in aqueous solution gave, at various *p*H, the following values for the ratio total N (Dumas)/amino N (Van Slyke), which corresponds to the degree of polymerization (buffer concentration *M*/30, concentration of substance, 100 mg. in 5 cc. of buffer solution).

*p*H	9	6	5	4
Dumas N/Van Slyke N	1.5	3	4	20 (insol. product)

Chromatographic analysis showed that all the polyleucines were mixtures of at least 3 substances. Each of them gave pure leucine on hydrolysis.

B. **Aspartyl Phosphate.** (1) **Carbobenzyloxyaspartic Acid-α-benzyl Ester β-Dibenzylphosphate.**—Carbobenzyloxy-*l*-aspartic-α-benzyl ester was prepared according to Bergmann, *et al.*[10,8] 5.4 g. of carbobenzyloxy-1-aspartic acid was boiled quickly with 16 ml. of acetic anhydride, immediately cooled, and absolute ether and petrol ether were added. Four grams of carbobenzyloxy-1-aspartic acid anhydride precipitated as white crystals, which had, after washing with ether, a melting point of 124°.

The carbobenzyloxyaspartic acid-α-benzyl ester was prepared by heating 3 g. of the above anhydride with 1.9 g. of freshly distilled benzyl alcohol for 3.5 hours in a closed tube kept in boiling water. The oil formed was extracted with ether, and the etheric solution washed twice with a solution of bicarbonate. The bicarbonate solution was then washed with ether; upon the addition of diluted hydrochloric acid, the benzyl ester precipitated as an oil which quickly crystallized. Recrystallization from ether–petrol ether gave a melting point of 84°.

Five equivalents of the silver salt of the benzyl ester to one of dibenzyl chlorophosphate was used for the preparation of the carbobenzyloxyaspartic acid-α-benzyl ester β-dibenzylphosphate. The method used to prepare carbobenzyloxyleucine dibenzylphosphate was followed, yielding a heavy, yellowish oil. Calculated on the amount of dibenzyl chlorophosphate, a 100% yield was obtained.

Anal. Calcd. for $C_{33}H_{31}O_9NP$: C, 64.1; H, 5.0; N, 2.3; P, 5.2. Found: C, 63.7; H, 5.4; N, 2.3; P, 4.9.

(2) **β-Aspartyl Phosphate.**—As with leucine phosphate, the β-aspartyl phosphate was prepared by passing a stream of dry hydrogen bromide through a water-free carbon tetrachloride solution of carbobenzyloxyaspartic acid-α-benzyl ester β-dibenzylphosphate. In this case the carbobenzyloxy and three benzyl groups were removed, producing an oil which was 92% pure, according to the colorimetric analysis described above. The yield, calculated on the weight of carbobenzyloxyaspartic acid benzyl ester dibenzylphosphate, was 80%.

(3) **Asparagine Formation.**—One hundred mg. of β-aspartyl phosphate was added to 5 ml. of 1 *M* aqueous ammonia. After standing for about 5 minutes, the solution was concentrated by distillation to 1 ml. The phosphate salt of *asparagine* precipitated as a white crystalline substance; m.p. 168°, mixed melting point with the phosphate salt of natural asparagine 168°.

Anal. Calcd. for $C_4H_{11}O_7N_2P$: C, 20.5; H, 4.8; N, 12.1; P, 13.5. Found: C, 19.8; H, 5.5; N, 12.1; P, 13.6.

C. **Glutamyl Phosphate.** (1) **Carbobenzyloxy-*l*-glutamyl-α-benzyl Ester γ-Dibenzylphosphate.**—This substance was prepared according to the procedure used to obtain the aspartic acid derivative, yielding the carbobenzyloxy-*l*-glutamic acid-α-benzyl ester as a colorless oil. Only a slight excess of its silver salt was required to obtain a good yield of carbobenzyloxy-*l*-glutamyl-α-benzyl ester γ-dibenzylphosphate in the form of a heavy oil.

Anal. Calcd. for $C_{34}H_{32}O_9NP$: C, 64.6; H, 5.1; N, 2.2; P, 4.9. Found: C, 64.2; H, 5.9; N, 2.3; P, 5.2.

(2) **γ-Glutamyl Phosphate.**—The γ-glutamyl phosphate was prepared in the same way as the β-aspartyl phosphate. The yield was 85%. The reaction with hydroxylamine showed that the product was 92% pure.

(3) **Glutamine Formation.**—One hundred mg. of γ-glutamyl phosphate was dissolved in 1 ml. of 1 *M* aqueous ammonia. *Glutamine* immediately precipitated; m.p. 185°. On heating, the glutamine was converted into the ammonium salt of pyrrolidone carboxylic acid, which has the same melting point as glutamine.[13] The amino nitrogen of the latter was 6.3%, while its total nitrogen was 19.0% (theoretical 19.2%). The amino nitrogen of the glutamine, air-dried, was 18.9%.

(13) N. Lichtenstein, THIS JOURNAL, **64**, 1021 (1942).

REHOVOT, ISRAEL

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Streaming Orientation Studies on Denatured Proteins. V. Bovine Serum Albumin[1]

BY JOSEPH F. FOSTER,[2] EDWARD G. SAMSA AND G. FREDERICK HANNA

RECEIVED JUNE 7, 1954

The denaturation of bovine serum albumin under various conditions has been investigated by flow birefringence. No common pattern of alteration, under the various denaturing conditions, is evident. Heat denaturation in either acid or alkaline aqueous solution leads to moderately highly birefringent solutions, probably largely through aggregation as previously concluded by Joly and Barbu. Samples heat denatured in the presence of 80% glycerol possess relatively large rotary diffusion constants suggesting neither appreciable aggregation nor drastic unfolding of the molecule. The rotary diffusion behavior following heat denaturation is not appreciably altered by inclusion of anionic detergent ions (in alkaline solutions) or cationic detergent ions (in acid solutions). Denaturation by guanidine hydrochloride or thiocyanate at 37° leads to measurable, though relatively weak, birefringence of flow. Guanidine thiocyanate (3.0 *M*) in alkaline solution and in absence of buffer ions appears to be particularly effective in minimizing aggregation. The ability of caprylate ions to repress or modify denaturation is not significant unless prolonged interaction with the protein is permitted prior to subjection of the protein to the denaturing conditions. It is tentatively suggested that the primary denaturation process in the case of this protein is essentially an expansion of the molecule, rather than an unfolding such as has been suggested for ovalbumin in previous studies of this series.

Previous contributions in this series[3] have been concerned with an attempt to clarify, through studies of streaming birefringence behavior, the character of the configurational changes involved in the denaturation of ovalbumin under a variety of conditions. It has been shown that any interpretation of such results on a molecular level is complicated by the tendency of denatured ovalbumin to aggregate. Nevertheless, evidence has been presented to show that under conditions which minimize intermolecular effects similar rotary diffusion constants are obtained under a variety of denaturing conditions including heating aqueous

(1) Journal Paper Number J-2538 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project Number 1223. A portion of this research was conducted under Contract Nonr-803 (00) with the Office of Naval Research.

(2) To whom correspondence concerning this contribution should be addressed. Present address: Department of Chemistry, Purdue University, Lafayette, Indiana.

(3) (a) J. F. Foster and E. G. Samsa, THIS JOURNAL, **73**, 3187 (1951); (b) E. G. Samsa and J. F. Foster, *ibid.*, **73**, 3190 (1951); (c) J. F. Foster and E. G. Samsa, *ibid.*, **73**, 5388 (1951); (d) G. F. Hanna and J. F. Foster, *J. Phys. Chem.*, **57**, 614 (1953).

solutions at low *p*H, urea denaturation at either low or elevated temperature, and denaturation with surface active cations. These rotary diffusion constants have been tentatively interpreted in terms of unfolded, rod-like molecules approximately 600 Å. in length.

In parallel with the reported studies on ovalbumin, many experiments also have been carried out on bovine serum albumin. None of these results have been previously reported, largely because it has not been possible to integrate them into any satisfactory picture. It is the purpose of this paper to summarize these results briefly, emphasizing some obvious points of contrast between the behavior of this protein and that of ovalbumin.

Previous studies by streaming birefringence of the effect of heat denaturation on the rotary diffusion behavior of serum albumin have been reported by Joly and Barbu.[4] These workers demonstrated the presence of readily orientable material under a variety of experimental conditions and attributed the effects entirely to aggregation, concluding molecular unfolding to be absent or minor.

Experimental

Crystalline bovine serum albumin was supplied through the courtesy of the Armour Laboratories. All other chemicals used were of reagent grade and were not further purified.

Solutions were prepared by dissolving the weighed protein sample in water or buffer, or in the aqueous solution of the denaturant using a rubber-stoppered test-tube. In some cases heat denaturation was carried out at this stage, in other cases following dilution with glycerol. The required amount of glycerol (95%) was added by weight and the contents mixed thoroughly by inversion of the tube. In most experiments the final glycerol concentration was 80% by weight. Prior to flow birefringence runs the solutions were filtered through a coarse sintered-glass filter to remove any traces of debris and coagulated protein and in some cases also centrifuged at 20,000 times gravity[5] if the filtered solution was not perfectly clear. Finally, just before running the solutions into the cylinders of the apparatus, they were deaerated for 20–30 minutes under reduced pressure (water aspirator).

The instrument used and experimental procedure for making the measurements of birefringence (Δ) and extinction angle (χ), were essentially as previously described.[3] The only important modification was the substitution of a new outer cylinder (stator) providing a gap width of 0.51 mm. in place of the 0.99 mm. gap used previously.

In heat denaturation experiments, the time of heating was measured from the moment of immersion in the boiling water-bath. A period of approximately 2 minutes was required for the temperature of the protein solution to come to bath temperature.

The following solvent viscosities were employed in calculations: for 80% glycerol in absence of guanidine salts, 0.46 poise; for 3 *M* guanidine salt in 80% glycerol, 0.63 poise; for 4 *M* guanidine salt in 80% glycerol, 0.67 poise. The latter two values were determined in an Ostwald viscometer.

Results and Discussion

For brevity, the most significant experimental results are summarized in a series of tables which include the following data: (1) Protein concentration, reported as the final concentration after dilution with glycerol in g. per 100 ml. (2) *p*H of aqueous solution (prior to dilution with glycerol). (3) Heating time. (4) Range of gradients covered in the experiment in terms of the quantity $G\eta/T$ (gradient in sec.$^{-1}$ times solvent viscosity in poises divided by absolute temperature). (5) Range of birefringence measured in terms of Δ, the measured rotation of the Senarmont compensator. The actual birefringence in the instrument used is related to Δ through the relation $n_e - n_0 = 4.8 \times 10^{-8}\ \Delta$. (6) Range of observed extinction angle (χ). (7) Range of the reduced birefringence function,[3] Δ/fC (where C is the protein concentration and f the orientation function). (8) Range of the reduced rotary diffusion constant, $\eta\theta/T$ (where θ is the rotary diffusion constant, η and T are as defined under (4) above). (9) Any special comments concerning the method of denaturation or observed results.

Heat Denaturation.—Results on heat denaturation are summarized in Tables I and II. Table I is concerned with denaturation experiments on the acid side of the isoelectric point, Table II on the alkaline side.

The most salient feature of the results of denaturation in acid media is the marked difference between solutions heated prior to and after addition of glycerol. It seems probable that aggregation is serious in the former case. Nevertheless, the results do not indicate the development of extremely elongated aggregates during denaturation since even in the extreme cases $\eta\theta/T$ values do not fall below about 0.2 corresponding to lengths, in terms of rigid prolate ellipsoids, of the order of 1500 Å. In the case of samples heated in the presence of glycerol, the $\eta\theta/T$ values are higher than observed under comparable conditions with ovalbumin[3] and it is concluded that neither unfolding nor drastic aggregation has taken place. The concentration effect is actually in the opposite direction to that expected if aggregation were an important factor, the values increasing significantly with increasing concentration. Further, comparison of samples heated 5 and 60 minutes indicates that the transition which yields birefringence is rapid and essentially complete in 5 minutes.

In the samples heated in alkaline solution no marked difference is observed under the two heating conditions. The $\eta\theta/T$ values here are of the same order as those observed with ovalbumin but show no obvious consistency.

In view of the very interesting experiments reported by Boyer, *et al.*,[6] which suggested that fatty acid anions such as caprylate can inhibit, or even reverse, the denaturation of serum albumin by heat, urea and guanidine salts (as judged by viscosity and turbidity studies), it was of interest to test the effect of caprylate on the flow birefringence behavior. The two such results shown in Table II indicate that caprylate has no effect whatsoever on the flow birefringence behavior under these conditions. It is, of course, conceivable that the anion combination does not take place in the presence of the glycerol-rich solvent or that sufficient time was not permitted for combination to take place (see below).

It should be pointed out that all of the heat denatured systems, as well as most of the other de-

(4) M. Joly and E. Barbu, *Bull. soc. chim. biol.*, **31**, 1642 (1949); *ibid.*, **32**, 908 (1950).

(5) Sorvall SS-1 centrifuge.

(6) P. Boyer, F. Lum, G. Ballou, J. Luck and R. Rice, *J. Biol. Chem.*, **162**, 181 (1946); P. Boyer, G. Ballou and J. Luck, *ibid.*, **162**, 199 (1946).

TABLE I

SUMMARY OF RESULTS, HEAT DENATURATION (100°) IN ACID SOLUTION

Prot. concn., %	pH[a]	Heat. time, min.	Range $G\eta/T$	Range Δ	Range χ	Range Δ/fC	Range $\eta\theta/T$	Comments
Heated prior to addition of glycerol								
0.72	2.57N	5		1.7– 9.0	22.2– 2.2		0.125–	Solution hazy
.36	1.5 N	65	1.80– 9.0	3.0–10	16.2– 8.5		0.21– 0.10	
.54	G	20	1.80–12	14 –33	22 –12.5		0.24– 0.53	
Heated in the presence of glycerol								
.36	3.3 G	5	3.0–12.0	2.5– 4.7	39.9–37.1	100–130	7.03– 6.92	
.36	3.3 G	60[b]	3.0– 6.0	2.0– 3.5	40.9–39.7	100–120	7.10– 8.22	[b]
.72	3.3 G	5	6.0–12.0	3.3– 6.3	44.2–38.1	160–95	13.4 – 8.15	
.72	3.3 G	60[b]	3.0–12.0	2.0– 7.3	44.0–39.2	200–130	13.7 – 9.90	[b]
1.08	3.6 G	5	3.0–12.0	2.7–10.5	43.2–39.9	100–140	7.85–11.3	
1.08	3.6 G	60[b]	6.0–12.0	6.0–12.0	43.5–41.1	400–220	18.3 –14.9	[b]
0.72	2.8 G	5	3.0–12.0	8.5–15.0	43.5–42.4	570–600	18.3 –22.0	

[a] N = pH adjusted with HCl, no buffer present. G = glycine buffer, ionic strength 0.04. [b] Preceding sample reheated 55 minutes in presence of glycerol.

TABLE II

SUMMARY OF RESULTS, HEAT DENATURATION (100°) IN ALKALINE SOLUTION

Prot. concn., %	pH[a]	Heat. time, min.	Range $G\eta/T$	Range Δ	Range χ	Range Δ/fC	Range $\eta\theta/T$	Comments
Solutions were heated prior to dilution with glycerol								
0.36	7.7N	5		Birefringence too low for study				
0.72	7.7N	5	3.15– 6.30	3.5– 7.5	36.5–33.6	40–70	1.70–2.40	
1.08	7.7N	5	0.95– 6.30	6.0–29.7	39.5–29.2	80–130	0.82–1.58	
0.36	7.7N	30						
(80°)—Birefringence too low for study								
.36	8.9V	5	0.95– 6.30	2.2– 8.1	30.9–22.1	34–65	0.28–0.62	Solution hazy
.54	7.8P	60	3.0 – 8.9	10 –20	15.6–15.0	49–95	0.20–0.54	Solution very hazy
Solutions were heated in presence of glycerol								
.36	7.2N	15	0.40– 6.30	16.7–43	18.5–14.4	130–280	0.036–0.35	
.36	8.9V	5	3.15– 6.30	3.0– 6.4	37.1–35.5	80–140	1.79 –3.08	
.36	8.9V	15	1.58– 6.30	1.6– 9.5	37.6–33.2	80–170	0.96 –2.32	
.36	9.2V	5	0.95– 6.30	3.3–15.1	39.7–28.0	130–190	1.65 –2.72	
.36	8.8V	5	1.8 –12.0	2.5–13.0	38.6–25.2	80–140	1.33 –2.11	Caprylate added
.36	8.8V	60	1.8 –12.0	7.7–26.7	32.3–20.0	130–220	0.61 –1.29	Caprylate added

[a] N = pH adjusted with NaOH, no buffer present. V = Veronal buffer, ionic strength 0.10. P = phosphate buffer, ionic strength 0.20.

TABLE III

SUMMARY OF RESULTS, HEAT DENATURATION IN PRESENCE OF CATIONIC DETERGENT IONS

Prot. concn., %	pH[a]	Heat. time, min.	Range $G\eta/T$	Range Δ	Range χ	Range Δ/fC	Range $\eta\theta/T$	Comments
0.36	2.5G	5	9.0–12.0	3.5–4.3	39.4–38.2	130–130	7.2– 8.3	
.36	2.5G	60	6.0–12.0	2.4–4.7	40.1–36.0	100–110	5.9– 6.0	Preceding sample reheated 55 min.
.72	1.5G	5	3.0 –9.0	3.0–8.1	43.9–42.1	280–310	12.1–15.1	
.72	1.5G	60	6.0– 9.0	3.6–5.3	42.7–41.9	170–190	12.6–13.9	Preceding sample reheated 55 min.

[a] Glycine buffer, ionic strength 0.04.

natured systems reported below, are obviously heterogeneous as judged by the pronounced gradient dependence of the results.

Heat Denaturation in the Presence of Detergent Ions.—In an attempt to reduce the possibility of aggregation, a number of studies was carried out in the presence of detergent ions (to enhance the molecular charge). Results in the presence of a cationic detergent, dodecyldimethylbenzylammonium chloride,[7] in acid solution are given in Table III and with sodium dodecylbenzenesulfonate[8] in alkaline solution in Table IV. In all cases, the detergent to protein ratio was the same, 1:6 by weight. It seems probable that both the anionic and cationic detergents are largely bound, even in the presence of the glycerol in view of the high affinity of such ions for this protein.[9] In both cases results are in rather close agreement with comparable results in the absence of detergent. It is of interest that neither detergent produced any measurable birefringence in absence of heating. Indeed Table IV shows the marked effect of temperature between 80 and 100° at pH 7.0, substantially no birefringence being obtained at the lower

(7) Purchased from Onyx Oil and Chemical Co.

(8) Santomerse No. 3, supplied by Monsanto Chemical Co.

(9) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954); J. F. Foster and J. T. Yang, *ibid.*, **76**, 1015 (1954).

TABLE IV

SUMMARY OF RESULTS, HEAT DENATURATION IN PRESENCE OF ANIONIC DETERGENT IONS

Prot. concn., %	pH[a]	Heat.[b] time, min.	Range $G\eta/T$	Range Δ	Range x	Range Δ/fC	Range $\eta\theta/T$	Comments
1.08	8.1N	5[c]	3.15– 6.30	2 – 4.3	38.7–39.0	20–50	2.32 –4.96	Heated before glycerol dilution
1.08	8.1N	10[c]	0.95– 6.30	2 –13	39.0–36.0	20–100	0.75 –3.14	Previous sample htd. additional 5 min.
1.08	8.1N	25[c]	0.95– 6.30	22 –80	35.5–33.7	160–270	0.43 –0.96	Previous sample htd. additional 15 min.
0.80	7.8P	60	3.0 – 8.9	3 –14	27.5–26.9	16–76	0.65 –1.82	
.36	7.4N	15	0.38– 6.30	17 –64	22.0–12.0	160–350	0.056–0.16	
.36	7.7N	15	0.38– 6.30	2.3–24	39.0–26.2	80–270	0.30 –1.18	
.36	7.7N	30	0.35– 6.30	4.0–31	38.1–23.0	120–290	0.24 –0.90	
.36	8.9V	5	6.0 –12.0	3.1– 4.2	37.6–32.7	90–70	3.73 –4.23	
.36	8.9V	15	3.15– 6.30	2.2– 4.2	36.4–36.1	80–100	2.52 –3.15	
.36	8.9V	60	1.58– 6.30	2.0– 7.5	35.9–33.0	50–130	0.87 –2.25	
.36	8.9V	60	3.0 –12.0	2.0– 6.7	38.2–30.9	60–100	2.05 –3.52	
.54	8.9V	5	4.73– 6.30	2.0– 2.7	34.9–35.6	30–40	2.07 –2.96	
.54	8.9V	15	3.15– 6.30	2.5– 5.1	37.0–35.5	40–80	1.77 –2.96	
.54	8.9V	60	0.95– 6.30	2.4–12.5	37.5–31.0	40–130	0.57 –1.85	
.72	8.9V	5	3.15– 6.30	2.5– 4.7	37.6–36.7	40–60	1.93 –3.40	
.72	8.9V	15	1.58– 6.30	2.7–10	38.5–34.5	40–100	1.12 –2.63	
.72	8.9V	60	0.95– 6.30	3.7–18	39.5–30.5	70–140	0.82 –1.77	
1.08	8.9V	5	3.15– 6.30	3.0– 6.2	37.9–37.2	30–60	2.03 –3.65	
1.08	8.9V	17	0.95– 6.30	2.2–13	40.9–32.5	40–70	1.11 –2.14	
1.08	8.9V	60	0.95– 6.30	6.6–32	39.4–29.6	80–150	0.81 –1.62	
0.72	9.1V	5	6.0 –12.0	3.5– 7.0	39.0–33.6	40–60	4.75 –4.62	
.72	9.1V	60	1.80–12.0	6.5–20.1	37.7–27.7	90–120	1.13 –1.98	
.36	7.0V	5	0.54–12.0	17 –61	23.5–11.5	170–325	0.082–0.40	
.36	7.0V	5[c]	0.65–12.0	1.8–13.0	35.2–14.7	40–85	0.27 –0.69	Heated at 90°
.36	7.0V	5[c]	No birefringence					Heated at 80°
.36	7.0V	60[c]	Birefringence too low to study					Heated at 80°

[a] N = no buffer present, V = veronal buffer, ionic strength 0.10, P = phosphate buffer, ionic strength 0.20. [b] All samples heated at 100° in presence of glycerol unless otherwise stated. [c] See comments for heating procedure.

temperature. It is concluded that the flow birefringence behavior, whether due to intramolecular alterations or aggregation, is little modified by the combination with detergent ions.

Denaturation with Guanidine Salts.—Guanidine salts are well known to be among the strongest of protein denaturing agents. It is further known that the character of the anion is of importance, the thiocyanate salt being particularly effective.

Table V summarizes some results with guanidine hydrochloride, while Table VI is concerned with guanidine thiocyanate, in all cases in isoelectric or slightly alkaline solution.

The most obvious feature of these results is the relatively low birefringence obtained in all cases. This is best seen in the low values of the Δ/fC function which is of the order of 100 or less in most cases. This suggests that either (1) only a fraction of the protein is modified or that (2) the optical situation is not favorable for birefringence studies (see below). In the absence of buffer the systems are relatively homogeneous as judged by the substantial constancy (with changing gradient) of $\eta\theta/T$ in several cases. Upon addition of buffer ions (Veronal) the rotary diffusion constants tend in general toward lower values suggesting an aggregation effect. The fact that time of exposure to the reagent is not an important variable in the unbuffered experiments is in further accord with the view that aggregation is not significant. Unfortunately, the birefringence was too low to extend the studies to lower concentration to test for the absence of aggregation effects.

A few light scattering studies were carried out on these systems to test further for absence of aggregation. Results of such measurements at various protein concentrations were plotted as HC/τ *vs.* C in the usual fashion and extrapolated to infinite dilution. Resulting estimates of weight-average particle weight are included in Table VI, and suggest that in general aggregation was not severe. It must be emphasized that these values refer to the aqueous solution prior to dilution with glycerol and do not rule out the possibility of aggregation following such dilution. However, all results tend to suggest that glycerol prevents rather than encourages aggregation. A more serious limitation in the interpretation of these light scattering results arises from the uncertainty as to the relative binding of the two solvent components, water and guanidine thiocyanate.

Finally, it was of interest to test the effect of caprylate on denaturation by guanidine thiocyanate for reasons already discussed. Table VII summarizes such studies. When compared with results on Veronal buffered systems in absence of caprylate these data indicate no striking modification of the reaction by this agent unless it is permitted to interact for a prolonged period of time with the protein prior to addition of the denaturing agent. Thus there is a progressive increase in rotary diffusion constant obtained with increasing contact time with caprylate. It is of interest that the values tend to approach those obtained in absence of buffer, which condition has been suggested above to lead to minimal aggregation.

TABLE V

SUMMARY OF RESULTS, DENATURATION IN PRESENCE OF GUANIDINE HYDROCHLORIDE

Prot. concn., %	pH[a]	Heat.[b] time, min.	Range $G\eta/T$	Range Δ	Range x	Range Δ/fC	Range $\eta\theta/T$	Comments
0.72	5.2	60[c]	−90	Birefringence very weak				4.0 *M* guanidine Room temp. (25°)
.36	~5	30	4.5–9.0	2.3– 4.5	34.4–28.5	45–55	2.39–2.72	3.0 *M* guanidine
.54	~5	30	1.8–9.0	3.0– 9.0	33.2–23.7	35–60	0.85–1.27	3.0 *M* guanidine
.72	~5	35	1.8–9.0	5.5–14.5	27.6–21.2	35–65	0.51–1.40	3.0 *M* guanidine
.72	~5	60	1.8–9.0	9.7–19.2	19.6–16.0	40–65	0.24–0.79	3.0 *M* guanidine
.72	~5	120	Sample gelled					
.72	8.5V	5	1.8–9.0	11.3–21	18.5–16.6	45–75	0.24–0.96	4.0 *M* guanidine Soln. slt. hazy
.72	8.5V	30	1.8–9.0	13.4–23	17.0–15.2	50–80	0.18–0.71	3.0 *M* guanidine Soln. hazy

[a] V represents veronal buffer, ionic strength 0.10. Other solutions contained no buffer ions. [b] All samples heated at 37° with guanidine salt prior to dilution with glycerol unless noted otherwise. [c] Room temperature.

TABLE VI

SUMMARY OF RESULTS, DENATURATION IN PRESENCE OF GUANIDINE THIOCYANATE[a]

Prot. concn., %	pH[b]	Heat.[c] time, min.	Range $G\eta/T$	Range Δ	Range x	Range Δ/fC	Range $\eta\theta/T$	Comments
0.72	7.6N	30	8.2 –19.7	2.3– 5.5	39.4–29.2	63–54	6.96– 4.92	M.W. 150,000
.72	8.2N	30	4.5 – 9.0	2.5– 5.3	40.4–34.2	55–50	6.4 – 5.0	
.72	8.2N	60	4.5 – 9.0	2.6– 5.5	42.1–39.7	95–110	10.2 –11.2	
.72	8.2N	120	3.0 – 9.0	2.2– 6.7	39.7–36.5	42–85	5.3 – 6.5	
.72	8.3N	60	3.0 – 9.0	1.6– 6.2	40.4–37.5	35–82	6.9 – 6.7	M.W. 90,000
.72	8.1N	60	4.1 –16.4	9.5–15	36.8–32.0	84–83	2.3 – 5.36	M.W. 150,000
.54	8.4V	30	3.0 – 9.0	1.8– 4.6	40.9–35.1	60–70	4.82– 5.50	
.54	8.4V	60	3.0 – 9.0	2.6– 6.5	41.2–32.9	95–75	5.20– 4.36	
.54	8.4V	120	3.0 – 9.0	2.4– 6.6	40.1–32.0	70–70	3.98– 4.01	
.72	8.4V	30	1.8 – 9.0	2.5– 7.0	39.4–32.4	40–60	2.13– 4.18	
.72	8.4V	60	1.8 – 9.0	3.8–10.8	39.4–26.1	60–60	2.11– 2.33	
.72	8.4V	120	1.8 – 9.0	8.0–18	24.2–19.4	40–70	0.40– 1.23	
.72	8.4V	19 hr.	1.8 – 9.0	2.0– 9.3	39.4–33.2	40–85	2.11– 4.50	Room temp. 60 min. prior to glycerol addn. 18 hr. in presence of glycerol
1.08	8.4V	30	1.8 – 9.0	6.7– 18	32.6–25.1	40–65	0.85– 2.13	
0.50	9.0V	60	2.47–12.3	3.0– 7.5	37.3–27.4	52–54	1.5 – 2.62	
0.72	9.0V	30	3.0 –12.0	2.8– 9.8	39.0–31.5	46–73	3.21– 5.06	M.W. ~270,000
0.72	8.4V	60[d]	1.8 – 9.0	2.2– 8.0	42.2–34.7	80–80	4.26– 5.23	Room temperature
0.32	8.9V	~100[d]	6.0 –12.0	2.1– 4.1	33.6–23.1	41–41	3.14– 2.36	Room temperature M.W. ~270,000
0.54	9.0V	30	3.0 – 9.0	2.0– 4.8	44.0–40.9	250–160	13.7 –10.7	1.50 M Guanidine salt

[a] 3.0 *M* unless otherwise noted under comments. [b] N = no buffer present, V = veronal buffer, ionic strength 0.10. [c] Heated at 37° in absence of glycerol unless otherwise noted. [d] Held at room temperature.

TABLE VII

SUMMARY OF RESULTS, DENATURATION WITH GUANIDINE THIOCYANATE IN PRESENCE OF CAPRYLATE[a]

Heating time,[b] min.	Caprylate contact time[c]	Range $G\eta/T$	Range Δ	Range x	Range Δ/fC	Range $\eta\theta/T$
120	10 min.	1.8– 9.0	6.3–18.3	28.0–21.7	40–80	0.56–1.6
60	20 min.	3.0– 9.0	4.0– 9.7	39.1–30.0	70–70	3.3 –3.3
35	3 hr.	6.0–12.0	4.4– 8.0	40.8–31.4	110–60	9.4 –5.1
120	3 hr.	1.8– 9.0	4.8–12.8	33.4–24.2	45–65	0.92–2.0
120	18 hr.	1.8– 9.0	3.7–11.5	37.7–29.1	55–75	1.6 –3.0
120	48 hr.	4.5– 9.0	2.5– 4.5	42.7–38.2	110–70	13 –8.4
240	48 hr.	3.0– 9.0	2.5– 7.0	41.2–35.0	70–75	5.1 –5.5
240	95 hr.	6.0– 9.0	3.7– 5.7	39.4–37.3	70–80	7.0 –7.7

[a] The final protein concentration was 0.72% in all cases (approximately 4.0% at the denaturation stage. All samples were buffered at *p*H 8.5 with veronal buffer of ionic strength 0.10. [b] Time of contact with guanidine thiocyanate at 37° in presence of 0.05 *M* caprylate. [c] Time of contact with 0.25 *M* caprylate at room temperature (25°) prior to addition of guanidine thiocyanate.

Conclusions.—In a general way the results obtained are in agreement with the conclusion of Joly and Barbu[4] that aggregation is the important contributory factor toward flow birefringence in heat denaturation of this protein. On the other hand, they indicate the possibility that streaming orientation may be attainable in absence of aggregation at lower temperatures in the presence of

strong denaturing agents. The rotary diffusion constants corresponding to this denatured state are, however, relatively high, $\eta\theta/T$ ranging from about 5 to perhaps 20. These relatively high rotary diffusion constants suggest rather strongly that no drastic unfolding of the protein molecule has occurred under such conditions, but at most only an expansion. To actually draw any conclusions as to the nature of the change would require additional physical data such as intrinsic viscosity, as has been emphasized recently by Scheraga and Mandelkern.[10] It would be of considerable interest to obtain such data in the systems used, but measurement of intrinsic viscosity in a mixed solvent such as the glycerol–water systems employed poses a considerable problem.[11]

One final argument, favoring the idea that no substantial unfolding takes place in the case of serum albumin, appears worth mentioning. The low values of the reduced birefringence in the case of guanidine denaturation have been pointed out above. One can generalize further and say that in only a few cases are these figures comparable to those obtained with ovalbumin denatured under various conditions.[3] Interpretation of such data is uncertain since they depend on not only the intrinsic anisotropy of the molecule being oriented but also on the form birefringence. On the other hand, the intrinsic anisotropy of a swollen molecule such as envisaged here would almost certainly be less than that of an elongated, more highly ordered molecule. The form birefringence, too, would be less, other factors being the same. The low reduced birefringence is thus in accord, at least, with the swelling concept.

Acknowledgment.—We are indebted to Dr. Lawrence L. Lachat of Armour and Company for generous donations of bovine serum albumin.

AMES, IOWA

(10) H. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953).

(11) A few such experiments have been carried out in our laboratory on denatured ovalbumin and have yielded results in close agreement with those obtained in absence of glycerol.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICAL CHEMISTRY, HARVARD UNIVERSITY]

Preparation and Properties of Serum and Plasma Proteins. Plasma Cholinesterase[1]

BY DOUGLAS M. SURGENOR AND DRUMMOND ELLIS[2]

RECEIVED APRIL 2, 1954

Under controlled conditions of *p*H, ionic strength, ethanol concentration and temperature, plasma cholinestarase has been concentrated 3400 times over plasma on a protein basis. Although not yet pure, it has been estimated from ultracentrifugal analyses that the pure enzyme has an activity at least 11,000 times that of plasma, and comprises not more than 0.01% of the plasma proteins.

Among the enzymes found in human plasma is an esterase which catalyzes the hydrolysis of choline esters. In this respect, as well as in other physical properties, the plasma enzyme is distinguishable from the acetylcholinesterases which are found in nerve tissue and in intimate association with the envelope of the red cell,[3] both of which split acetylcholine faster than any other choline ester. The physiological function of the nerve enzyme is undoubtedly concerned with the hydrolysis of acetylcholine. The role of the plasma enzyme is, however, unknown.

In the fractionation of plasma, cholinesterase was separated into fraction IV-4,[4] together with the β_1-metal-combining protein. In the subfractionation of fraction IV-4, the enzyme was concentrated into fraction IV-6, which was rich in α_2-globulins.[5] Since our earlier studies had indicated that some denaturation of the rather labile carbohydrate-containing proteins in this fraction had occurred, we have confined our present efforts to concentration of the enzyme whose activity appeared to have been maintained in these separations. Preparations of the enzyme have been obtained which were over three thousand-fold as active, on a dry weight basis, as plasma; it has not yet been obtained in the pure state, however. The newer methods, recently described,[6] for the fractionation of proteins, which open up several new avenues of approach to difficult separations, while at the same time maintaining optimal conditions for protein stability, thus averting the danger of partial denaturation of at least certain of the components in the concentrates here described, make possible considerable improvement in the yields and purifications of this as well as other enzymes.

(1) This work has been supported by funds of Harvard University and the Eugene Higgins Trust, by grants from the Rockefeller Foundation and the National Institutes of Health, and by contributions from industry. It was presented at the 119th Meeting of the American Chemical Society, April 2, 1951, Boston, Massachusetts.

(2) The award to one of us (D. E.) of a Travelling Fellowship by the Kemsley Foundation, Glasgow, Scotland, and of a Fellowship Grant by the Cross Trust, Edinburgh, Scotland, in 1947–1948, is gratefully acknowledged. Present address: Blood Transfusion Service, Royal Infirmary, Edinburgh 9, Scotland.

(3) K. Augustinson, *Acta Physiol. Scand.*, **15**, Suppl. 52 (1948).

(4) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(5) D. M. Surgenor, L. E. Strong, H. L. Taylor, R. S. Gordon, Jr., and D. M. Gibson, *ibid.*, **71**, 1223 (1949).

Materials and Methods

Fraction IV-6 was the starting materials in these studies. It was prepared by the method and from the sources described previously.[5]

Plasma esterase determinations were carried out by Miss M. Dougherty, working under the direction of Dr. Avram Goldstein in the Department of Pharmacology, Harvard Medical School, to whom we are indebted for continued interest. They measure release of CO_2 from a bicarbonate–Ringer solution (0.025 *M* bicarbonate), buffered to *p*H 7.8

(6) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma, *ibid.*, **72**, 465 (1950).