Shugar, D., and Fox, J. J. (1952), *Biochim. Biophys. Acta 9*, 199.
Smith, J. D., and Dunn, D. B. (1959), *Biochem. J. 72*, 294.
Spahr, P. F., and Tissières, A. (1959), *J. Mol. Biol. 1*, 237.
Terayama, H. (1952), *J. Polymer Sci. 8*, 243.
Ulbricht, T. L. V. (1962), *Angew. Chem. 74*, 767.
Volkin, E., and Cohn, W. E. (1954), *in* Methods of Biochemical Analysis, Vol. I, Glick, D., ed., New York, Interscience, p. 299.
Wheeler, G. P. (1962), *Cancer Res. 22*, 651.
Zamecnik, P. (1962), *Biochem. J. 85*, 257.
Zubay, G., and Doty, P. (1958), *Biochim. Biophys. Acta 29*, 47.

The Stability and Rearrangement of ε-*N*-Glutamyl-Lysines*

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Two model compounds, ε-*N*-(α-glutamyl)-lysine and ε-*N*-(γ-glutamyl)-lysine, have been synthesized and their stability has been studied in acid solutions. At intermediate stages of hydrolysis each compound forms the isomeric ε-*N*-glutamyl-lysine through the same transient intermediate, ε-*N*-(αγ-glutamyl)-lysine. This cyclic imide has been isolated from the partial hydrolysate of ε-*N*-(α-glutamyl)-lysine and obtained by chemical synthesis. ε-*N*-(α-Glutamyl)-lysine is hydrolyzed more slowly at all HCl concentrations and yields optimally 25 mole per cent of the cyclic imide and 10 mole per cent of ε-*N*-(γ-glutamyl)-lysine in 11 N HCl. The γ-compound forms only up to 2 mole per cent of the cyclic imide in 11 N HCl, but its product of rearrangement, ε-*N*-(α-glutamyl)-lysine continues to persist throughout the course of hydrolysis because of its greater stability. Evidence of an α,γ-rearrangement has also been obtained with ε-*N*-(glycyl-α-L-glutamyl)-α-*N*-L-toluene-sulfonyl-lysine, its γ-isomer, α-glutamyl glycine and α-glutamyl alanine. It is concluded that in view of the possible α,γ-glutamyl rearrangement and γ-glutamyl bond instability, partial acid hydrolysis is not suitable for the investigation of the presence of an ε-*N*-glutamyl-lysine sequence in protein.

Work in this laboratory has shown that the enzyme transglutaminase catalyzes the hydrolysis of the amide group of protein-bound glutamine or its exchange with ammonia, hydroxylamine, and a variety of primary amines (Clarke *et al.*, 1959; Mycek *et al.*, 1959; Mycek and Waelsch, 1960). There is also tentative evidence indicating that the ε-amino group of protein-bound lysine can act as a replacing amine, resulting in the crosslinking of proteins (Neidle *et al.*, 1958). These and other experiments[1] suggest the possibility that an amide linkage, involving the γ-carboxyl of glutamic acid and the ε-amino group of lysine, may occur in native proteins. It has been suggested that a linkage between one of the carboxyl groups of glutamic acid and the ε-amino group of lysine occurs in collagen (Mechanic and Levy, 1959).

Prior to an investigation of the possible occurrence of an ε-*N*-(γ-glutamyl)-lysine sequence in proteins, a study of the relative stability of the γ-glutamyl bond under various hydrolytic conditions was undertaken. Such a study was indicated particularly since an α,γ-glutamyl rearrangement under conditions of hydrolysis could be visualized on the basis of behavior of aspartyl peptides (Swallow and Abraham, 1958). In addition, the cyclization of the glutamyl residue, a likely intermediate step in an α,γ-rearrangement, has been observed in nonaqueous systems (Sondheimer and Holley, 1954; Kovacs *et al.*, 1955; Clayton *et al.*, 1956).

In order to study glutamyl amide bond stability and possible rearrangement, two model compounds, ε-*N*-(α-glutamyl)-lysine and ε-*N*-(γ-glutamyl)-lysine were synthesized and their properties examined in acid solutions. The present communication describes the interconversion of the two model compounds via a common cyclic intermediate, ε-*N*-(αγ-glutamyl)-lysine, and presents a comparison of their rates of hydrolysis.

EXPERIMENTAL

α-N-Cbz-ε-N-(cbz-α-L-Glutamyl)-L-Lysine Dibenzyl Ester.[2]—Cbz-γ-L-glutamyl benzyl ester, prepared from γ-benzyl-glutamate (Hanby *et al.*, 1950) by the procedure described for cbz-α-glutamyl benzyl ester (Sachs and Brand, 1953), and α-cbz-L-lysine benzyl ester benzene sulfonate (Bezas and Zervas, 1961) were coupled by the mixed anhydride procedure (Boissonnas, 1951). Ethyl chlorocarbonate, 0.72 ml., was added dropwise to a cooled solution of cbz-γ-L-glutamyl benzyl ester, 2.8 g (0.0075 mole), and tri-*n*-butylamine, 1.78 ml (0.0075 mole), in 10 ml dimethylformamide–10 ml tetrahydrofuran. After 20 minutes at 0°, a chilled solution containing 4.0 g (0.0075 mole) of α-cbz-lysine benzyl ester benzene sulfonate and 1.78 ml of tri-*n*-butylamine (0.0075 mole), in 20 ml of the same solvent was added. The reaction mixture was kept for 30 minutes at 0° and for 2 additional hours at room temperature. The solvent was evaporated *in vacuo*. The residue was taken up in ethyl acetate, and the organic solution was extracted twice with 2 N HCl, once with water, twice with 5% K_2CO_3 solution, and with water. The ethyl acetate was evaporated *in vacuo*. The yield was 4.2 g (78%). The product, after recrystallization from ethanol-water, melted at 103–105°.

Anal. Calcd. for $C_{41}H_{45}O_9N_3$: C, 68.03; H, 6.27; N, 5.80. Found: C, 68.31; H, 6.38; N, 5.92.[3]

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[1] Kornguth, S. E., and Waelsch, H., manuscript in preparation.

[2] Abbreviations used: cbz, carbobenzoxy; tosyl, *para*-toluene sulfonyl.

[3] We are indebted to Mr. T. Zelmenis for the nitrogen analyses. Carbon, hydrogen, and bromine analyses were carried out by Schwarzkopf Microanalytical Laboratory, Woodside, New York.

α-N-Cbz-ε-N-(cbz-γ-L-Glutamyl)-L-Lysine Dibenzyl Ester.—Cbz-α-L-glutamyl benzyl ester (Sachs and Brand, 1953), 2.8 g (0.0075 mole) was coupled with α-cbz-L-lysine benzyl ester benzene sulfonate, 4.0 g (0.0075 mole) as described above. The yield was 5.0 g (92%). The product was recrystallized from dimethylformamide-water, and chloroform-ligroin. Mp. 77–78°.

Anal. Calcd. for $C_{41}H_{45}O_9N_3$: C, 68.03; H, 6.27; N, 5.80. Found: C, 68.26; H, 6.48; N, 5.89.

ε-N-(α-L-Glutamyl)-L-Lysine.—Three g of α-*N*-cbz-ε-*N*-(cbz-α-L-glutamyl)-L-lysine dibenzyl ester were hydrogenated in ethanol-water with palladium oxide as catalyst. The catalyst was filtered off and the solvent removed *in vacuo*. The yield was 1.05 g (92%). The product was recrystallized from water-ethanol. $[\alpha]^{29°}_{D}$ + 26.50 (*c*, 2 in water). It migrated as a single spot on paper electropherograms.

Anal. Calcd. for $C_{11}H_{21}O_5N_3$: C, 47.99; H, 7.69; N, 15.26. Found: C, 48.24; H, 7.63; N, 15.33.

ε-N-(γ-L-Glutamyl)-L-Lysine.—One g of α-*N*-cbz-ε-*N*-(cbz-γ-L-glutamyl)-L-lysine dibenzyl ester in 20 ml of glacial acetic acid was hydrogenated with palladium oxide as catalyst. After filtration, the solvent was removed by lyophilization. The crude product weighed 350 mg (92%). It was recrystallized from water-ethanol. $[\alpha]^{29°}_{D}$ + 11.5° (*c*, 2 in water). The compound migrated as a single spot on paper electropherograms.

Anal. Calcd. for $C_{11}H_{21}O_5N_3$: C, 47.99; H, 7.69; N, 15.26. Found: C, 48.22; H, 7.71; N, 14.97.

ε-N-(αγ-L-Glutamyl)-L-Lysine Dibromobenzene Sulfonate (Cyclic Imide).—α-*N*-Cbz-ε-*N*-(cbz-α-L-glutamyl)-L-lysine dibenzyl ester, 1.5 g (0.0021 mole) was dissolved in 30 ml of anhydrous acetic acid. The flask, with an attached $CaCl_2$ tube, containing the solution was kept in a water bath maintained at 68°. Gaseous HBr was bubbled through the solution for 5 hours. The reaction mixture was poured into 300 ml of anhydrous ethyl ether and the precipitated crude product washed several times with ether by decantation. Yield: 920 mg. To 500 mg of the crude product, 2.8 g of dibromobenzene sulfonic acid in 20 ml of water was added and the solution was cooled. The crystals which separated out were collected and washed with ether. After recrystallization from water 440 mg of ε-*N*-(αγ-L-glutamyl)-lysine dibromobenzene sulfonate were obtained. Addition of further dibromobenzene sulfonic acid to the mother liquors yielded an additional crop of crystals weighing 125 mg. Total yield: 565 mg (55% calculated on the basis of the starting material). Mp. 173–174°. $[\alpha]^{29°}_{D}$ − 11.5° (*c*, 2 in water). The compound migrated toward the negative pole on paper electropherograms (*p*H 4 and 7) as a single spot and gave a characteristic red-purple color with ninhydrin.

Anal. Calcd. for $C_{23}H_{27}O_{10}N_3S_2Br_4$: C, 31.06; H, 3.06; N, 4.72; Br, 35.94. Found: C, 31.00; H, 3.31; N, 4.72; Br, 35.61.

In order to obtain the free ε-*N*-(αγ-glutamyl)-lysine, the dibromobenzene sulfonate salt of the compound was passed through a Dowex 1–acetate column and the effluent containing the cyclic imide was evaporated. Since the free compound proved to be unstable upon storage, the bromobenzene sulfonate was utilized for further experiments.

Acid Hydrolysis of ε-N-Glutamyl-Lysines.—Samples of ε-*N*-(α-glutamyl)- or ε-*N*-(γ-glutamyl)-lysine (0.75 to 1.00 mg) were dissolved in 0.5 ml of HCl of various molarities and heated in a sealed tube at 100° for the desired length of time. The solution was cooled in ice and evaporated under a stream of air. Excess HCl was removed by addition and evaporation of water. The residue was taken up in the appropriate buffer and an aliquot of the solution was analyzed either by column chromatography or by electrophoresis. As controls, samples of the two compounds were dissolved in HCl of the appropriate concentration and treated as the experimental samples with the omission of heating.

Electrophoresis.—A modification of the apparatus originally described by Kunkel and Tiselius (1951) was employed. For paper electrophoresis, Whatman No. 3 mm strips, 32 × 10 cm, were set between silicone-treated glass plates. The buffer systems used were: pyridine; acetic acid (2.5 ml: 10 ml per l), *p*H 4.0; sodium phosphate, 0.1 M, *p*H 6.0, 6.6, 7.0. A potential of 25 v/cm (6–8 ma) was applied for 1–1.5 hours. The electropherograms were developed with ninhydrin.

Column Chromatography.—This was carried out according to the general procedure of Moore *et al.* (1958). Columns, 30 × 0.9 cm of Dowex 50-X2 or Dowex 50-X4 were used. The partial acid hydrolysates, corresponding to approximately 3 μmoles of either of the ε-*N*-glutamyl-lysine isomers, were applied in citrate buffer. For the elution of the columns two procedures were employed, depending upon the expected amounts of glutamic acid and ε-*N*-(γ-glutamyl)-lysine. In procedure (a) the elution was initiated with sodium citrate, 0.2 N, *p*H 3.25. At fraction 42 (approx. 60 ml) the eluting solution was changed to sodium citrate, 0.38 N, *p*H 4.25, and at fraction 95 (approx. 133 ml) to sodium citrate, 0.38 N, *p*H 6.5–7.2. In scheme (b) the column was first eluted with the *p*H 4.25 buffer, followed by the *p*H 6.5–7.2 buffer beginning with fraction 42 (60 ml). Quantitative ninhydrin determinations were carried out on the effluent solution (Moore and Stein, 1954). The optical density was read at 580 mμ in a Coleman Junior spectrophotometer. Leucine was used as the standard for the conversion of optical density to micromoles; leucine, 1.0; glutamic acid, 1.0; lysine, 1.1; ε-*N*-(α-glutamyl)-lysine, 1.8; ε-*N*-(γ-glutamyl)-lysine, 1.8; ε-*N*-(αγ-glutamyl)-lysine, 1.7.

Isolation of ε-N-(αγ-Glutamyl)-Lysine (Cyclic Imide).—A suspension of Geon 427 (B. F. Goodrich Chemical Co.) in phosphate buffer, *p*H 6.6, 0.01 M, was poured into a 25 × 12 × 0.5 cm glass mold equipped with gauze wicks. The partial acid hydrolysate (11 N HCl, 100°, 1 hour) of 2 mg of ε-*N*-(α-glutamyl)-lysine was streaked across the damp Geon block and electrophoresis was carried out using the same phosphate buffer in the electrode compartments (25 v/cm; 6–10 ma; 1.5 hour). The position of ε-*N*-(γ-glutamyl)-lysine at the end of the run was located by pressing a damp filter paper against the block and developing the paper with ninhydrin. The portion of the medium corresponding to the cyclic imide was cut out and eluted with water. The aqueous solutions derived from several runs were pooled, reduced in volume, and used for further experiments.

Hydroxylamine Reaction.—This reaction was carried out according to the general procedure of Goldenberg and Spoerri (1958) with minor modifications. To a solution of the compound, 0.5 ml, an equal volume of alkaline NH_2OH reagent was added (*p*H 12 or *p*H 10.2). After allowing the reaction to proceed at 25°, 2 ml of 5% $FeCl_3$ in 2.5 N HCl were added. Optical density was read at 525 mμ in a Coleman Junior colorimeter.

Results

Interconversion of ε-N-Glutamyl-Lysines.—When the partial acid hydrolysates of either ε-*N*-(α-glutamyl)-

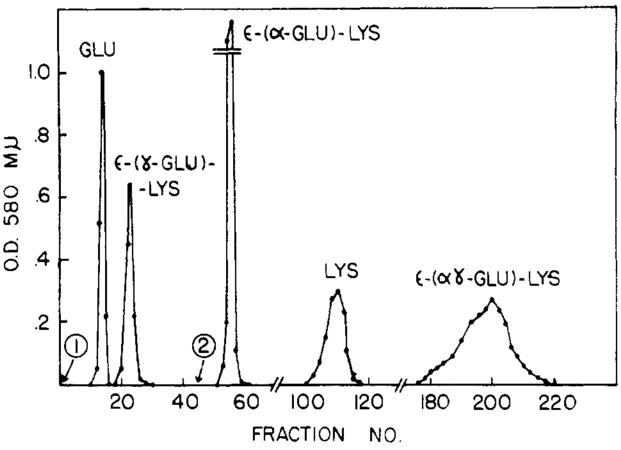

FIG. 1.—The partial hydrolysate of ε-N-(α-L-glutamyl)-L-lysine. Conditions of hydrolysis: 11 N HCl, 100°, 1.5 hour (0.75 mg of the compound). Elution pattern from Dowex 50-X2 column, 0.9 × 30 cm. The optical density of ninhydrin color at 580 mμ is plotted against the number of effluent fractions (1.4 ml). Sodium citrate buffer changes: (1) *p*H 4.25, 0.2 N; (2) *p*H 6.7, 0.38 N.

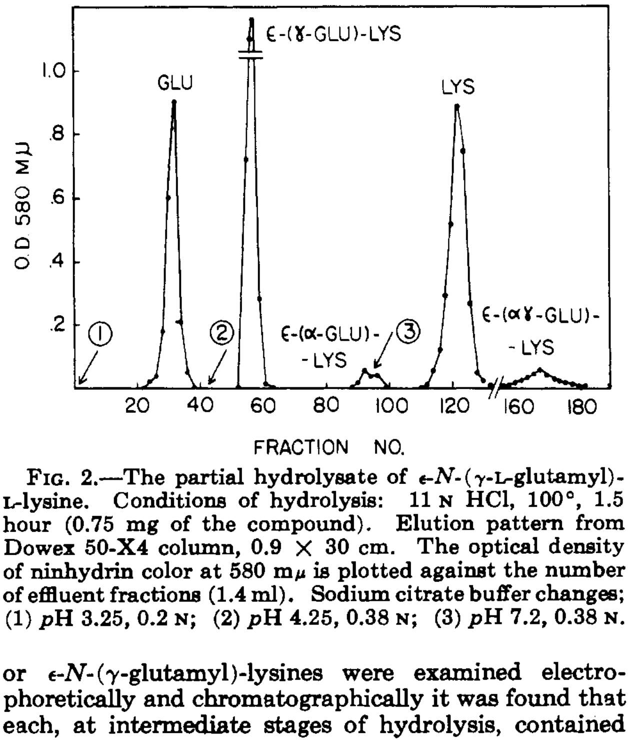

FIG. 2.—The partial hydrolysate of ε-*N*-(γ-L-glutamyl)-L-lysine. Conditions of hydrolysis: 11 N HCl, 100°, 1.5 hour (0.75 mg of the compound). Elution pattern from Dowex 50-X4 column, 0.9 × 30 cm. The optical density of ninhydrin color at 580 mμ is plotted against the number of effluent fractions (1.4 ml). Sodium citrate buffer changes; (1) *p*H 3.25, 0.2 N; (2) *p*H 4.25, 0.38 N; (3) *p*H 7.2, 0.38 N.

or ε-*N*-(γ-glutamyl)-lysines were examined electrophoretically and chromatographically it was found that each, at intermediate stages of hydrolysis, contained the isomeric ε-*N*-glutamyl-lysine in addition to the starting material and free lysine and glutamic acid. Upon treatment with 11 N HCl at 100° for 1.5 hour, ε-N-(α-glutamyl)-lysine yielded an appreciable amount of the γ-compound (Fig. 1). In a similar way the conversion of ε-*N*-(γ-glutamyl)-lysine to the α-glutamyl isomer was demonstrated (Fig. 2). The partial hydrolysates of both compounds also contained a common basic substance, which electrophoretically migrated closely behind lysine, and on Dowex 50 columns was eluted after lysine (Figs. 1 and 2). Prolonged hydrolysis of either of the ε-*N*-glutamyl-lysines resulted in the disappearance of the basic compound and yielded only glutamic acid and lysine.

It seemed probable that the basic substance common to both ε-*N*-glutamyl-lysines was the cyclic imide, ε-*N*-(αγ-glutamyl)-lysine. This compound could arise from either ε-*N*-(α-glutamyl)- or ε-*N*-(γ-glutamyl)-lysine by the formation of a new bond between the carbon derived from the free carboxyl group of the glutamyl moiety and the amide nitrogen atom. The cyclic imide, once formed, would provide a means for α,γ-glutamyl interconversion, since one of the two carbonyl-amide bonds could be hydrolytically cleaved to yield either ε-*N*-(α-glutamyl)- or ε-*N*-(γ-glutamyl)-lysine.

The Cyclic Imide, ε-N-(αγ-Glutamyl)-Lysine.—The basic compound formed at intermediate stages of acid hydrolysis from ε-*N*-(α-glutamyl)-lysine was isolated by means of electrophoresis at *p*H 6.6 using Geon 427 resin as supporting medium. The isolated compound yielded both ε-*N*-(α-glutamyl)- and ε-*N*-(γ-glutamyl)-lysine upon acid or base treatment. Its chromatographic and electrophoretic migration was also consistent with that expected for the postulated cyclic imide. In order to achieve complete characterization, ε-*N*-(αγ-glutamyl)-lysine (obtained in the crystalline form as the dibromobenzene sulfonate salt) was synthesized by treating α-*N*-cbz-ε-*N*-(α-glutamyl)-lysine dibenzyl ester with HBr in glacial acetic acid. Since under the above described conditions the properties of the synthetic and isolated compounds proved to be identical, it was concluded that the basic compound formed from either of the ε-*N*-glutamyl-lysine isomers is, in fact, the cyclic imide, ε-*N*-(αγ-glutamyl)-lysine.

The electrophoretic migration of the cyclic imide at *p*H 4.0 is shown in Figure 3, and is compared with that of lysine, glutamic acid, and the two ε-*N*-glutamyl-lysines. In this system, as well as at *p*H 6 and 7, the cyclic compound migrated toward the negative pole behind lysine. In the *p*H region 6.2–7.0 its mobility was particularly sensitive to small *p*H changes. This migratory behavior suggests that the *p*K of one functional group occurs in this *p*H region. Since the *pK* values of the α-carboxyl and α-amino groups of the lysine residue of the cyclic imide would be expected to be similar to those of free lysine, 2.18 and 8.95, respectively (Cohn and Edsall, 1943), the functional group involved probably is the amino group of the glutamyl residue.

The cyclic compound, ε-*N*-(αγ-glutamyl)-lysine, at intermediate stages of acid hydrolysis yielded the two ε-*N*-glutamyl lysines as well as the two component amino acids (Fig. 3). Quantitative analysis by column chromatography indicated that the composition of the partial hydrolysate of the cyclic imide after 0.5 hour, 6 N HCl, 100°, was as follows: ε-*N*-(αγ-glutamyl)-lysine, 47%; ε-*N*-(α-glutamyl)-lysine, 34%; ε-*N*-(γ-glutamyl)-lysine, 9.2%; glutamic acid or lysine, 9.8%. Since in this system the total hydrolysis of ε-*N*-(α-glutamyl)-lysine is small as compared with that of the γ-compound (Figs. 5 and 7), it can be assumed that the free glutamic acid and lysine arise primarily from the latter. Thus the opening of the imide ring in acid is 2:1 in favor of ε-*N*-(α-glutamyl)-lysine over the γ-compound. The complete hydrolysis of ε-*N*-(αγ-glutamyl)-lysine yielded equimolar amounts of glutamic acid and lysine.

The cyclic imide was found to be very sensitive to alkaline hydrolysis. After 3 hours in 1 N KOH, 38°, it was completely cleaved to ε-*N*-(α-glutamyl)- and ε-*N*(γ-glutamyl)-lysine. Negligible total hydrolysis occurred during this time as determined by column chromatography. Of the two linear compounds formed ε-*N*-(γ-glutamyl)-lysine predominated over ε-*N*-(α-glutamyl)-lysine at a ratio 2:1.

The cyclic glutarimide formed hydroxamic acid upon treatment with alkaline hydroxylamine. At *p*H 12 the reaction was maximal within 15 minutes, at *p*H 10.2 it occurred to a negligible extent even after 3 hours. The optical density of the ferric hydroxamate

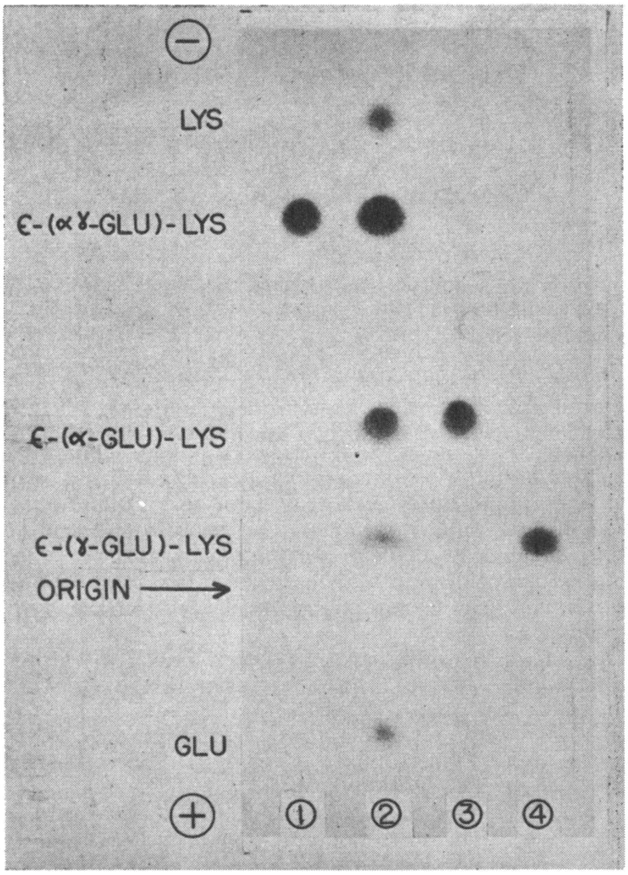

FIG. 3.—The products of partial acid hydrolysis of ε-*N*-(αγ-L-glutamyl)-L-lysine (cyclic imide). Paper electrophoresis in pyridine: acetic acid buffer (2.5 ml: 10 ml per l) *p*H 4.0. (1) ε-N-(αγ-L-glutamyl)-L-lysine; (2) partial hydrolysate of ε-N-(αγ-L-glutamyl)-L-lysine, 6 N HCl, 100°, 0.5 hour; (3) ε-*N*-(α-L-glutamyl)-L-lysine; (4) ε-N-(γ-L-glutamyl)-L-lysine. Electropherogram was developed with ninhydrin.

complex derived from the cyclic imide was approximately one-third of that obtained from an equimolar amount of γ-glutamyl hydroxamic acid.

Rates of Hydrolysis.—The kinetics of hydrolysis of ε-*N*-(α-glutamyl)-lysine in 11 N and 6 N HCl were investigated. Initially in 11 N HCl the predominant reaction is the cyclization resulting in an accumulation of the cyclic imide and the isomeric ε-*N*-(γ-glutamyl)-lysine (Fig. 4). Since the latter is hydrolyzed at a faster rate than the α-compound, its formation leads to an enhanced rate of appearance of glutamic acid and lysine. In the 6 N HCl system the formation of the cyclic imide and the linear isomer occur to a considerably lesser extent (Fig. 5). The rate of disappearance of the α-compound in this system is lower than in 11 N HCl.

In contrast to the α-isomer, ε-*N*-(γ-glutamyl)-lysine is hydrolyzed at least 3 times faster in 6 N than in 11 N HCl (Fig. 6 and 7). At both acid concentrations the hydrolytic cleavage of the amide bond is the major reaction. In 11 N HCl the cyclic imide peak was detected by column chromatography. Since the cyclic imide usually gives a broad peak upon column chromatography, the smaller amount of it formed in 6 N HCl was not detected in this system, however it was readily demonstrated on paper electropherograms. The sharp elution peak from columns due to the isomeric ε-*N*-(α-glutamyl)-lysine formed from the γ-compound was evident in both 6 N and 11 N HCl. Due to its greater stability it showed evidence of accumulating during the course of hydrolysis.

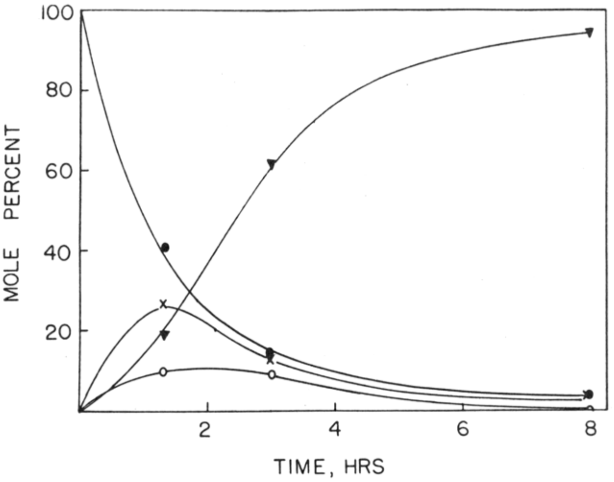

FIG. 4.—Hydrolysis of ε-N-(α-L-glutamyl)-L-lysine, 11 N HCl 100°. The ordinate represents the composition of the partial hydrolysate in mole per cent, the abscissa represents the time of hydrolysis. The extent of hydrolysis at each given time was determined by analysis on Dowex 50 ion-exchange columns. ●——●, ε-*N*-(α-glutamyl)-lysine; ○——○, ε-*N*-(γ-glutamyl)-lysine; ×——×, ε-*N*-(αγ-glutamyl)-lysine; ▼——▼, lysine or glutamic acid.

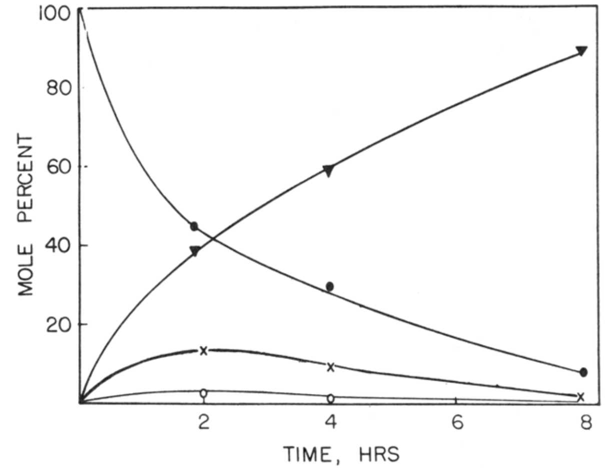

FIG. 5.—Hydrolysis of ε-*N*-(α-L-glutamyl)-L-lysine, 6 N HCl, 100°. The ordinate represents the composition of the partial hydrolysate in mole per cent, the abscissa represents the time of hydrolysis. Extent of hydrolysis at each given time was determined by analysis on Dowex 50 ion-exchange columns. ●——●, ε-*N*-(α-glutamyl)-lysine; ○——○, ε-*N*-(γ-glutamyl)-lysine; ×——×, ε-*N*-(αγ-glutamyl)-lysine; ▼——▼, lysine or glutamic acid.

Glutamyl Rearrangement under Other Conditions.—When the concentration of HCl was lowered to 2 N, both ε-*N*-glutamyl-lysine isomers gave evidence of cyclization and rearrangement to a lesser extent than in 6 N, as determined by paper electrophoresis. A lowering of the temperature from 100° to 65° or 25° decreased the rates of hydrolysis of the ε-*N*-glutamyl-lysines, but did not significantly alter the relative amounts of the cyclic imide and the rearranged product.

Pyrrolidone Carboxylic Acid Formation.—No evidence of pyrrolidone carboxylic acid formation was obtained when the two ε-*N*-glutamyl-lysines were heated at 100° in 2 N, 6 N, and 11 N HCl. This result is to be expected since pyrrolidone carboxylic acid formation from glutamic acid occurs only in the *p*H range 3–10 (Wilson and Cannan, 1937). It was of interest to

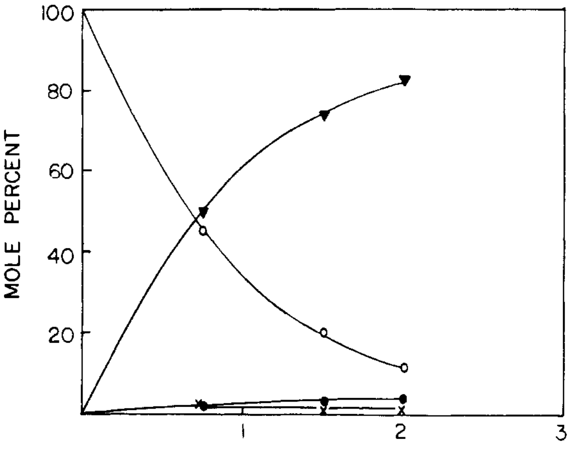

FIG. 6.—Hydrolysis of ε-*N*-(γ-L-glutamyl)-L-lysine, 11 N HCl, 100°. The ordinate represents the composition of the partial hydrolysate in mole per cent, the abscissa represents the time of hydrolysis. Extent of hydrolysis at each given time determined by analysis on Dowex-50 ion-exchange columns. O——O, ε-*N*-(γ-glutamyl)-lysine; ●——●, ε-*N*-(α-glutamyl)-lysine; ×——×, ε-*N*-(αγ-glutamyl)-lysine; ▼——▼, lysine or glutamic acid.

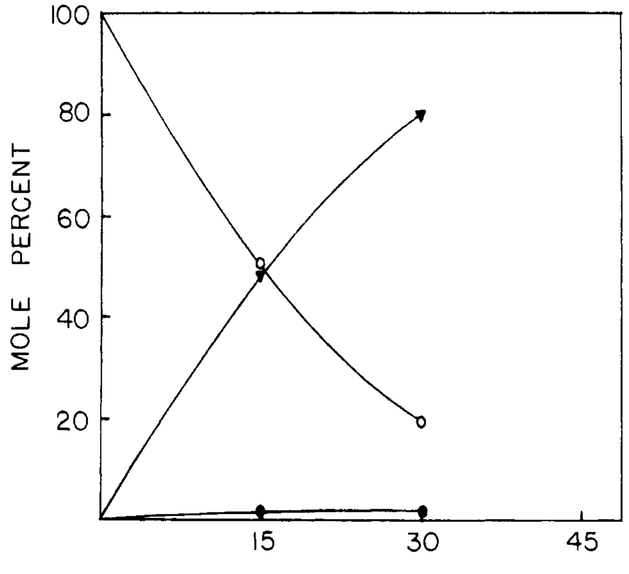

FIG. 7.—Hydrolysis of ε-*N*-(γ-L-glutamyl)-L-lysine, 6 N HCl, 100°. The ordinate represents the composition of the partial hydrolysate in mole per cent, the abscissa represents the time of hydrolysis. Extent on hydrolysis at each given time determined by analysis on Dowex-50 ion-exchange columns. O——O, ε-*N*-(γ-glutamyl)-lysine; ●——●, ε-*N*-(α-glutamyl)-lysine; ▼——▼, lysine or glutamic acid. The presence of ε-*N*-(αγ-glutamyl)-lysine in the hydrolysate was demonstrated on paper electropherograms (see Results).

establish whether within this *p*H range the cyclization to the imide could compete to any extent with pyrrolidone formation. When the ε-*N*-glutamyl-lysines were heated in aqueous solution or in glacial acetic acid no cyclic imide was formed, but both compounds gave evidence of pyrrolidone carboxylic acid formation, as determined by paper electrophoresis. With the α-compound the cyclization of the glutamyl moiety occurred without hydrolysis, the product being ε-*N*-(pyrrolidone-2-carbonyl)-lysine (cf. Le Quesne and Young, 1952). The identity of the compound was confirmed by its electrophoretic migration at several *p*H values and by treatment with HCl, which regenerated the original starting material, ε-*N*-(α-glutamyl)-lysine. The formation of the ε-*N*-(pyrrolidone-2-carbonyl)-lysine was virtually quantitative in glacial acetic acid after 5 hours. The γ-compound in water and in glacial acetic acid at 100° yielded lysine and pyrrolidone carboxylic acid, but no free glutamic acid, indicating that pyrrolidone carboxylic acid formation occurred concurrently with hydrolysis.

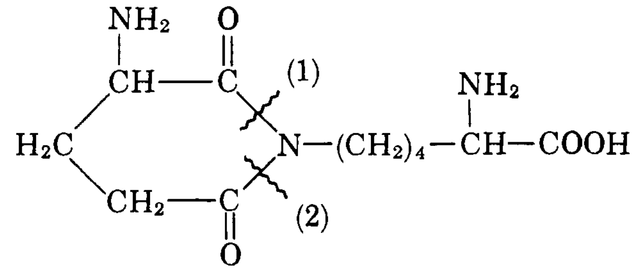

Fig. 8.—ε-*N*-(αγ-glutamyl)-lysine. Cleavage at (1) yields ε-*N*-(γ-glutamyl)-lysine; cleavage at (2) yields ε-*N*-(α-glutamyl)-lysine.

Glutamyl Rearrangement in Other Peptides.—Other compounds, besides the ε-*N*-glutamyl-lysines, were found to rearrange. A partial hydrolysate (6 N HCl, 60°, 12 hours) of ε-*N*-(glycyl-α-glutamyl)-α-*N*-tosyl-lysine upon electrophoresis showed the presence of ε-*N*-(glycyl-γ-glutamyl)-α-*N*-tosyl-lysine and ε-*N*-(γ-glutamyl)-α-*N*-tosyl-lysine as well as other hydrolysis fragments. The corresponding γ-glutamyl peptide, in turn, yielded peptides containing an α-glutamyl linkage. The peptides, α-glutamyl glycine and α-glutamyl alanine, when treated with 11 N HCl (85°, 4 hours) showed the presence of small amounts of the corresponding γ-glutamyl peptides. The γ-glutamyl peptides of alanine and glycine under identical conditions were rapidly cleaved and no glutamyl rearrangement could be demonstrated.

DISCUSSION

The two model compounds, ε-*N*-(α-glutamyl)-lysine and ε-*N*-(γ-glutamyl)-lysine, when heated in 6 N and 11 N HCl, in addition to hydrolysis, undergo cyclization to produce the same transient intermediate, ε-*N*-(αγ-glutamyl)-lysine. The formation of this cyclic imide provides a means for interconverting the linear ε-*N*-glutamyl-lysines at intermediate stages of hydrolysis (Fig. 8).

Alkaline hydrolysis of the cyclic imide favors the formation of ε-*N*-(γ-glutamyl)-lysine over ε-*N*-(α-glutamyl)-lysine at a ratio 2:1. This observation is in general agreement with other data regarding the alkaline hydrolysis of some αγ-glutamyl derivatives such as cbz-αγ-glutamyl glycine and benzoyl-αγ-glutamyl glycine cyclohexylamide (Kovacs *et al.*, 1955; Clayton *et al.*, 1956). The preferential cleavage of the imide ring at the α-carbonyl-amide bond under these conditions is to be expected, since of the two carbonyl groups the α, due to the inductive effect of the adjacent amino group, would be more susceptible to nucleophilic attack. In acid solution (6 N HCl) the cyclic imide yields the α-compound in preference to the γ-compound in a ratio 2:1. The reactivity toward hydroxylamine of the cyclic ε-*N*-(αγ-glutamyl)-lysine is in keeping with the results obtained with phthalimide and its derivatives (Fishman-Goldenberg and Spoerri, 1959).

Of the two linear compounds, ε-*N*-(α-glutamyl)-lysine was hydrolyzed slower at all concentrations of HCl used, and it yielded a larger amount of the cyclic imide and the rearranged isomer at intermediate stages of hydrolysis. Acid catalyzed hydrolysis of an amide bond is believed to be initiated by the addition of a proton, followed by a nucleophilic attack on the partially positive carbon (Bender, 1960). It has also been postulated that a positively charged amino group, adjacent to a peptide bond, hinders the approach of a hydrogen ion (Gordon *et al.*, 1941). Since in the γ-compound the positively charged amino group is two carbons removed from the amide group, it might be expected that the protonation (either on nitrogen or oxygen) in this case would occur with greater ease than in the case of the α-compound, and thus would account for the higher rate of hydrolysis observed.

The cyclization reaction requires the formation of a new bond between the nucleophilic amide nitrogen and the partially positive carbonyl group of glutamate. The observed ease of cyclization of the α-compound as compared to the γ-isomer points to the favorable electronic and steric factors existing in the former case. In the α-compound a larger proportion of the amide nitrogen would be expected to be in its unprotonated form, the carbonyl group would carry a greater fractional positive charge due to the absence of an adjacent α-amino group, and the potential steric interference of the amino group is absent. The lower activity of water at 11 N, as compared with 6 N HCl, serves to enhance the new carbon-nitrogen bond formation in the ε-*N*-(α-glutamyl)-lysine molecule, and leads to a twofold increase in its rate of disappearance. These results are in contrast with those found for ε-*N*-(γ-glutamyl)-lysine, which cyclizes to a limited extent at both acid concentrations and follows the general pattern of amide hydrolysis (Krieble and Holst, 1938). Its rate of disappearance decreases at least by a factor of 3 going from 6 N to 11 N HCl. In this case the rearranged product, ε-*N*-(α-glutamyl)-lysine, is more resistant to hydrolysis than the starting material and persists until the terminal stages of hydrolysis. Although the experimental conditions are somewhat different, these findings are of interest in view of the reported occurrence of an ε-*N*-(α-glutamyl)-lysine sequence in the acid hydrolysate of collagen (Mechanic and Levy, 1959).

Other glutamic acid derivatives, besides the two ε-*N*-glutamyl-lysines, have been demonstrated to undergo an α,γ-rearrangement during acid hydrolysis. In the hydrolysate of the tripeptide ε-*N*-(glycyl-α-glutamyl)-α-*N*-tosyl-lysine or its γ-glutamyl analog the rearranged products have been detected. Also dipeptides, such as α-glutamyl glycine and α-glutamyl alanine, in which the glutamyl residue is bound to an α-amino instead of an ε-amino group, rearrange to some extent in 11 N HCl. In view of these results, demonstrating the reactivity of the free carboxyl group of peptide-bound glutamate, it appears likely that some α,γ-interconversion could take place during the acid hydrolysis of proteins. The extent of the reaction in part would be determined by the nieghboring amino acids, since they would introduce new steric and electronic factors. As a consequence of the experiments presented here it seems apparent that partial acid hydrolysis is not suitable for the investigation of possible ε-*N*-(γ-glutamyl)-lysine sequences in proteins. The use of proteolytic enzymes offers an alternative approach. Preliminary experiments have shown that a number of proteolytic enzymes (trypsin, chymotrypsin, papain, leucine amino peptidase, and pronase) do not attack the amide bond of ε-*N*-(γ-glutamyl)-lysine.

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References

Bender, M. L. (1960), *Chem. Rev. 60*, 53.

Bezas, B., and Zervas, L. (1961), *J. Am. Chem. Soc. 83*, 719.

Boissonnas, R. A. (1951), *Helv. Chim. Acta 34*, 874.

Clarke, D. D., Mycek, M. J., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 79*, 338.

Clayton, D. W., Kenner, G. W., and Sheppard, R. C. (1956), *J. Chem. Soc. 1956*, 371.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, Reinhold, p. 85.

Fishman-Goldenberg, V., and Spoerri, P. E. (1959), *Anal. Chem. 31*, 1735.

Goldenberg, V., and Spoerri, P. E. (1958), *Anal. Chem. 30*, 1327.

Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1941), *Biochem. J. 35*, 1369.

Hanby, W. E., Waley, S. G., and Watson, J. (1950), *J. Chem. Soc. 1950*, 3239.

Kornguth, M. L., Neidle, A., and Waelsch, H. (1962), *Fed. Proc. 21*, A404e.

Kovacs, J., Medzihradszky, K., and Bruckner, V. (1955), *Acta Chim. Acad. Sci. Hung. 6*, 183.

Krieble, V. K., and Holst, K. A. (1938), *J. Am. Chem. Soc. 60*, 2976.

Kunkel, H. G., and Tiselus, A. (1951), *J. Gen. Physiol. 35*, 89.

Le Quesne, W. J., and Young, G. T. (1952), *J. Chem. Soc. 1952*, 594.

Mechanic, G. L., and Levy, M. (1959), *J. Am. Chem. Soc. 81*, 1889.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.

Mycek, M. J., Clarke, D. D., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 84*, 528

Mycek, M. J., and Waelsch, H. (1960), *J. Biol. Chem. 235*, 3513.

Neidle, A., Mycek, M. J., Clarke, D. D., and Waelsch, H. (1958), *Arch. Biochem. Biophys. 77*, 227.

Sachs, H., and Brand, E. (1953), *J. Am. Chem. Soc. 75*, 4610.

Sondheimer, E., and Holley, R. W. (1954), *J. Am. Chem. Soc. 76*, 2467.

Swallow, D. L., and Abraham, E. P. (1958), *Biochem. J. 70*, 364.

Wilson, H., and Cannan, R. K. (1937), *J. Biol. Chem. 119*, 309.