9.75	1.0
.02	65.5	9.75	6.00
.01	65.5	9.75	3.33
.004	65.5	9.75	1.77
...	65.5	9.75	0.37
...	65.5	9.75	0.34
.02	55	9.75	2.03
...	55	9.75	0.27
.02	40	9.75	0.441
.02	25[b]	9.75	0.103
.02	65.5	10.25	10.3
.02	65.5	10.25	9.80
.01	65.5	10.25	5.05
.004	65.5	10.25	3.53
...	65.5	10.25	1.4

[a] *p*H was maintained by 0.4 *M* Na_2CO_3–$NaHCO_3$ buffers. [b] Run was made at room temperature.

The copper ion catalyzed reactions led to satisfactory first-order rate constants, but the uncatalyzed runs gave first-order "constants" which showed a considerable drift toward lower values as the reaction progressed; this drift was especially important for the reactions carried out at the lowest *p*H. The constants recorded in Table I are those obtained after the first 20% of the reaction, when the drift had practically ceased. The reason for this drift has not yet been established, but presumably a second-order reaction competes with the first (*i.e.*, presumably, the amino group of glycine amide, as well as hydroxide ion, can attack the carboxamide group). The rate constants, therefore, must be regarded as approximate, although they clearly show that the hydrolysis is strongly promoted by cupric ion.

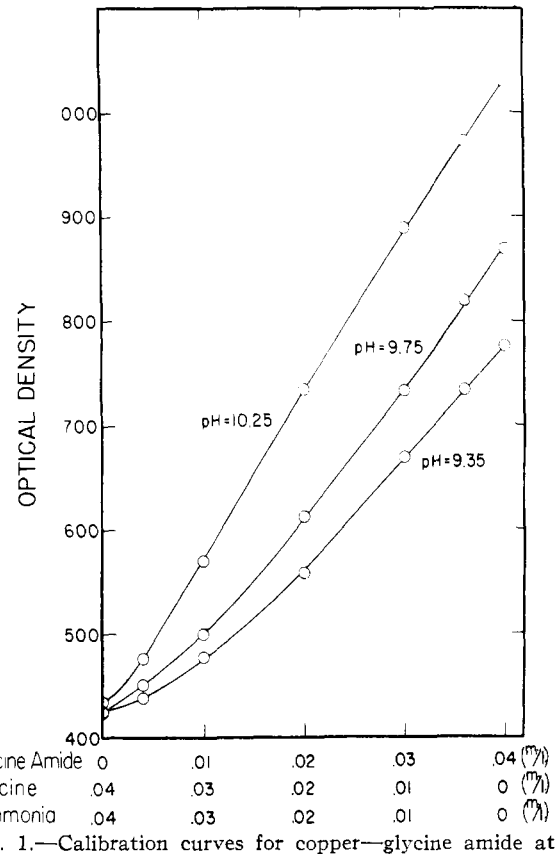

Fig. 1.—Calibration curves for copper—glycine amide at 545 mμ (0.02 *M* Cu^{++}).

Some qualitative experiments carried out by chromatography were conducted with 0.04 *M* glycine amide and 0.02 *M* metal ions at 75° in veronal buffer at *p*H 7.95. After 24 hr., the amount of glycine formed was estimated from the intensity of color of the ninhydrin spots on paper chromatograms. Roughly, the percentages of hydrolysis were as follows: no metal, 10%; Cu^{++}, 95%; Co^{++}, 70%; and Ni^{++}, 60%. No acceleration was observed with Zn^{++}, although the comparison is limited by the fact that, with this metal, the *p*H could not be raised above 7.1.

Phenylalanylglycine Amide Hydrochloride.—The hydrolysis of phenylalanylglycine amide is described by the following set of equations.

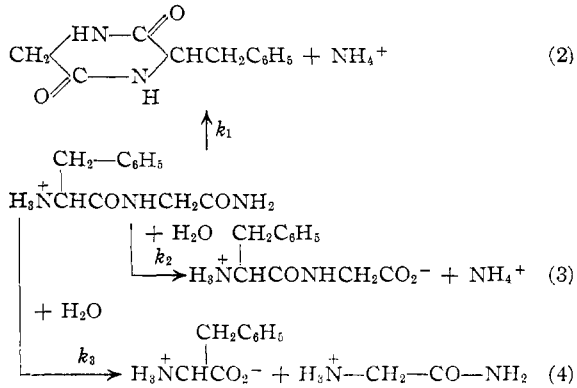

TABLE II[a]

HYDROLYSIS OF 0.02 *M* PHENYLALANYLGLYCINE AMIDE HYDROCHLORIDE IN LUTIDINE BUFFER AT 75°

Promoter	None		0.02 *M* Cu^{++}[b]		0.02 *M* Ni^{++}		0.02 *M* Co^{++}	
Time, hr.	72	144	72	144	72	144	72	144
Gly	tr.	tr.	+	++	+++	++++	++	++++
Gly–Am	+	+	++	++	++	tr.	+	tr.
Gly–Phe	+	++	tr.	+	+	+	tr.	++
Phe–Gly	tr.	+	+++	++++	++	++	+++	++++
Phe	+	+	+++	++++	+++	+++	+	+++
Phe–Gly–Am	+++	++	++	+	+++++	++++	+++	++

[a] The relative intensities of the spots on the chromatograms are represented thus: ... = no spot; tr. = trace, just visible; + to +++++, increasing intensity of visible spots. [b] Cu^{++} was partially reduced to Cu^{+}.

The pK of phenylalanylglycine amide is 6.72 (potentiometric titration). Therefore, at pH 5 the major species is the protonated form of the substrate; at pH > 7, the unprotonated substrate predominates.

Semi-quantitative data were obtained by separating the amino acids and peptides chromatographically and estimating the intensity of the color of the spots developed by ninhydrin. The results show strong Cu^{++} promoted hydrolysis of phenylalanylglycine amide hydrochloride at pH 5 and catalysis by Cu^{++}, Ni^{++} and Co^{++} at higher pH values The data for cupric ion catalysis is partially obscured by the reduction of Cu^{++} to Cu^{+} or Cu_2O; the reduction was slight at the lower pH range. At high pH, the hydrolysis of phenylalanylglycine amide yielded, in addition to phenylalanylglycine, almost equal quantities of glycylphenylalanine. Presumably, the "inverted" dipeptide arises from the hydrolysis of the mixed diketopiperazine. Typical sets of data are shown in Tables II and III.

TABLE III[a]

HYDROLYSIS OF 0.02 *M* PHENYLALANYLGLYCINE AMIDE HYDROCHLORIDE IN CARBONATE BUFFER AT 75°

Promoter	None		0.02 *M* Cu^{++}	
Time, hr.	0.5	4	0.5	4
Gly	Spots obliterated completely by buffer			
Gly–Am	...	...	...	...
Gly–Phe	tr.	+++	+	tr.
Phe–Gly	+	+++	++	++++
Phe	tr.	tr.	tr.	+
Phe–Gly–Am	+++++	++++	+++++	+++

[a] See footnote *a*, Table II.

Phenylalanylglycine Amide Sulfate.—The hydrolysis of the dipeptide amide sulfate was carried out around pH 5 in acetate buffers. Aliquots of the reaction mixture were pipetted into small reaction tubes, which were evacuated and sealed, and then placed in a thermostat at 75°. At the longest times, a small amount of brown precipitate sometimes formed in the tubes; this material was identified as Cu_2O by an X-ray powder photograph.[22] Although Cu^{++} is extensively reduced by phenylalanylglycine amide hydrochloride, the reduction by the dipeptide amide sulfate was slight; it did not exceed 2% of the total cupric ion present during a period of time sufficient to hydrolyze more than 80% of the substrate. Furthermore, no Cu^{+} remains in solution at pH 5. This fact was established by showing that the optical density over the range 540–625 mμ was unaffected by aeration. The reduction of Cu^{++} was therefore an unimportant side reaction, grossly insufficient to account for the changes which occurred in the solution.

The reaction, in the absence of cupric ion, gave an almost quantitative yield of the mixed diketopiperazine, as shown in equation 2. In the presence of cupric ion, the formation of the diketopiperazine was accompanied by the cleavage of both the peptide and of the amide bond, according to equations 3 and 4; even with 0.1 *M* Cu^{++}, however, the diketopiperazine was the major product. The rates of the various processes are shown in Table IV. Here the rate of formation of the diketopiperazine was measured both gravimetrically and spectrophotometrically. The rate of disappearance of phenylalanylglycine amide, the rate of appearance of the dipeptide and the rate of appearance of phenylalanine were measured by the dinitrophenylation procedure. The rate constants k_2 and k_3 were determined from "product plots,"[23] *i.e.*, from a plot of the concentration of phenylalanylglycine against $1 - e^{-kt}$ and of the concentration of phenylalanine against $1 - e^{-kt}$, respectively, where k is the average constant for the disappearance of the dipeptide amide.

In order to find the extent of copper catalysis, the rate of cleavage in the absence of the metal ion was needed. Unfortunately, no true measure of this cleavage has yet been obtained. However, an upper limit could be established for these rate constants. The least amount of each product which could be detected by the ninhydrin technique was determined, and it was then assumed that this amount of product had in fact been formed during the uncatalyzed cleavage. These limits of detection, by the ninhydrin method, led to the rate constants recorded in Table IV.

Phenylalanylglycine, Etc.—The hydrolyses of phenylalanylglycine, of glycyl phenylalanine and of the mixed diketopiperazine are only slightly promoted by nickelous or cupric ions.

Discussion

The data here presented show that cupric ion,

(22) The authors wish to express their appreciation to Professor C. Frondel of the Geology Department at Harvard for this identification of Cu_2O.

(23) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, N. Y., 1953, p. 149.

TABLE IV

RATES OF THE CUPRIC ION CATALYZED HYDROLYSIS OF PHENYLALANYLGLYCINE AMIDE SULFATE AT 75° AND μ 0.60

PGA, m./l.	$CuSO_4$, m./l.	pH[a]	10^5k_{obs}, sec.$^{-1}$ DKP – Spec.[b]	10^5k_{obs}, sec.$^{-1}$ DKP – Grav.[c]	10^5k_{obs}, sec.$^{-1}$ PGA[d]	10^5k_{obs}, sec.$^{-1}$ Ave.	10^5k_2,[e] sec.$^{-1}$	10^5k_3,[f] sec.$^{-1}$
0.02	...	4.66	2.03	2.25	2.11	2.14	<0.002	<0.0006
.02	0.004	4.63	1.81	1.92	1.83	1.86	.014	....[g]
.02	.01	4.62	1.47	1.33	1.56	1.44	.033	....
.02	.02	4.59	1.25	..	1.31	1.28	....[g]	....
.02	.02	4.61	1.14	1.25	1.39	1.25	0.072	0.017
.02	.10	4.58	..[g]	..	0.97	0.97	.23	.050
.02[h]	.10	4.58	..	..	0.83	0.83	.21	.047
.10	...	4.67	1.89	2.36	..[g]	2.12	< .002	< .0006
.10	.02	4.58	0.86	0.72	1.00	0.86	.0069	....
.02[i]	...	5.07	2.70	..	2.61	2.67	....	....
.02	...	5.16	2.62	2.84	..	2.72	< .005	< .0006
.02	.02	5.07	..	0.64	0.72	0.69	.13	.042
.02	...	4.06	1.45	1.50	1.19	1.39	< .002	< .0006
.02	.02	4.12	0.92	0.86	1.00	0.92	.044	....

[a] pH was maintained with 0.5 M NaOAc–HOAc buffers. [b] Calculated from the spectrophotometric determinations of the diketopiperazine, assuming that it is the only product formed. [c] Calculated from the gravimetric determinations of the diketopiperazine. [d] Calculated from the chromatographic determination of dinitrophenyl PheGlyAm. [e] k_2 as shown in equation 3. [f] k_3 as shown in equation 4. [g] Constant could not be obtained because of limitations in the analytical procedure. [h] Degassed three times and sealed in vacuum. [i] PheGlyAm hydrochloride; no added salt.

and to a smaller extent cobaltous and nickelous ion, promote the hydrolysis of glycine amide; 0.02 M Cu^{++} at pH 7.9–9.25 increases the rate of hydrolysis by a factor of about 30. At pH 5, cupric ion directs the hydrolysis of phenylalanylglycine amide to cleavage products rather than to the mixed diketopiperazine. The increase in rate for the cleavage reactions in the presence of 0.1 M Cu^{++} amounts at least to a factor of 100. A part of the effect of Cu^{++} in this reaction consists in complexing the dipeptide amide at the amino group and therefore decreasing the rate at which it forms diketopiperazine. However, the rate of formation of the mixed diketopiperazine is diminished, in the presence of copper, by a factor of only about two. The greatly increased hydrolysis of the peptide and amide bonds, therefore, cannot be a simple concentration effect, *i.e.*, the dipeptide amide is not present in high concentration for a long period of time even in the presence of copper ions. The relatively rapid rate of formation of the diketopiperazine from the cupric ion complex of phenylalanylglycine amide has not been explained. Preliminary measurements of the complexing constants[24] for phenylalanylglycine amide with cupric ion suggest that at pH 5 and 0.02 M peptide amide almost all of the dipeptide amide is complexed, probably in a two-to-one complex. The structure of this complex has not yet been elucidated, although earlier work on the complexes of amino acid amides[25] and of peptides[26] suggests that in alkaline solution the formation of the complex requires that a proton be ionized from the amide or peptide bond. In the absence of a knowledge of the structure of the complexes, and of more precise kinetics, no mechanism for the hydrolytic reactions can be advanced with confidence. However, the positive charge of the metal ion probably is responsible for facilitating the attack of water or hydroxide ion on the amide bond (*i.e.*, the metal ion is acting as a Lewis acid in aqueous solution).[27]

The effect of metal ions on the hydrolysis of glycine amide and of phenylalanylglycine amide, although large, compares very unfavorably with the enormous catalytic effects obtained with metal-ion promoted peptidases. In these enzymatic reactions the enzyme may employ the metal cation, as in the examples here cited, to produce part of the acceleration in hydrolysis, but the largest part of the catalytic effect of the enzymatic systems remains to be explained.

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CAMBRIDGE, MASS.
CHICAGO, ILLINOIS

(24) L. Meriwether (Dissertation, University of Chicago, 1956) has determined the equilibrium constants for the reactions: $M^{++} + B \rightleftarrows MB^{++}$ and $MB^{++} + B \rightleftarrows MB_2^{++}$ where B represents PheGly–Am and MB^{++} and MB_2^{++} are the complexes of cupric ion with one and with two equivalents of the dipeptide amide. In the pH range from 3 to 5, both K_1 and K_2 have values around 3×10^4 (m./l.)$^{-1}$.

(25) M. M. Rising and P. S. Yang, *J. Biol. Chem.*, **99**, 755 (1933).

(26) M. M. Rising, F. M. Parker and D. R. Gaston, THIS JOURNAL, **56**, 1178 (1934); P. Pfeiffer and S. Saure, *J. prakt. Chem.*, **157**, 97 (1941).

(27) *Cf.* F. H. Westheimer, Abstracts of Papers, 120th Meeting of the American Chemical Society, New York, N. Y., 1951, p. 7-P.