[CONTRIBUTION FROM THE CHEMISTRY LABORATORY, NAGOYA CITY UNIVERSITY]

Interactions of Egg Albumin with Detergents

BY KOICHIRO AOKI AND JOJI HORI

RECEIVED JULY 31, 1958

The interaction between egg albumin (EA) and a cationic detergent, dodecylpyridinium bromide (DPB), was studied over the *p*H range 3.2–10.6. Precipitation took place when the *p*H was on the alkaline side of the isoelectric point. There was no precipitate when the *p*H was on the acid side. In the electrophoretic pattern of the solution at *p*H 3.2 and at EA/DPB = 80/20 there were two boundaries, those of EA and the complex. The viscosity of the solution increased with time, and after a day or two the solution changed to a gel. The ultracentrifugal pattern of a fresh solution at *p*H 3.2 and at EA/DPB = 80/20 had two boundaries. The sedimentation coefficient of the slower boundary corresponded to that of EA, hence the faster boundary having a sedimentation coefficient of *ca.* 20 *S* was interpreted to be the complex. Taking into consideration the viscosity behavior, the faster component was interpreted to be "aggregated complex" which is an intermediate in gel formation. In contrast to serum albumin-detergent interaction, the mode of interaction between egg albumin and detergent did not change when the *p*H was changed. This means that egg albumin would have no configurational change associated with *p*H. Sedimentation coefficients of the system egg albumin-sodium dodecyl sulfate were determined at *p*H 6.8. There was no appreciable difference in them when the weight mixing ratio was changed.

Introduction

In former papers[1-4] the interaction between egg albumin (EA) and anionic detergent, sodium dodecyl sulfate (SDS), was studied at various *p*H values. It was found by electrophoresis that a discrete complex AD_n (A:EA, D:SDS and n was approximately 40) was formed in the *p*H region between 5.4 and 10.8. The precipitation curve suggested that there is also a complex AD_n on the acid side of the isoelectric point.

Recently one of the authors studied[5] the interaction of horse serum albumin and SDS as a function of *p*H and found that the mode of interaction changed discontinuously at several *p*H values. The present study was undertaken to obtain enough data to conclude whether or not the mode of interaction between EA and detergent is changed by *p*H. Another object was to study fully the interaction between the protein and the cationic detergent, since only a few studies have been made[6-8] on the protein–cationic detergent interaction. Using dodecylpyridinium bromide (DPB) the study was made over the *p*H range 3.2–10.6.

Furthermore, two additional studies were made at *p*H 6.8. One of them is the ultracentrifugal study of the system EA–SDS; the other is a preliminary study of the interaction between EA and sodium octyl sulfate (SOS).

Experimental

Materials.—Egg albumin was prepared from fresh egg white by ammonium sulfate salting out at the isoelectric point and was recrystallized three times and dialyzed free of salt.[1] Dodecylpyridinium bromide was synthesized from dodecyl bromide and pyridine.[5] Sodium dodecyl and octyl sulfates were the same as used previously.[1] Following buffer solutions were used: *p*H 6.8, sodium monohydrogen phosphate and sodium dihydrogen phosphate; *p*H 8.9 and 10.6, sodium carbonate and sodium bicarbonate; *p*H 4.4 and 3.5, sodium acetate and hydrochloric acid; *p*H 3.2, glycine, hydrochloric acid and sodium chloride. The ionic strength was 0.10 in all cases.

Procedure.—The precipitation curves of the system EA–DPB was determined in the same way as previously described.[1] After mixing EA and DPB solutions, a series of mixtures in which weight mixing ratios were changed successively were allowed to stand overnight at room temperature. Then supernatants were separated from precipitate by centrifuge. Knowing the original amount of protein and finding the amount in the supernatant after centrifuge by micro-Kjeldahl analysis, the percentage of the EA which had turned into precipitate was found. The percentage of nitrogen in EA was assumed to be N = 15.67%.[9] Control run showed that 20% of the nitrogen in DPB solution had decomposed in the same procedure of Kjeldahl analysis as applied to the EA–DPB mixtures.

Hitachi's electrophoretic apparatus (Tokyo), Model HT-B, equipped with the schlieren diagonal system was used. All the experiments were carried out in a thermostat at 25 ± 0.01°. A series of samples, the weight mixing ratio (EA/DPB) of which were changed keeping the total sum of concentrations of EA and of DPB (total concentration) constant at 1.0%, were analyzed electrophoretically. The electrophoresis was carried out 1 hour after mixing solutions of EA and DPB. The conductivity of the sample was measured at 25.00°.

The relative viscosity of the mixtures of EA and DPB were measured using an Ostwald viscometer at 25 ± 0.01°. Distilled water took 143.2 sec. at this temperature by this viscometer. After EA and DPB solutions were mixed, the mixture was permitted to stand in the thermostat at 25.00° for 20 min.; then the viscosity was measured.

Sedimentation measurements were conducted at room temperature at 59,780 r.p.m. using a Spinco Model E ultracentrifuge. To avoid the possible aggregation of protein,[10] "Kel-F" cell was used. Values of the partial specific volume ($\bar{v}$) were determined by measuring the density of the mixture at 25.00°. The total concentration of samples was 1.0% in measurements of both sedimentation and density.

Results and Discussion

EA–DPB. Precipitation Curve.—When EA and DPB were mixed, there was an instant precipitate at the alkaline side of the isoelectric point and no precipitate at the acid side. Hence precipitation curves were determined at *p*H 6.8, 8.9 and 10.6 (Fig. 1). These curves have the same shape as those in the system EA–SDS,[1] and plots fall on a common curve when EA is in excess. It is seen that the percentage values are negative when they are small. This is because some of the DPB in the supernatant was decomposed in the course of Kjeldahl analysis. Thus it is thought that precipitation curves are shifted downward a little, Fig. 1 having only a qualitative meaning. However, it is noted that the weight ratio value at point A is regardless of *p*H.

(1) K. Aoki and J. Hori, *Bull. Chem. Soc. Japan*, **29**, 104 (1956).
(2) K. Aoki, *ibid.*, **29**, 369 (1956).
(3) K. Aoki, J. Hori and K. Sakurai, *ibid.*, **29**, 758 (1956).
(4) K. Aoki and Y. Suzuki, *ibid.*, **30**, 53 (1957).
(5) K. Aoki, THIS JOURNAL, **80**, 4904 (1958).
(6) H. N. Glassman and D. M. Molner, *Arch. Biochem. Biophys.*, **32**, 170 (1951).
(7) S. N. Timasheff and F. F. Nord, *ibid.*, **31**, 309 (1951).
(8) J. F. Foster and J. T. Yang, THIS JOURNAL, **76**, 1015 (1954).
(9) "The Proteins," Vol. I, edited by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, p. 217.
(10) M. J. Kronman and J. F. Foster, *Arch. Biochem. Biophys.*, **72**, 205 (1957).

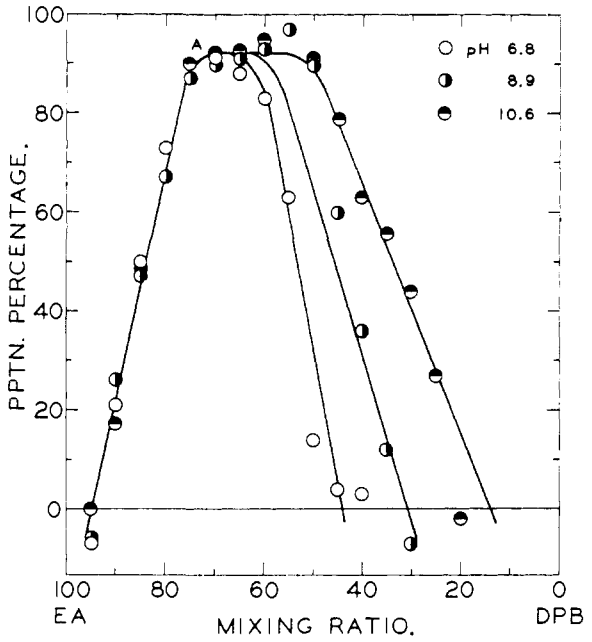


Fig. 1.—Precipitation curves of the system egg albumin-DPB. Total concentration was 1.0% and ionic strength of supporting media was 0.10.

Electrophoresis and Viscosity Measurements.—When the *p*H was on the acid side of the isoelectric point, the electrophoresis and viscosity measurements were conducted. Typical electrophoretic patterns are shown in Fig. 2. The upper patterns are those shown by EA at *p*H 4.4. These patterns show two moving boundaries, a major slow-moving one and a minor fast-moving one, designated as *I*. According to Cann and Phelps,[11] these two boundaries correspond to isomeric forms of EA in slow-adjusted equilibrium with one another. Although the electrophoresis of the system EA–DPB was conducted under the same conditions as in the study of the system EA–SDS,[2] the patterns obtained were not ideal. It is thought that the unbound DPB disturbed the pattern. (As was deduced in a former paper,[5] the affinity of the cationic detergent with the protein is less than that of the anionic detergent with protein.) In the region of weight mixing ratio EA/DPB = 95/5 – 70/30 there were two boundaries. While the two boundaries on the ascending side were good, only the slower boundary on the descending side was good.[12] The faster moving boundary is interpreted as the EA–DPB complex and the slower one as EA. The relative area of each boundary changed continuously with the weight ratio. When the weight ratio was between 65/35 and 40/60, the complex boundary only was observed, and when the ratio was less than 30/70, the electrophoresis could not be conducted because of a serious convection. The

(11) R. A. Phelps and J. R. Cann, THIS JOURNAL, **78**, 3539 (1956); J. R. Cann and R. A. Phelps, *ibid.*, **79**, 4672 (1957).

(12) The faster boundary on the descending pattern was flat. When the faster boundary on the descending leg of the electrophoretic cell was actually observed, it was not perpendicular to the wall of the cell; it was curved. The other three boundaries were perpendicular to the wall of the cell. Curvature of faster descending boundary may be due to convection or to low interfacial tension at the boundary. In Fig. 2, the δ-boundary is clearly seen in the middle and lower patterns.

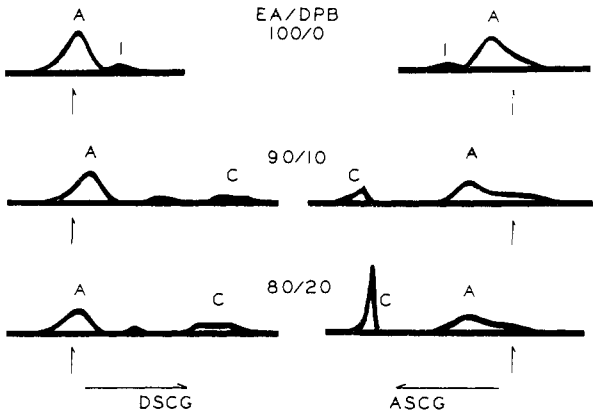


Fig. 2.—Electrophoretic patterns of the system egg albumin-DPB at *p*H 4.4. Total concentration was 1.0% and ionic strength was 0.10. A, egg albumin, C, complex, *I*, isomerized form of egg albumin. Upper pattern, 3.0 ma. and 3600 sec.; middle pattern, 5.0 ma. and 4800 sec.; lower pattern, 3.0 ma. and 3600 sec. The δ-boundary is clearly seen in middle and lower patterns. Arrows indicate the position of the initial boundaries.

mobility of EA measured on the ascending pattern was constant in the region 100/0 – 75/25 and was 4 × 10^{-5} cm.²/volt.sec. (The mobility of the boundary designated as *I* was 9 × 10^{-5} cm.²/volt.-sec.) The mobility of the complex measured on the ascending side was 14 × 10^{-5} at 95/5 and increased continuously with the weight ratio, being *ca.* 22 × 10^{-5} cm.²/volt.sec. at 40/60.

Viscosity data are given in Fig. 3. The position of viscosity maximum at *p*H 4.4 is at EA/DPB = 75/25, and at *p*H 3.5 and 3.2 it is 70/30. During

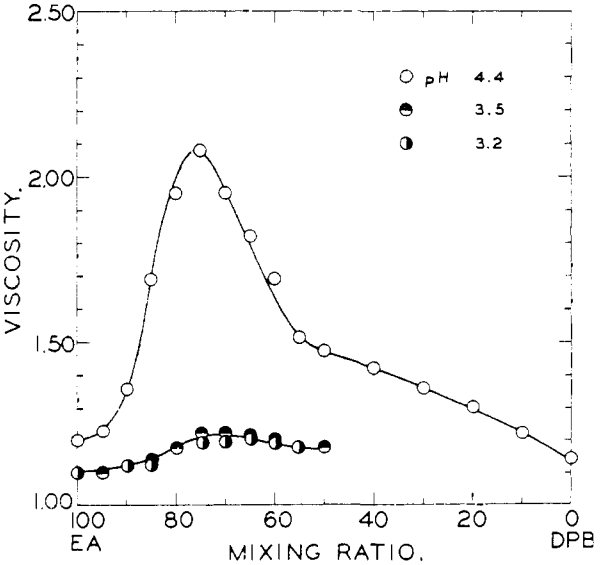


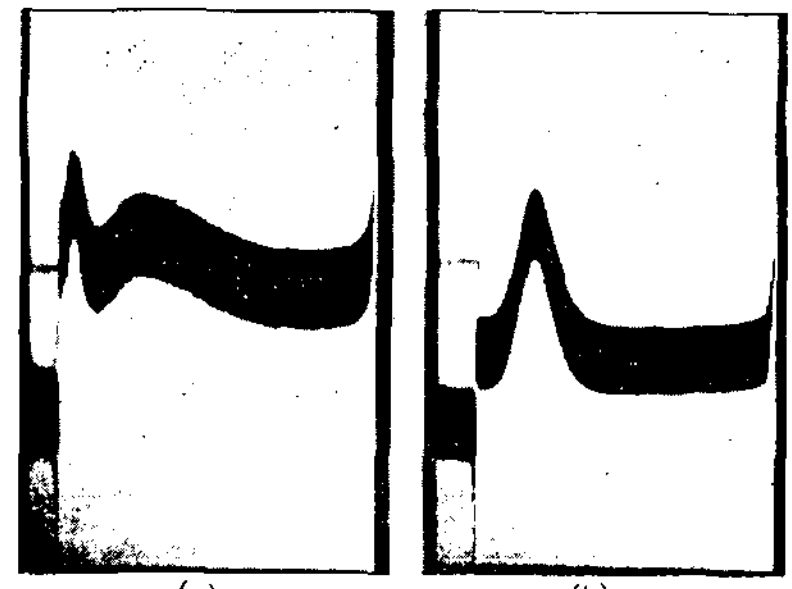
Fig. 3.—Relative viscosity *vs.* weight mixing ratio egg albumin/DPB. Total concentration was 4.0% at *p*H 4.4 and 2.0% at *p*H 3.5 and 3.2. Ionic strength was 0.10.

the experiment it was noticed that the effusion time of the solution increased gradually when the measurement was repeated and that after standing a day or two at room temperature, the solution changed to gel and not to the usual precipitate.

Solution changed easily to gel when the weight mixing ratio was between 80/20 and 60/40. Data shown in Fig. 3 are values of the first measurement. The 4% solution was used when the *p*H was 4.4, and 2% solution was used at *p*H 3.5 and 3.2 because the gel was formed more easily when the concentration was high.

Number of boundaries in the electrophoretic pattern changed from two to one at EA/DPB = 70/30 at *p*H 4.4. Two boundaries were observed at *p*H 3.5 and 3.2 also, when EA/DPB was 90/10. The maximum of the viscosity, as seen in Fig. 3, exists at EA/DPB = 75/25 at *p*H 4.4 and at 70/30 at other *p*H values. In the system EA–anionic detergent,[2,13] it was found that the weight ratio value at which the viscosity was maximum corresponded to that at which the number of boundaries in electrophoretic pattern changed from two to one. In the present system this was true at *p*H 4.4. Therefore it is supposed that the number of boundaries in the electrophoretic pattern at *p*H 3.2 would change near EA/DPB = 70/30. This indicates that the EA–DPB interaction also is stepwise in the *p*H region 3.2–4.4.[14]

Sedimentation and Gelation.—Using a fresh solution at *p*H 3.2 and at EA/DPB = 80/20, where gelation takes place easily, an ultracentrifugal run was made. (Description on the sedimentation experiment is given later in a section EA–SDS). The pattern shown in Fig. 4a was taken 15 min. after ultracentrifugal drive reached full speed. This was 80 min. after EA and DPB solutions were mixed. There are two boundaries; sedimentation coefficients $s_{20,w}$ are *ca.* 3 *S* and *ca.* 20 *S*. The slower component is found to be EA by the sedimentation coefficient value. Therefore the faster component is the complex. When the ultracentrifugal pattern is compared with the electrophoretic pattern at the same weight ratio, it is seen that the area composition of the two components is almost the same. The value of the sedimentation coefficient of the faster boundary is too large. As was described in a former section, the viscosity of the present system increased with time, and the solution changed gradually to gel. The ultracentrifugal pattern can be interpreted in connection with this viscosity behavior. It seems unreasonable that EA–DPB complex has such a large sedimentation coefficient, since the $s_{20,w}$ value of the complex in the system EA–SDS did not differ greatly from that of EA (Table I). Rather we are inclined to conclude that the complex forms an aggregate, which is an intermediate in the process of gel formation. Hence the faster boundary in the electrophoretic pattern and that in the ultracentrifugal



(a) (b)

Fig. 4.—Ultracentrifugal patterns. Total concentration was 1.0% and ionic strength was 0.10. Sedimentation is from left to right. Bar angle 65°. (a) *p*H 3.2, EA/DPB = 80/20 and 900 sec. after reaching full speed (59,780 r.p.m.). (b) *p*H 6.8, EA/SDS = 90/10 and 3600 sec. after reaching full speed (59,780 r.p.m.).

pattern is "aggregated complex." The electrophoretic mobility of the complex is larger than that of EA. This seems to indicate that the charge of the aggregated complex is considerable, since this complex is heavier than the "monomeric complex."[15]

When the EA–DPB complex is permitted to stand, aggregates of the complex are formed; then the aggregates grow up to form the gel. When the electrophoretic mobility was measured carefully (on the ascending) as a function of time, it was found that the mobility of the complex was not constant but slightly increased with time, while the mobility of EA was entirely constant during that time. This indicates that the gel changes its size and/or charge during the time of electrophoresis and that the speed of gel formation under this condition is fairly fast.

It was noticed that the gel formation was faster when the *p*H was lower. Further, it was found that the higher the protein concentration and the higher the ionic strength, the faster the gel was formed.[16] Foster and Yang also observed the gel formation.[8]

Mode of EA–Detergent Interaction.—In a previous study,[2] it was found in the system EA–SDS that the discrete complex AD_n existed in the *p*H region between 5.4 and 10.8. In the present work it was found that plots of precipitation percentage values fell on a common curve when the concentration of EA was high in the *p*H region between 6.8 and 10.6. This indicates that the interaction between EA and detergent occurs with a common mechanism at the alkaline *p*H. On the system

(13) H. P. Lundgren and R. A. O'Connell, *Ind. Eng. Chem., Ind. Ed.*, **36**, 370 (1944).

(14) If it is assumed that the molecular weights of EA and DPB are 46,000 and 328, respectively, and that all the detergents used were bound to EA, the composition of the complex at the weight ratio EA/DPB = 75/25 would be AD_{16} and at 70/30 it would be AD_{40}. Assuming that the total number of carboxyl groups is 51[9] and its *p*K value is 4.0, the number of negative charges on EA could be calculated as 7 and 35 at *p*H 3.2 and 4.4, respectively. It is supposed that interactions occur between one of the negative sites on EA and a DPB cation. However, there is no correspondence between the number of detergents in the formula of the complex (*n* in AD_n) and the number of negative charges at a given *p*H. One reason is that only a certain number of detergent cations used were bound to EA.

(15) Roughly speaking, the electrophoretic mobility of a particle is proportional to the net charge (Z) and reversely proportional to the effective radius (r). Thus it is seen, although qualitatively, that the Z value of the complex is larger than that of EA, since r of the complex is larger than r of EA.

(16) The same effect of protein concentration and ionic strength on the gelation of EA was observed by Jirgensons[17] and by Myers, *et al.*[18] The former studied the gel formation of EA in presence of 40–60% *n*-propyl alcohol and the latter by 30% acetic acid.

(17) B. Jirgensons, *Kolloid Z.*, **74**, 300 (1936).

(18) W. G. Myers and W. G. France, *J. Phys. Chem.*, **44**, 1113 (1940).

EA–SDS it was found that the precipitation percentage values were plotted on a common curve when the EA concentration was high.[1,3] Now it was found that the electrophoretic pattern of the system EA–DPB had a discrete complex in the *p*H region between 3.2 and 4.4. This means that the interaction occurs through a common mechanism in this *p*H region also.

In a study of the titration curve of egg albumin[19] it was found that the titration curve of this protein can be expressed by the theoretical Linderstrøm-Lang equation with two empirical corrections. That the titration curve is expressed by the theoretical equation means that the titration behavior can be explained only if the electrostatic interaction is considered and hence means that there is no configurational change which is associated with *p*H. Longsworth[20] studied the electrophoretic behavior of egg albumin and found that there was a proportionality between the electrophoretic mobility–*p*H curve and the titration curve in the *p*H range 3–12. Today it is known that some anions in the supporting medium are bound to EA.[21] There would be a better agreement between experimental results and theoretical values, if the anion binding were taken into consideration.

The sedimentation coefficient of EA was measured[22] as a function of the *p*H in the region between *p*H 2 and 10. It was found that the $s_{20,w}$ value was constant over that *p*H range except a slight change at *p*H 3.5. Optical rotation of EA was measured,[23] and it was found that the value of the specific rotation was constant in the *p*H region 3.5–11. The value changed sharply when the *p*H was above 11 and changed gradually when the *p*H was below 3.5. These results seem to indicate that the configurational change in EA does not occur in the *p*H region between 3.5 and 11.[24] Above *p*H 11, partial denaturation will occur. Longsworth[20] conducted the electrophoresis of EA at *p*H 12.81, and obtained two boundaries. He interpreted the faster boundary to be the native EA and the slower one the denatured. The behavior of EA below *p*H 3.5 is not obvious, because the sedimentation and optical rotational behaviors changed slightly, while the electrophoretic and titration behaviors were the same below and above *p*H 3.5. Thus there is a clear difference in the configurational change between EA and serum albumin. As was summarized in a previous paper,[5] serum albumin undergoes various configurational changes. The conclusion in that paper was that the mode of interaction of serum albumin with detergents reflected these configurational changes. In contrast to this result, EA which has no remarkable configurational change associated with *p*H reacted with DPB and with SDS by identical mechanisms in the *p*H region 3.2–10.8. Thus it seems possible to generalize the idea that the mechanism of the protein–detergent interaction reflects the change in the inner structure of the protein.

EA–SDS.—The ultracentrifugal study of this system was carried out at *p*H 6.8. Ultracentrifugal drive was continued for 60 min., and all the patterns had a single boundary. Values of the sedimentation coefficient are given in Table I. It is emphasized that these values are not those obtained by extrapolating to the zero concentration. The sedimentation coefficient of EA was 3.2–3.3 *S* when 0.3% solution was used. This is in agreement with the value found by Charlwood and Ens.[22] Values of $\bar{v}$ for complexes were determined by simply assuming that all the SDS used was bound to EA to form a complex. The value was a linear function of the weight mixing ratio in the region between EA/SDS = 100/0 and 40/60. Values $\bar{v}$ are 0.740[25] at 100/0 and 0.815 at 40/60.

TABLE I

SEDIMENTATION COEFFICIENT AND INTRINSIC VISCOSITY OF THE SYSTEM EGG ALBUMIN–SODIUM DODECYL SULFATE

*p*H	Weight ratio EA/SDS	$s_{20,w}$	$[\eta]$
6.8	100/0	$2.8\text{–}2.9 \times 10^{-13}$	0.042
6.8	90/10	2.7	.042
6.8	80/20	2.9	.042
6.8	70/30	2.9	.049
6.8	60/40	2.6	.058
6.8	40/60	2.5	.065
10.6	80/20	2.8	

The ultracentrifugal pattern had a single boundary at the weight ratio EA/SDS = 90/10 (Fig. 4b), while there were two boundaries, those of EA and AD_{40}, in the electrophoretic pattern. This means that the $s_{20,w}$ value of EA and of the complex AD_{40} are the same or that the difference between them is so small that it is within the resolving power of the ultracentrifuge.[26] At the weight ratio 80/20, where only AD_{40} existed in the electrophoretic pattern, the $s_{20,w}$ value was almost the same as that of EA.

The relative viscosity of the system EA–SDS had been measured as a function of the weight mixing ratio.[2] Viscosity values were determined at total concentrations 4.0 and 1.0%. Connecting these two values by a straight line, the intrinsic viscosity was calculated. The value of EA was 0.042, agreeing with the value found in the literature.[27] As is seen in Table I, the intrinsic viscosity was constant, 0.042, in the region EA/SDS = 100/0 – 80/20. Since $s_{20,w}$ and the intrinsic viscosity are the same for EA and AD_{40} despite a significant change in molecular weight, it is concluded that changes in molecular weight, volume and shape of the protein molecule as a result of

(19) R. K. Cannan, A. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

(20) L. G. Longsworth, *ibid.*, **41**, 267 (1941).

(21) C. W. Carr, *Arch. Biochem. Biophys.*, **40**, 286 (1952); **46**, 417 (1953).

(22) P. A. Charlwood and A. Ens, *Can. J. Chem.*, **35**, 99 (1957).

(23) H. J. Almquist and D. M. Greenberg, *J. Biol. Chem.*, **105**, 519 (1934).

(24) According to Cann and Phelps,[11] two components of EA at *p*H 4.4 are in isomerization equilibrium. But the isomerization is not considered here.

(25) P. A. Charlwood, THIS JOURNAL, **79**, 776 (1957).

(26) Approximate calculation showed that, if the difference in distance sedimentated by two boundaries having $s_{20,w}$ value *ca.* 2–3 *S* is 1.0 mm. on the ultracentrifugal pattern after 60 minutes' drive at 59,780 r.p.m., the difference in the sedimentation coefficient is 0.6 *S*. It seems impossible that two boundaries are separated at a distance of 1.0 *mm.* by the present ultracentrifuge. Thus, if the difference in sedimentation coefficient of the two components is within 0.6 *S*, the two boundaries would not be separated by 60 minutes' drive at 59,780 r.p.m.

(27) "The Proteins," Vol. I, edited by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y. 1953, p. 692.

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.

Acknowledgment.—The authors wish to express their sincere thanks to Dr. Rempei Goto of Kyoto University for his interest and encouragement.

MIZUHO-KU, NAGOYA, JAPAN

(28) K. Aoki, unpublished.

(29) G. F. Hanna and J. F. Foster, *J. Phys. Chem.*, **57**, 614 (1953).

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

An ε-Lysine Tripeptide Obtained from Collagen

BY GERALD L. MECHANIC[1] AND MILTON LEVY[2]

RECEIVED SEPTEMBER 30, 1958

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-

(1) From the thesis submitted by Gerald L. Mechanic to New York University in partial fulfillment of the requirements for the Ph.D. A preliminary report appeared in *Federation Proc.*, **16**, 220 (1957). The authors are grateful for aid provided by The American Cancer Society through an institutional grant to New York University-Bellevue Medical Center and by USPHS through a research grant #RG.-4902.

(2) To whom enquiries should be addressed at New York University College of Dentistry.

(3) P. Desnuelle in H. Neurath and K. Bailey, "The Proteins," Vol. I, Academic Press, New York, N. Y., 1953, p. 134.

(4) We thank Dr. Maxwell Schubert for this preparation.

(5) M. S. Dunn and A. Loshakoff, *J. Biol. Chem.*, **113**, 359 (1936).

(6) D. D. Van Slyke, *ibid.*, **83**, 425 (1929).

(7) W. Troll and R. K. Cannan, *ibid.*, **200**, 803 (1953).