complexing with detergent are compensating within experimental error.

Even at the weight ratios 70/30 and 60/40, there was no appreciable difference in sedimentation coefficient values, although intrinsic viscosity increased. As was concluded in a previous paper,[2] the mechanism of interaction in the region EA/SDS = 100/0 − 80/20 differed from that in the region 80/20 − 35/65. Nevertheless, the $s_{20,w}$ value did not change considerably. This is in contrast to the result in the system horse serum albumin–SDS.[28] In this system the $s_{20,w}$ value changed stepwisely when AD_{12} and AD_{2n} ($2n$ = 105) were completed.

It was described above that the EA–DPB complex aggregates before the gel is formed, but the EA–SDS complex does not. A similar difference between the anionic and cationic detergents in the interaction with EA was observed through the flow birefringence study by Hanna and Foster.[29]

The sedimentation coefficient at EA/SDS = 80/20 and at *p*H 10.6 was 2.8 *S*, being the same value as that at *p*H 6.8 and at the same weight mixing ratio. This indicates that $s_{20,w}$ value of AD_n would be constant in the *p*H region 6.8–10.6.

EA–SOS.—The electrophoretic study was made on the system EA–SOS at *p*H 6.8. It cannot be stated that the patterns were the same as those obtained in the system EA–SDS.[2] Although the study was made in the same condition as in the study of the system EA–SDS, the boundaries were not easily resolved. Although there were two boundaries in the weight ratio region EA/SOS = 90/10 − 70/30, a quantitative interpretation was difficult to make because of the poor resolution. Therefore the relative viscosity of this system was measured at *p*H 6.8 using solutions having a total concentration of 3.5%. There were two maxima of viscosity at EA/SOS = 65/35 and 25/75, and these mixing ratio values were different from those in the system EA–SDS. The detailed mechanism of the interaction is not clear now.

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[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGES OF MEDICINE AND OF DENTISTRY]

An ε-Lysine Tripeptide Obtained from Collagen

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Achilles tendon collagen subjected to incomplete hydrolysis in strong hydrochloric acid at 24° yields a small amount of a tripeptide of glycine, glutamic acid and lysine. Its structure is proven by degradation and synthesis to be L,L-N$^{\epsilon}$-(glycyl-α-glutamyl)-lysine. Collagen therefore contains a peptide bond involving the ε-amino group of lysine. It is proposed that this be considered a general possibility for branching of peptide chains in collagen, gelatin, and, perhaps, other proteins.

It is generally held that the ε-amino groups of lysine in proteins are not involved in peptide linkage. The admittedly inconclusive evidence for this belief is summarized by Desnuelle.[3]

The present paper is a description of the isolation and identification, by comparison with a synthesized product, of L,L-N$^{\epsilon}$-(glycyl-α-glutamyl)-lysine. It was found in cattle achilles tendon partially hydrolyzed by treatment with strong acid at room temperature. We maintain that the isolation of this peptide demonstrates the existence of a peptide chain beginning at an ε-amino group of lysine in collagen and justifies serious consideration of the hyopthesis that lysine may serve as a branching point in protein structure.

Experimental

Hydrolysis of Collagen.—Bovine achilles tendon collagen[4] (10.2 g.) was suspended in 200 ml. of glass distilled 7 *M* hydrochloric acid. It soon dispersed in the acid and was kept at 24° for 48 hr. with shaking. The solution was then evaporated (below 30°) to dryness. Water was added and the evaporation was repeated three times. After eight days over calcium chloride and soda-lime *in vacuo*, the residue was pulverized and stored at room temperature over calcium chloride. The product contained 14.3% of nitrogen by Kjeldahl, 2.56% formol titratable[5] nitrogen and 2.23% of nitrogen reacting with nitrous acid[6] to give N_2. After correcting for the expected amino nitrogen (0.47%), these figures indicate an average peptide size of 6.5 residues with 84% of the peptides terminating in with amino groups and 16% with imino groups.

Chromatography of Hydrolysate.—A glass column 20 mm. in diameter was filled with 70 g. of recycled air-dry ammonium Dowex 50-X-4 (200–400 mesh) suspended in 250 ml. of 0.2 *N* ammonium formate adjusted to *p*H 3.12 by adding formic acid. One hundred ml. of the same buffer was passed through the column before charging it with 2.91 g. of the hydrolysate dissolved in 24 ml. of the buffer. The flow rate was adjusted to 2 ml. per hr. and 1 or 2-ml. samples were collected. The load gave ninhydrin color equivalent to 4.2 millimoles of leucine using the Troll–Cannan[7] method.

A small amount (0.096 ml.) of each sample was evaporated at 40° *in vacuo* overnight to remove water, formic acid and ammonium formate. 0.967 ml. of water was added to dissolve the residue. A sample of this solution (0.096 ml.) was used for ninhydrin analysis.[7] The adequacy of removal of ammonia was checked by running a blank of the eluting buffer with each batch of samples. The chromato-

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gram was complex and failed, as shown by paper chromatograms, to make complete separations. The material of interest appeared in greatest quantity at 435 ml. and was present from 432 to 437 ml., inclusive. The buffer *p*H was changed from 3.12 to 3.38 at 340 ml. The fractions of this peak were combined, taken to dryness *in vacuo* at 40° and chromatographed on paper as described below.

Paper Chromatography.—Whatman #1 paper was washed with 0.2% ethylenediaminetetraacetic acid adjusted to *p*H 8.5 with sodium hydroxide. It was next soaked for six periods of one-half hr. each with distilled water and then it was air-dried. The most useful solvent among those tried was the butanol-rich layer from a one to one mixture of δ[1]-butanol with 0.05 *M* trichloroacetic acid in water (butanol-TCA). About 0.15 leucine microequivalents of the samples were spotted at the origin and run by descent for 16 hr. Color development was done with 0.3% ninhydrin in butanol containing 5% of 2,4,6-collidine.[8] Fractions 432–7 showed two spots with butanol-TCA and with phenol–ammonia. These spots were designated 435A (R_f = 0.47 in butanol-TCA and 0.62 in phenol–ammonia) and 435B (R_fs 0.34 and 0.48, in the same solvents).

The residue of 432–7 was dissolved in a small amount of water, streaked across 12 sheets (18 × 58 cm.) of paper, which were then developed for 16 hr. with butanol-TCA. Guide strips were cut from the center and edges of each sheet and treated with ninhydrin to locate the positions of 435A and B. The fractions were separated with shears and eluted from the papers with water. In this way ten leucine microequivalents of 435A and 5.3 leucine microequivalents of 435B were collected. Each chromatographed as a single spot with the two solvents mentioned as well as in butanol–acetic acid–water(4/1/5)[9] and on a two dimensional chromatogram using isoamyl alcohol, pyridine, water (35/35/30) in one direction and phenol-water in the second.

Degradation Studies.—Aliquots of 435A and 435B were hydrolyzed in 6 *N* hydrochloric acid in sealed tubes for 16 hr. at 105°. After removal of the acid, chromatography according to Levy and Chung[10] showed that 435A contained only alanine. The ninhydrin color was not increased by hydrolysis nor was the R_f of the material changed. 435A is therefore alanine.

The hydrolysate of 435B showed glycine, glutamic acid and lysine in about equal amounts. Proline and hydroxyproline were absent as shown by specific tests.[7,11] The hydrolysate showed 40% more color with ninhydrin in the Troll–Cannan method than the original material. In this method the nonamino acids give colors equal to leucine but lysine gives 10% more color than equimolar amounts of leucine. The ninhydrin colors given by ε-aminocaproic acid[12] and by benzyl-Nε-tosyllysinate are 0.67 of the leucine equivalent. We therefore expected an increase from 1 + 0.67 to 1 + 1 + 1.10 or 86% for the normal α-linked tripeptide. Nε-Carbobenzyloxylysine gives color equivalent to leucine. A tripeptide in which the ε-amino group is blocked by a peptide linkage should therefore show an increase from 1 + 1 to 1 + 1 + 1.10 or 55% on hydrolysis. The behavior of the ninhydrin color indicated that 435B was not a completely α-linked tripeptide of the three amino acids found on complete hydrolysis.

Amino Terminus.—One leucine microequivalent of 435B was converted to S^{35}-labeled *p*-iodophenylsulfonyl (pipsyl) derivative by vibrating 0.5 ml. of its solution containing 17 mg. of disodium phosphate with 18 mg. of S^{35} labeled pipsyl chloride[13] at 80° until the pipsyl chloride had disappeared (three minutes). The approximately neutral solution was extracted four times with wet ether, and the water layer was evaporated to dryness. One ml. of 3 *N* hydrochloric acid was added, the solution was sealed in glass and heated for 3 hr. at 103°. These conditions hydrolyze peptide bonds but leave pipsylamide bonds practically intact.[14] After opening the tube 1.5 ml. of water was added, and the solution was extracted with four one-ml. portions of wet ether. The combined extract was evaporated and the residue was dissolved in a little ammonia. The solution was applied to a 10 × 37.5 cm. strip of Whatman #1 paper. Samples of pipsylglycine and pipsylglutamic acid were also applied. After development with *n*-propanol–concentrated ammonia (85/15), the presence of pipsylglycine in the unknown was demonstrated by ultraviolet absorption and radioactivity. This establishes glycine as a nitrogen terminus. It also established that glutamic acid is not nitrogen-terminal and that lysine does not have both amino groups free in the peptide. The monopipsyllysines are not extracted by ether from acid solution, but dipipsyllysine is extracted. The aqueous residue of the experiment was chromatographed on paper using butanol–acetic acid–water (4/1/5)[9] with control applications of glycine, glutamic acid, lysine and ε-pipsyllysine. Upon treating the chromatogram with ninhydrin a spot with the R_f of glutamic acid appeared. An identical experiment with non-radioactive pipsyl chloride except that the pipsylated and hydrolyzed mixture was not extracted with ether showed an ultraviolet absorbing spot which was also colored by ninhydrin. Its location differed from that of ε-pipsyllysine, and we assumed that this was α-pipsyllysine. Glutamic acid also appeared on this chromatogram. Pipsylglycine travels to the solvent front.

Carboxyl Terminus.—The method of Akabori[15] was applied to 0.5 leucine microequivalent[15] of the peptide. The hydrazinolysis residue after removal of excess hydrazine was dissolved in 0.4 ml. of water and applied to a 0.9 × 9 cm. column of Amberlite IRC-50 in the acid form. Water was passed through until 30 ml. was collected. This solution was concentrated to a small volume and chromatographed according to Redfield.[16] Controls of glycine, glutamic acid and lysine were run simultaneously. Only lysine survived the hydrazinolysis. Therefore, it is the only amino acid in the peptide with no carboxyl group in peptide linkage.

Nitrous Acid Treatment.—Oratz[17] has shown that glycyl peptides deaminated by nitrous acid using the method of Hamilton and Ortiz[18] lose both the N-terminal glycine and the amino group to which it was bound. Sachs and Brand[19] state that the γ-carboxyl of glutamic acid in peptide linkage does not protect the amino group involved from deamination by nitrous acid. This reagent should therefore remove free amino groups, as well as amino groups covered by N-terminal glycine, or by the γ-carboxyl of glutamic acid.

Sodium nitrite (4.5 m*M*) and hydrochloric acid (30 m*M*) were mixed in 5 ml. of solution. After obvious gas evolution had ceased, one ml. of the solution was added to 0.2 leucine microequivalent of 435B. The mixture was heated for three minutes in boiling water. One ml. of 3 *M* urea was added. The mixture was evaporated to dryness and the residue was hydrolyzed with 0.5 ml. of 6 *N* hydrochloric acid in a sealed tube for 16 hr. at 103°. After opening the tube and evaporating *in vacuo*, the residue was extracted with a 1/1 mixture of 95% ethanol and concentrated hydrochloric acid. The extract was evaporated, the residue was dissolved in a 0.2 ml. of water and chromatographed.[17] Glycine, lysine and glutamic acid were absent. A new spot appeared. This was identified as 2 hydroxy-6-aminohexanoic acid by comparison with a sample of L-Nε-benzoyllysine deaminated by the procedure detailed above. Thus the only amino group surviving deamination by nitrous acid is the ε-group of lysine.

Summary of Degradation Studies.—From the data given the structure is evidently Nε-(glycyl-α-glutamyl)-lysine. The nitrogen terminal glycyl does not protect glutamic acid from deamination. The glutamyl group is linked through its α-carboxyl group because linkage through its γ-carboxyl group would not protect the ε-amino group of lysine. The yield of pipsylglycine and of α-pipsyllysine on hydrolysis of the pipsylated peptide conforms to the free amino groups in the proposed structure. The observed increase of ninhydrin color on hydrolysis (40%) is comparable to the 55% predicted, if allowance is made for probable errors in the prediction of the color expected from the original compound. If more than three peptide linked residues were

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present the increase should be larger. Thus we are justified in assuming that no amino acid was missed.

Synthesis of the Tripeptide

To confirm the postulated structure the tripeptide L,L-N$^{\epsilon}$-(glycyl-α-glutamyl)-lysine and two related tripeptides were synthesized by standard methods using protecting groups which could be removed differentially.[20] The final products confirm the stucture proposed as shown by identity of chromatographic behavior on paper, of hydrolysis products, color development with ninhydrin before and after hydrolysis, and the results of treatment with nitrous acid.

Benzyl-Nα-tosyl-N$^{\epsilon}$-carbobenzyloxyl-L-lysinate.—Benzyl-N$^{\epsilon}$-carbobenzyloxy-L-lysinate hydrochloride[21] was prepared by the simpler method[22] in 90% yield, m.p. 139–140°. A suspension of 11.5 g. (0.028 mole) of this material in 300 ml. of ethyl acetate was heated under reflux with triethylamine (8.2 ml., 0.058 mole) and tosyl chloride (5.72 g., 0.03 mole). After 2 hr. the solution was cooled and the precipitate of triethylamine hydrochloride was removed. The solution was washed with *N* hydrochloric acid and water, and the organic layer was dried over sodium sulfate. The ethyl acetate was removed at reduced pressure. The compound crystallized during evaporation. The product was recrystallized from ethyl acetate–ether mixture; yield 12.3 g. (85%), m.p. 86–87°.

Benzyl-Nα-tosyl-L-lysinate Hydrobromide.—The carbobenzyloxy group of the preceding compound (12.3 g., 0.024 mole) was removed by mixing it with 40 ml. of 2 *M* hydrogen bromide in glacial acetic acid.[23] After 2 hr. at room temperature the product had precipitated. Ten volumes of ether were added, and the precipitate was collected by suction filtration. It was slurried five times with ether and air-dried; yield 10 g. (88%), m.p. 160–161°.

L,L-Benzyl-Nα-tosyl-N$^{\epsilon}$-(γ-benzyl-carbobenzyloxy-α-glutamyl)-lysinate.—γ-Benzylcarbobenzyloxy-L-glutamate[24] (3.71 g., 0.01 mole), the preceding compound (5.12 g., 0.01 mole), triethylamine (1.5 ml., 0.01 mole) and dicyclohexylcarbodiimide (2.06 g., 0.01 mole) were stirred with 100 ml. of methylene chloride for 5 hr. at room temperature.[25] After filtration the organic layer was washed with *N* hydrochloric acid, water, cold 0.5 *N* sodium hydroxide and water successively. After drying over sodium sulfate and distillation, the oily residue crystallized on trituration with petroleum ether. The compound was recrystallized from a mixture of ethyl acetate and petroleum ether; yield 5 g. (67%), m.p. 74–76°.

L,L-Benzyl-Nα-tosyl-N$^{\epsilon}$-(γ-benzyl-glutamyl)-lysinate Hydrobromide.—The carbobenzyloxy group of the preceding compound was removed by the use of 2 *M* hydrogen bromide in glacial acetic acid[23] as described above. Ether added to the solution precipitated an oil which crystallized from methanol–ether mixtures, but no m.p. was obtained because the compound was extremely hygroscopic; yield 2.74 g. (60%).

L,L-Benzyl-Nα-tosyl-N$^{\epsilon}$-(tosylglycyl-γ-benzyl-α-glutamyl)-lysinate.—An ethyl acetate solution of tosylglycylazide was prepared[26] from 0.784 g. (0.039 mole) of tosylglycylhydrazide. The compound of the preceding paragraph was converted to the free amine by treating 2.4 g. (0.039 mole) with 10 ml. of saturated potassium carbonate solution and 50 ml. of cold ethyl acetate. After shaking vigorously the organic layer was separated, washed with ice-water and dried over sodium sulfate. The two ethyl acetate solutions were mixed cold and allowed to stand for 16 hr. at room temperature. The mixture was washed with *N* hydrochloric acid and water and then dried over sodium sulfate. The solvent was removed at low pressure and the product was recrystallized from an ethyl acetate–ether mixture; yield 1 g. (32%), m.p. 116–118°.

L,L-N$^{\epsilon}$-(glycyl-α-glutamyl)-lysine.—The protected tripeptide was dissolved in liquid ammonia and reduced by addition of sodium until the solution remained blue for ten minutes.[27,28] The excess sodium was removed by the use[28] of MH_4^+ Dowex 50 (3.5 g.). The ammonia was allowed to evaporate at room temperature, and the residues were subjected to high vacuum to remove residual ammonia. The resin was suspended in 10 ml. of water, filtered and washed with an additional 10 ml. of water. The combined extracts were adjusted to *p*H 1 with hydrochloric acid and applied to a 4 g. column of Dowex-50-X-8 in the hydrogen form. The column was washed with water to remove chlorides and eluted with 0.2 *N* ammonia. Two ml. of effluent were collected beginning with the first appearance of alkalinity and evaporated to dryness.

This material was chromatographed on paper with isoamyl alcohol–pyridine–water (35/35/30). Three spots were revealed by the ninhydrin spray. The one traveling with the largest R_f was glycine. The maximum color appeared at an intermediate spot with the R_f of the natural product. It was isolated from a large scale chromatogram by elution with water. Hydrolysis of this product gave the three expected amino acids and no others; nitrous acid treatment followed by hydrolysis gave 2-hydroxy-6-aminohexanoic acid as the only ninhydrin reactive spot; and the ninhydrin color value increased 47% on hydrolysis. On this basis we conclude that the isolated material is the expected product of the synthesis.

L-Benzyl-Nα-tosylglycyl-N$^{\epsilon}$-carbobenzyloxylysinate.—L-Benzyl-N$^{\epsilon}$-carbobenzyloxylysine hydrochloride[21] (18.9 g., 0.047 mole) was coupled with tosylglycylazide[26] as indicated above. The reaction product isolated as described was recrystallized from ethyl acetate–petroleum ether; yield 15.6 g. (65%), m.p. 85–87°.

L-Benzyl-Nα-tosylglycyl-lysinate Hydrobromide.—Treatment of the preceding compound with 2 *M* hydrogen bromide in glacial acetic acid[23] removed the carbobenzyloxy group; yield 6.8 g. (74%). The compound was too hygroscopic to obtain a m.p.

L,L-Benzyl-Nα-tosylglycyl-N$^{\epsilon}$-(tosyl-γ-glutamyl)-lysine.—The preceding compound (3.5 g., 0.0066 mole) was refluxed for 5 hr. in 15 ml. of acetonitrile with 2 ml. of triethylamine (0.014 mole) and 1.8 g. (0.0062 mole) of L,1-tosyl-2-pyrrolidone-5-carboxylic acid.[29] The liquids were evaporated at reduced pressure, and the residue was dissolved in 60 ml. of cold 0.5 *N* sodium hydroxide. After extraction with ethyl acetate, the aqueous layer was cooled and acidified to Congo Red. The precipitated oil was dissolved in a minimal amount of ethanol and precipitated with ether. Upon trituration with ether it crystallized; yield 2.33 g (32%), m.p. 109–111°.

L,L-Nα-glycyl-N$^{\epsilon}$-(γ-glutamyl)-lysine.—The protective groups were removed from the preceding compound by reduction with sodium in liquid ammonia as described above. Hydrolysis of the peptide gave the expected increase in ninhydrin color and the expected three amino acids. It chromatographed as a single spot but differently from the natural peptide and from L,L-N$^{\epsilon}$-(glycyl-α-glutamyl)-lysine synthesized previously.

L,L-Benzyl-Nα-tosylglycyl-N$^{\epsilon}$-(γ-benzyl-carbobenzyloxy-α-glutamyl)-lysinate.—L-γ-Benzyl-carbobenzyloxy-glutamate 1.14 g. (0.003 mole) and 0.42 ml. of triethylamine were allowed to react[30] with 0.42 g. of isobutyl chloroformate for 0.5 hr. in 100 ml. of toluene at −5°. To the solution of the mixed anhydride an additional 0.42 ml. of triethylamine and 1.6 g. (0.003 mole) of L-benzyl-Nα-tosylglycyl-lysinate hydrobromide were added with stirring. The reaction mixture was withdrawn from the low temperature bath and stirred overnight at room temperature. The solution was washed with *N* hydrochloric acid, cold 0.5 *N* sodium hydroxide and cold water successively, and was then dried over sodium sulfate. The toluene was evaporated, and petroleum ether was added. A fine white precipitate was collected by filtration and air-dried; yield 1.47 g. (60%), m.p. 77–78°.

L,L-Nα-glycyl-N$^{\epsilon}$-(α-glutamyl)-lysine.—One gram (0.0012 mole) of the protected peptide was reduced with sodium in liquid ammonia[27] and isolated as detailed above

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for other peptides. The product chromatographed as a single spot but differently from the natural peptide. On hydrolysis it gave the expected products.

Comparison of Synthetic and Natural Peptides.—The synthetic and natural peptides were compared separately and as mixtures on paper chromatograms in a two dimensional system using isoamyl alcohol–pyridine–water (35/35/30) in one direction and phenol–water (70/30) in the second. One dimensional chromatograms were also run. Of the three peptides synthesized only L,L-N$^{\epsilon}$-(glycyl-α-glutamyl)-lysine behaved like the natural peptide.

Discussion

Peptide bonds involving the ϵ-amino groups of lysine are not novel in biochemical experience. Biocytin[31] and Bacitracin[32] contain such structures. Schweet[33] describes the incorporation of lysine into a soluble enzyme fraction of guinea pig liver through the epsilon amino group.

The isolation of the ϵ-lysine peptide from an acid hydrolysate of collagen indicates the existence of the structure in the protein. Strong acid hydrolysis at low temperature is not likely to produce artifacts. It seems likely that the α-carboxyl and the α-amino groups of lysine were originally part of a "normal" α-linked peptide chain, but of course this should be demonstrated by isolation of lysine peptides containing such linkages as well as the ϵ-linked amino acids. The amounts of peptide isolated accounts for only a fraction of a per cent. of the total lysine present, but the conditions of hydrolysis would be likely to yield only a little of any particular peptide, although there may be many more ϵ-amino branch points. If the deficit in the appearance of ϵ-dinitrophenyllysine on treatment of gelatin or collagen with dinitrobenzene fluoride[34] is accepted as evidence for protected ϵ-amino groups, about 40% of the lysine must be protected by peptide linkage. Bowes and Kenten[35] suggested the presence of peptide-bound ϵ-amino groups in collagen because of the agreement between titratable amino groups and the Van Slyke amino nitrogen and the deficit in both with respect to the total lysine contents. However, they express some doubts of the validity of the analyses for lysine with which they compare these figures. Revision of the analyses[36] still leaves a deficit of 31–37%. Courts and Stainsby[37] adduce evidence for the existence of multi-chain gelatin molecules and Pouradier[38] submits evidence that some gelatin molecules bear no terminal carboxyl groups. It is consistent with these findings and ours to propose that collagen contains peptide bonds formed between terminal carboxyls of α-linked chains and the ϵ-amino groups of lysine. The peptide we have isolated may be the residue of such a ring. The only direct evidence against the existence of covalently bound ϵ-amino groups is in the work of Bowes and Kenten[39] who found no lysine capable of yielding carbon dioxide with L-lysine decarboxylase after deamination of collagen with nitrous acid.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Denaturation and Electrophoretic Behavior of Lysozyme[1,2]

BY SHERMAN BEYCHOK[3] AND ROBERT C. WARNER

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The rate of denaturation of lysozyme has been studied as a function of *p*H at 94.3 and 84.6° at two ionic strengths. The change in rate constant as *p*H varies from 1 to 6.3 is unusually small. Using a set of postulates developed by Levy and Benaglia, two equilibrium constants, which govern the variation of rate constant with *p*H, were evaluated. Mobilities from electrophoretic measurements were employed in calculating the potential at the surface of shear of the protein as a function of *p*H. These potentials are used in a modified form of the usual titration equation for proteins and the electrostatic factor involved in the titration of lysozyme from *p*H 2.0 to 6.0 has been calculated. The construction of a theoretical titration curve requires that about half of the carboxyls have a pK_0 of 3.53, the other half a pK_0 of 5.08. The relation of these values to hydrogen bonds involving carboxyls is discussed. It is concluded that the extreme stability of lysozyme at acid *p*H's does not depend on the hydrogen bonded structure but on some other factor such as disulfide cross-linking. Small modification in the stability of this structure by rupture or formation of the weaker prototropic hydrogen bonds may be responsible for the small variation of rate of denaturation with *p*H.

The variation in the rate of denaturation with *p*H of several proteins has been accounted for by the assumptions that there are a limited number of proton dissociations which are critical for the stability of the molecule and that a different specific rate constant can be assigned to the dissociated and undissociated forms.[4–9] Such a dependence might arise from the existence of cross-linking hydrogen bonds involving these dissociating groups.

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