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The Thermal Condensation of Glutamic Acid and Glycine to Linear Peptides[1]

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Although glutamic acid is converted by heat virtually entirely to the inner lactam, it undergoes copolymerization with each of many amino acids to yield linear peptides. The effects of conditions of reaction have been studied for the copolymerization of glutamic acid and glycine. The dialyzed products, of average molecular weight 11,000–20,000, have also been characterized in yield, amino acid composition, N-terminal amino acid composition and infrared absorption spectra.

The heating of unsubstituted amino acids has typically yielded diketopiperazines, decarboxylation products and tars rather than linear peptides.[2–4] Such experiments have been carried out with single amino acids in almost all cases. When, however, amino acids are copolymerized by heat,[5] there often result linear peptides from amino acids which fail to yield peptides when heated individually.[6] Continuing investigation reveals different patterns of behavior for different combinations of amino acids. In this paper is described the formation of copolymers of glutamic acid and glycine.

The thermal behavior of glutamic acid has long been recognized as one which leads to α-pyrrolidonecarboxylic acid (pyroglutamic acid).[7] Confirmatory experiments in this Laboratory indicate no biuret-positive fraction after dialysis of heated glutamic acid and questionable amounts before dialysis. The other monomer, glycine, has been recognized as a single amino acid forming peptides, in part, on heating.[2] This behavior has been studied in detail recently by Meggy,[3] who, however, stated that other amino acids are not amenable to thermal formation of peptides. As the infrared examination, compositional studies and other data reveal (RESULTS) the heating of glutamic acid with glycine yields a polymer having the characteristics of a linear peptide composed of both amino acids.

Experiments revealed also that glutamic acid reacts more readily to form peptides if it is first converted to its lactam. Accordingly many of the experiments were performed by heating the glutamic acid alone prior to mixture with the other monomer. Testing of amino acids other than glycine with glutamic acid indicated that these also will form peptides with glutamic acid. Many of these coreactants are amino acids which, unlike glycine, will not form linear peptides when heated alone.

Experimental and Analytical

Copolymerizations of Glutamic Acid and Other Amino Acids and Derivatives.—In some cases glutamic acid, L or DL, was heated with another amino acid in equimolar amount, after the two had been ground together, typically for 30–120 min. at 160–190°. The cooled product was treated with 5 or 10 ml. of water. In most cases all of the product dissolved. The solution was subjected to biuret test with 10 volumes of 1 *N* NaOH and several drops of 0.3% $CuSO_4$ solution. In other cases, the glutamic acid was first liquefied as the lactam by heating, typically for 30 min. at 180°, and the other component(s) was then added in equimolar ratio. In these latter instances the glutamic acid is indicated in Table I as Pyroglutamic acid. Heating was continued usually for 50 min. in the range of 160–180°. All of the results are in Table I.

TABLE I

BIURET RESPONSES OF TWO OR MORE REACTANTS FOLLOWING HEATING

Reactants	Biuret response
Glutamic acid + glycine[a]	+
Glutamic acid + glycylglycine[a]	+
Glutamic acid + diketopiperazine[a]	+
Pyroglutamic acid + glycine[a]	+
Pyroglutamic acid + diketopiperazine	0
Pyroglutamic acid + diketopiperazine + water[b]	+
Glutamic acid + aspartic acid[a]	+
Glutamic acid + asparagine	+
Pyroglutamic acid + aspartic acid[a]	+
Pyroglutamic acid + alanine	+
Pyroglutamic acid + valine	+
Pyroglutamic acid + leucine	+
Pyroglutamic acid + phenylalanine	+
Pyroglutamic acid + serine	Product at 160° too dark to test
Pyroglutamic acid + lysine monohydrochloride	+ Intensely
Pyroglutamic acid + leucine + glycine[a]	+
Pyroglutamic acid + proline + glycine[a]	+
Pyroglutamic acid + aspartic acid + glycine[a]	+
Pyroglutamic acid + lysine mononydrochloride + glycine	+
Pyroglutamic acid + lysine monohydrochloride + cystine	+
Pyroglutamic acid + lysine monohydrochloride + glycine + cystine	+
Diketopiperazine + glycylglycine	0
Diketopiperazine + glycine + phenylalanine	0
Glycine + aspartic acid[a]	+ Weakly
Glutamine + aspartic acid	+
Glutamine + asparagine	+ Weakly

[a] On addition of water to the product, a biuret-positive precipitate separated. [b] 100 mg. of each reactant, plus few drops of water.

Copolymerizations of Glutamic Acid and Glycine.—In a typical preparation, 1.65 g. (0.01 mole) of DL-glutamic acid monohydrate was ground with 1.51 g. (0.02 mole) of glycine in a mortar. The mixture was heated in an open test-tube in an oil-bath at 175–180°. The mixture melted slowly and evolved a gas which turned litmus blue. When evolution of gas virtually ceased after 50 min., the heating was terminated and the brown liquid solidified on cooling. To this was added 10 ml. of water; a white to gray solid separated. After overnight standing, the solid was centrifuged and washed with 10 ml. of water and then with 10 ml. of ethanol. The dried polymer weighed 0.29 g. and gave an intense biuret reaction, as did the mother liquor. The solid was suspended and dialyzed for several days and then dried in a vacuum desiccator. A gelatin-like film was deposited, yield 0.09 g. Conducting the reaction under carbon dioxide or preferably nitrogen, minimized coloration which appeared

(1) Contribution No. 82 of the Oceanographic Institute, Aided by Grant RG-4666 of the National Institutes of Health, U. S. Public Health Service.

(2) E. Katchalski, *Advances Protein Chem.*, **6**, 123 (1951).

(3) A. B. Meggy, *J. Chem. Soc.*, 1444 (1956).

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(6) S. W. Fox, A. Vegotsky, K. Harada and P. D. Hoagland, *Ann. N. Y. Acad. Sci.*, **69**, 328 (1957).

(7) E. Fischer and T. Dörpinghaus, *Z. physiol. Chem.*, **36**, 476 (1902), and bibliography.

TABLE II

YIELDS, TOTAL COMPOSITIONS AND N-TERMINAL COMPOSITIONS OF GLUTAMIC ACID–GLYCINE POLYMER

Moles and form of glutamic acid	Moles of glycine	Temp., °C.	Time of heating, min.	Yield of polymer, g. Before dialysis	Yield of polymer, g. After dialysis	Total compn., % Glutamic acid	Total compn., % Glycine	N-Glutamic acid / ΣN-amino acid, %	N-Glycine / ΣN-amino acid, %
0.01 DL	0.01	175–180	50	..	..				
.01 L	.01	175–180	50	..	..				
.01 DL	.02	175–180	50	0.29	0.09			24	76
.01 L	.02	175–180	50	.29	.09	23.4	76.6		
.01 L	.03	175–180	60						
		+185	10	.55	.28	20.2	79.8	17	83
.02[a]	.04	175	60		.11				
.02[a]	.06	175	60		.55				

[a] Pyroglutamic acid from L-glutamic acid.

mainly at the surface. In most polymerizations 0.01 mole of L-glutamic acid was first heated alone at 180° for 30 min. to produce pyroglutamic acid.

When the polymerization was carried out for 4.5 hr. at 170° under CO_2 with 44.1 g. (0.3 mole) of L-glutamic acid preheated for 60 min. at 180° and 56.3 g. (0.75 mole) of glycine, the total yield of slightly colored polymer was 18.2 g. after dialysis.

In the reactions described in Table II, the heating was conducted in open test-tubes, and after heating the cooled product was treated with 10 ml. of water, left standing overnight, separated by centrifugation, and washed with 10 ml. each of water and ethanol. Dialysis was conducted in Visking cellophane tubing for 5 days with agitation of continuously changing bath by magnetic stirrer.

Elemental Analysis.—The elemental analysis was performed by Dr. T. Shiba of Osaka University. This material was from that prepared from 0.01 mole of L-glutamic acid and 0.02 mole of glycine as described in Table II. Before analysis, it was dried in a desiccator over sulfuric acid.

Calcd. for glutamic acid–glycine polymer (on basis of 23.4 glutamic acid: 76.6 glycine as reported in Table II): C, 43.14; H, 5.35; N, 21.30. Calcd. for polyglutamic acid: C, 46.51; H, 5.47; N, 10.86. Calcd. for polyglycine: C, 42.10; H, 5.31; N, 24.55. Found: C, 40.40; H, 5.53; N, 20.20. Electrometric titration gave equivalent weight values of 293 and 318.

Infrared Absorption Analysis.—The polymer was also subjected to infrared analysis by Miss N. Esquivel of Florida State University and by T. Inui of Osaka University, and the results interpreted from discussions by Bellamy[8] and Bamford, Elliott and Hanby.[9] The salient absorption maxima are depicted in Fig. 1. The spectra show that the material has a typical linear polypeptide structure and is not a diketopiperazine. The adsorption bands and their interpretations are: 3300 cm.$^{-1}$, NH stretching; 3080 cm.$^{-1}$, NH stretching; 1690 cm.$^{-1}$, CO of COOH; 1650 cm.$^{-1}$, CO stretching Amide I; 1550 cm.$^{-1}$, NH deformation, Amide II; 1250 cm.$^{-1}$, Amide III.

Amino Acid Composition.—Polymer (100 mg.) was hydrolyzed with 25 ml. of 6 *N* hydrochloric under reflux for 6 hr. After concentration under reduced pressure, water was added and the process repeated to remove unbound hydrochloric acid. The residue was brought to *p*H 7 and 5.0 ml. of volume. To this were added 0.4 g. of sodium bicarbonate, 0.4 g. (2.2 mmoles, 0.28 ml.) of 2,4-dinitrofluorobenzene (DNFB) and 10 ml. of ethanol.[10] After 2 hr. of mechanical shaking and 12 hr. of standing in the dark, the ethanol was evaporated under reduced pressure at 40° to approximately one-fourth of the original volume. The liquid was acidified with 2 *N* hydrochloric acid and extracted thrice with ethyl acetate. After the ethyl acetate was evaporated, 10 ml. of chloroform–ether (3:1) was added and the resulting dinitrophenyl (DNP)–amino acids and dinitrophenol were separated by chromatography on Hyflo Supercel.[11] The adsorbent was prepared by treating with 0.2 *M* buffer (sodium dihydrogen phosphate and citric acid; *p*H 4) and packed solidly into a tube of 8 mm. i.d. and length 25 cm. The column was charged with the chloroform–ether solution and the individual bands of DNP-glycine and DNP-glutamic acid were separated by cutting and the solvent was evaporated. Each fraction was eluted with 1.5% sodium bicarbonate solution and centrifuged to separate traces of Supercel carried along. The optical density of each of these solutions was read on a Beckman spectrophotometer at 360 mμ and compared with a standard solution of the DNP-amino acid in order to estimate the amount.

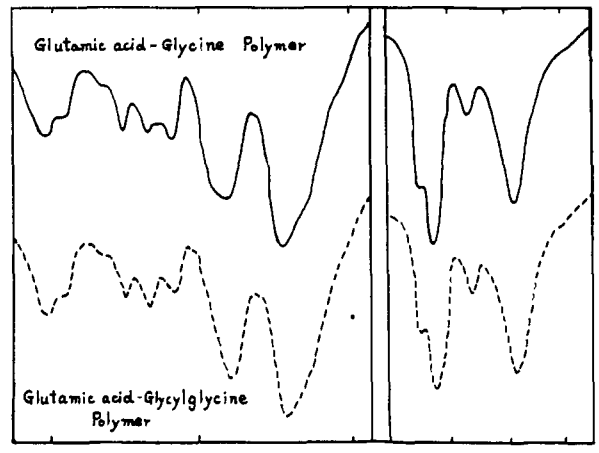

Fig. 1.

Estimation of Proportions of N-Glutamic Acid and N-Glycine.—DNP-Polymer (60 to 100 mg.) was hydrolyzed with 25 ml. of 6 *N* hydrochloric acid under reflux for 12 hr. in the dark. The solution was diluted with an equal volume of water, extracted by ethyl acetate, the ethyl acetate was evaporated and the residue was dissolved in chloroform–ether. The DNP-amino acids were separated on columns and estimated as described earlier, with the superposition of corrections calculated from recovery experiments with DNP-glutamic acid and DNP-glycine hydrolyzed under the same conditions as the DNP-polymer.[12]

Estimation of Average Molecular Weight of Peptide Chain.—The analytical procedure was the same as for assay of N-amino acids.[13] The molecular weights were calculated on the basis of a polymer consisting of 25% glutamic acid residue and 75% glycine residue. This calculation assumes no fragmentation of polymer molecule

(8) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," John Wiley and Sons, Inc., New York, N. Y., 1954, pp. 175–180.

(9) C. H. Bamford, A. Elliott and W. E. Hanby, "Synthetic Polypeptides," Academic Press, Inc., New York, N. Y., 1956, Chaps. 6, 7.

(10) K. R. Rao and H. A. Sober, THIS JOURNAL, **76**, 1328 (1954); S. Akabori and S. Mizushima, "Chemistry of Proteins," Vol. IV, Kyoritau Shuppan Co., Ltd. (Japan), 1956, p. 210.

(11) J. C. Perrone, *Nature*, **167**, 513 (1951); A. Courts, *Biochem. J.*, **58**, 70 (1954).

(12) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(13) W. R. Middlebrook, *Biochim. Biophys. Acta*, **7**, 547 (1951)

during the DNPylation, no cyclopeptide and no N-terminal pyroglutamic acid.[14]

In order to check on whether the amino group assessed by DNFB might arise from a pyroglutamyl residue opened by the DNP-ylation, the DNP procedure was carried out on an authentic sample of DL-pyroglutamic acid. No colored product other than dinitrophenol was obtained.

It is not known whether the glutamic residues are bonded α or γ or through some mixture of these two types. No attempt was made to determine the relative proportions of such bonds.

Results and Discussion

None of the products of the reactions in Table I left ninhydrin-positive residues in the dialysis bag after 5–7 days. The results of experiments in Table IV indicate that heating periods of longer than 1 hr. would have yielded more intense biuret tests and probably an increased proportion of insoluble peptides of higher molecular weight.

Of eight amino acids tested with pyroglutamic acid, seven yielded positive biuret tests. The eighth, serine, gave a product which was too dark for the test.

Glutamic acid was successfully copolymerized with diketopiperazine, an anhydride, and glycine was successfully copolymerized with pyroglutamic acid, also an anhydride. The heating of diketopiperazine and pyroglutamic acid together, however, failed to yield peptide. When water was added, a positive biuret reaction indicated peptide was formed (Table I). On one hand some water is necessary. At the other extreme a large proportion of water, as in a solution, favors hydrolysis of peptides.[3,6] At molar ratios of water:diketopiperazine which exceeded 3:1 Meggy observed no polymerization of glycine through its diketopiperazine.[3]

The readiness with which glycine undergoes polymerization is probably a manifestation of the fact that it has no side-chain to provide steric hindrance. This property is observed in glycine and its derivatives in acidic hydrolysis of dipeptides,[15] in the direct thermal polymerization[3] and in the polymerization of the N-carboxyamino acid anhydride,[16] although it is not observed generally in the more complex situations involving enzymic synthesis[17] and enzymic hydrolysis of such peptides as "sluggish" glycylglycine.[18] Steric hindrance by the isopropyl group of valine appears, on the other hand, to operate in both enzymic[17,19] and non-enzymic reactions.[15,20]

The intense biuret reaction with the products from lysine and from alanine is noteworthy and is scheduled for early additional study. It is also to be observed that pyroglutamic acid is involved in copolymerization with representatives of each of the three classes of acidic, basic and neutral amino acids.

Table II shows no significant difference in yield between reactions involving L- or DL-glutamic acid It is clear, however, that an increase in the proportion of glycine reacting results in an increase in the yield of the non-diffusible product. This occurs without a corresponding increase in the proportion of glycine in the product; in fact, the change in composition in the one comparison possible is of doubtful significance.

Table III presents the results of a study of the effect of temperature on the copolymerization. For 1 hr. of heating, the critical temperature is between 160 and 190°. This temperature is consistent with that observed to be critical by Maier in producing polymers from aspartic acid and leucine,[21] as well as in the reaction of pyroglutamic acid with each of several other amino acids. It should be stated, however, that other factors, notably the presence of phosphoric acid, can lower the threshold temperature in some cases.[22] As the temperature is increased, the proportion of non-diffusible (presumably higher molecular weight) material is increased. The average molecular weights found show a similar trend. Although a sufficient number of analyses of amino acid composition and N-terminal amino acids have not been run for statistical evaluation, it is believed that no single difference recorded in these figures in Table III is significantly larger than analytical variation. It seems safe to infer, however, that temperature does not appreciably affect composition. Perhaps increase in temperature increases the proportion of N-glycyl. The possibility of special reactions of the N-glutamic acid residue as by conversion to N-pyroglutamyl renders any such conclusion premature, especially inasmuch as N-glutamyl decreases with elevation of temperature.

In Table IV the effect of time of heating is presented. A considerable enhancement of yield results from carrying out the polymerization in the absence of oxygen.

As with increased temperature, increased time of heating produces a higher proportion of non-diffusible material. The effect upon molecular weight is pronounced. The composition is evidently significantly affected by time of heating, in contrast to the influence of elevated temperature.

It is of particular interest that the behavior of two amino acids in concerted heating is not the average nor the sum of their individual behaviors. The recognition of this principle in copolymerization of amino acids opens a vista for the relatively economical synthesis of peptides of a range of type not yet fully ascertained. The results of experiments in copolymerizing eighteen amino acids are being evaluated.

In attempting to visualize the mechanism of the reaction, it can be concluded that pyroglutamic acid, once formed, does not tend to homopolymerize. With other amino acids, however, glutamic acid, or its lactam, reacts as indicated in the equa-

(14) Pyroglutamyl peptides are known: T. Ohira, *J. Agric. Chem. (Japan)*, **15**, 370 (1939), and C. A. Dekker, D. D. Stone and J. S. Fruton, *J. Biol. Chem.*, **181**, 719 (1949). Poly-α-glutamic acid prepared through the Leuchs anhydride has no N-terminus, *cf.* H. Yuki, K. Okawa, T. Kimura and H. Tani, *J. Chem. Soc. Japan*, **72**, 264 (1957).

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

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(19) S. W. Fox, C. W. Pettinga, J. S. Halverson and H. Wax, *Arch. Biochem.*, **25**, 26 (1950).

(20) E. J. Harfenist, THIS JOURNAL, **75**, 5523 (1953).

(21) G. D. Maier, M.S. Thesis, Iowa State College, 1956.

(22) K. Harada, J. E. Johnson and S. W. Fox, unpublished experiments.

TABLE III

EFFECTS OF FOUR TEMPERATURES ON COPOLYMERIZATION OF GLUTAMIC ACID AND GLYCINE

Temp., °C.[a]	Yield of polymer, g. Before[b] dialysis	After[c] dialysis	Non-diffusible polymer (A) / Total polymer, %	Glutamic acid content of A, %	Glycine content of A, %	Av. mol. wt. of A	N-Glutamic acid / ΣN-amino acid, %	N-Glycine / ΣN-amino acid, %
160	0.06	Trace	..	..	..	....	..	..
170	.60	0.37[d]	61.7	24.1	75.9	15,500	23	77
180	.68	.47[e]	69.2	25.5	74.5	17,500	21	79
190	.70	.57[f]	81.4	25.7	74.3	20,000	19	81

[a] L-Glutamic acid (0.01 mole) was heated at 180° for 30 min., treated with 0.025 mole of glycine in an open tube with heating for 60 min. in an oil-bath at the temp. given. [b] The reaction mixture was taken up in 10 ml. of water, stood overnight, separated at the centrifuge and washed with 10 ml. of water and 10 ml. of ethanol. [c] After drying in vacuum desiccator following 7 days of dialysis. [d] Yellow film. [e] Grey film. [f] Grey-black film.

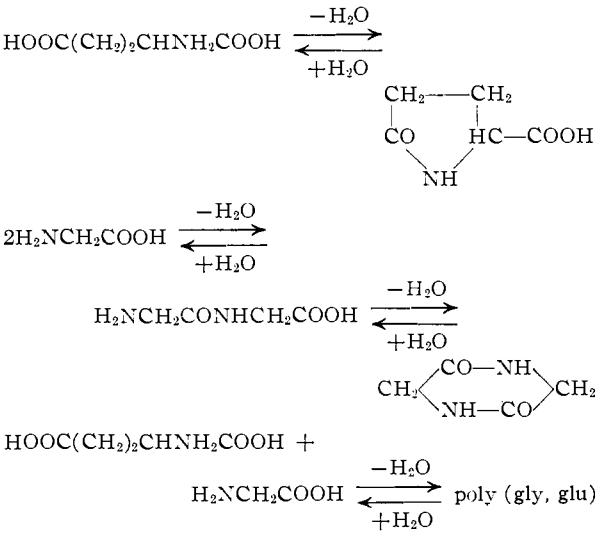
TABLE IV

EFFECT OF FOUR TIMES OF HEATING ON COPOLYMERIZATION OF GUTAMIC ACID AND GLYCINE

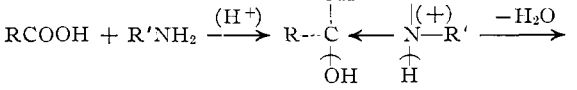
Reaction time,[a] hr.	Overlying atmosphere	Yield of polymer, g. Before dialysis	After dialysis	Non-diffusible polymer / Total polymer, %	Glutamic acid content, %	Glycine content, %	Av. mol. wt.	N-Glutamic acid / ΣN-amino acid, %	N-Glycine / ΣN-amino acid, %
1/2	CO_2	0.25	0.12[b]	48	22.6	77.4	12000	..	..
1	CO_2	.52	.39[c]	75	27.1	72.9	11000	20	80
2	CO_2	.62	.51[d]	82	26.7	73.3	18000	19	81
4	CO_2	.80	.71[e]	89	33.2	66.8	18000	17	83
1/2	Air	.26	.04[b]	15					
1	Air	.43	.15[d]	35					
2	Air	.63	.32[e]	51					

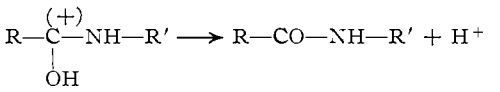
[a] All mixtures contained 0.01 mole of L-glutamic acid which was heated at 180° then treated with 0.025 mole of glycine at 170° for the time given, then processed as in Table IV. [b] Almost colorless film. [c] Slightly grey film. [d] Grey film. [e] Dark grey film.

tions. The ways in which water can influence the equilibria are illustrated in the equations.



Pyroglutamic acid is liquid at 170° in the presence of amino acids and therefore can function as a kind of solvent. The acidic property of this compound may also be significant in the polymerizations. The acidic condition suggests a proton-catalyzed formation of amide bonds.





This acid-catalysis may particularly be influenced by traces of water; the polymerization of caprolactam is known to be influenced in this way.[23]

The production of peptides by thermal copolymerization is of particular interest in attempting to understand prebiological origin of protein.[6] These experiments and others like them reveal in principle how a variegated peptide might have been generated spontaneously on the primitive earth. It is tempting to speculate that the particular function of glutamic acid in copolymerization explains its high proportion in all proteins[24] as the evolutionary consequence of an original biosynthesis in which the proportion of dicarboxylic acid was necessarily high. It will also be of interest to determine whether a real relationship exists between these manifestations of glutamic acid and its suggested key position in biosynthesis of protein.[25]

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