component designated as the 11S protein in this and earlier studies.[3-5,8]

Acknowledgments.—The authors are indebted to Mrs. Bonita Hopson and Mrs. Clara McGrew for performing the Kjeldahl analyses.

PEORIA, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE, AND THE MARINE BIOLOGICAL LABORATORY]

Denaturation of Bovine Plasma Albumin. II. Isolation of Intermediates and Mechanism of the Reaction at *p*H 7[1,2]

BY ROBERT C. WARNER AND MILTON LEVY[3]

RECEIVED MAY 5, 1958

The kinetics of denaturation of bovine plasma albumin between *p*H 4 and 12 have been analyzed by solubility methods. It was found that extensive aggregation occurs, but that by suitable choice of solubility conditions for determining the extent of denaturation, the reactions preceding aggregation could be kinetically identified. These reactions are first order with respect to protein concentration, but are complex in that the rate decreases with time. The reaction mechanism has been investigated by sedimentation of the reaction mixture and by solubility determinations over a wide range of salt concentration. The existence of intermediate species has been confirmed and some of these have been isolated and their properties investigated. The effect of sulfhydryl reagents and compounds and the determination of sulfhydryl groups indicates that the initial reactions involve an intramolecular sulfhydryl–disulfide exchange. A minimum kinetic scheme is proposed and the variation of the rate with *p*H is discussed.

The criteria for choosing solubility conditions that will make a kinetically useful distinction between native and denatured protein have been discussed in a previous paper.[4] It was there shown that under conditions chosen by the use of these criteria, the denaturation of bovine plasma albumin (BPA) from *p*H 0.8 to about *p*H 4 can be described by a first-order equation. At higher *p*H's, beginning abruptly at the point of maximum rate near *p*H 4, the use of the same solubility conditions yields a reaction which is first order with respect to protein concentration, but is kinetically complex in that the rate decreases as the reaction progresses. We are reporting here on the kinetics of denaturation between *p*H 4 and 12 and on studies of the properties of the products and intermediates designed to supplement the kinetic data in formulating the reactions involved in the denaturation of BPA.

Experimental

The kinetic experiments were carried out with crystalline BPA (Armour and Co.) using the methods previously described.[4] The conditions of salt concentration and *p*H employed for stopping the reaction and separating native from denatured protein, referred to as high salt and low salt stopping conditions, are those defined previously.[4] Rate constants are given in reciprocal sec. and natural logarithms.

The sedimentation studies were made in the Spinco model E ultracentrifuge. Solutions for these experiments were prepared by heating the reaction mixtures in sealed tubes.

The more complete analysis of the solubility behavior was carried out using the variable salt solubility method essentially as described by Derrien and Roche.[5] This consists of measuring the protein remaining in solution when the concentration of salt is systematically varied at constant *p*H. An acetate–acetic acid buffer of the desired *p*H and concentration was prepared. A concentrated ammonium sulfate or sodium trichloroacetate solution was prepared in this buffer. Mixtures were made by diluting the buffered salt solution with the buffer to known volumes. Aliquots of the solution to be tested were added to these mixtures usually in a volume ratio of 1 to 10, left at room temperature for 8 to 24 hours and then centrifuged at high speed. The protein concentration in the supernatant fluid was determined by reading the optical density at 278 mμ. The solutions were sufficiently well buffered with sodium acetate and acetic acid so that the *p*H in any experiment over the entire range of salt concentration did not vary more than 0.03 unit.

The amperometric titrations were carried out at *p*H 7.6 in phosphate buffer by the methods described by Kolthoff, Stricks and Morren[6] using $HgCl_2$ as the titrant. The use of nitrogen for the elimination of oxygen from the solutions during titration gave erratic results, but the enzymatic method of Benesch and Benesch[7] was entirely satisfactory. Glucose, enzyme and the protein sample were added to the buffer about 5 minutes before titration. Titrations on two samples of Armour BPA gave 0.70 mole of sulfhydryl group per 69,000 g. for lot N66706 and 0.69 mole for lot S68004. A sample of crystalline Hg dimer of mercaptalbumin,[8] after removal of the Hg on a thioglycolate resin column, gave 0.96 mole. Argentimetric titrations in ammoniacal alcohol were used in earlier experiments. These gave similar results, but lower values for the sulfhydryl content of BPA.

Results

Dependence of the Kinetics on the Stopping Conditions.—The effect of the use of different solubility conditions for following the formation of insoluble protein from BPA at neutral *p*H's can be seen from a comparison of Figs. 1 and 2. In the experiment in Fig. 1 the rate was determined by using the low salt stopping conditions. There was a lag phase during which no precipitation occurred followed by a reaction, the rate of which is clearly dependent on the initial protein concentration. The course of the reaction can be approximated by the modified second-order equation

$$(C_0 - C)/C = C_0kt - (b/C_0) \qquad (1)$$

The term containing b is included to account for the lag phase which is roughly inversely proportional to C_0. The solid lines in Fig. 1 are plots of

(1) This investigation was supported by a research grant, H-1642, from the National Heart Institute, Public Health Service.

(2) Preliminary reports of part of this work have appeared: M. Levy and R. C. Warner, *Federation Proc.*, **12**, 239 (1953); **15**, 300 (1956).

(3) Department of Biochemistry, New York University College of Dentistry.

(4) M. Levy and R. C. Warner, *J. Phys. Chem.*, **58**, 106 (1954).

(5) Y. Derrien and J. Roche, *Compt. rend. soc. biol.*, **142**, 1001 (1948).

(6) I. M. Kolthoff, W. Stricks and L. Morren, *Anal. Chem.*, **26**, 366 (1954).

(7) R. E. Benesch and R. Benesch, *Science*, **118**, 447 (1953).

(8) Kindly supplied by Prof. J. L. Oncley.

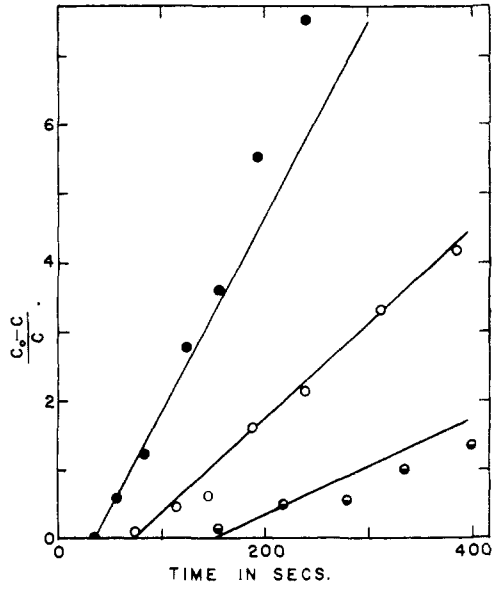

Fig. 1.—Rate of formation of precipitate at the low salt stopping conditions from BPA at 79.5° in a borate buffer, *p*H 7.95, ionic strength 0.2. Protein concentration: ●, 1 g./100 ml.; O, 0.5 g./100 ml.; ⊖, 0.25 g./100 ml. The solid lines are plots of eq. 1 with $k = 0.028$ and $b = 36$.

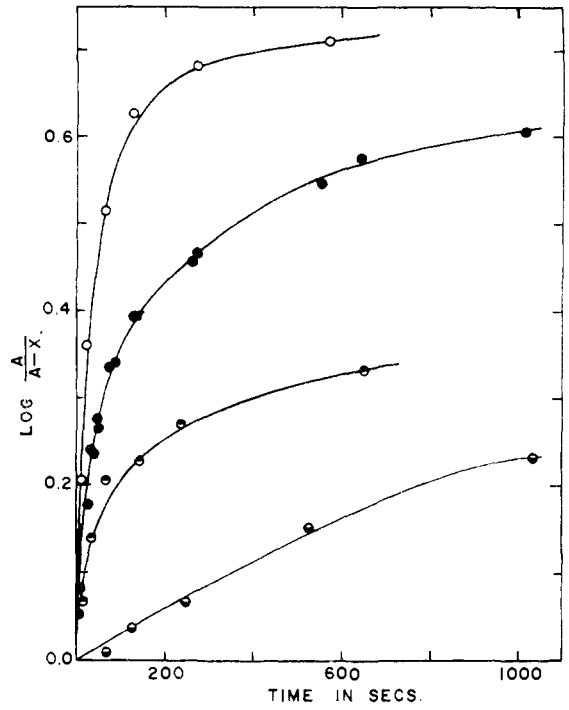

Fig. 2.—Rate of formation of precipitate at the high salt stopping conditions from BPA at 65.7° in a phosphate buffer, *p*H 7.00, ionic strength 0.2, protein concentration 0.5 g./100 ml.: ●, no additions, points are from several expts. over a concentration range of twofold; for extension of this curve to longer time intervals see Table I; O, 7×10^{-4} *M* cysteine added; ⊖, 10^{-3} *M* PSMB added; ◓, saturated with cystine.

this equation. No mechanistic significance can be attached to the description of the rate as a second-order process since ultracentrifugal analysis reported below has demonstrated aggregates of much greater size than dimers. It is presented here to emphasize the high degree of concentration dependence of the rate under these conditions. This is in contrast to the results obtained using the high salt stopping conditions which converted the rate of formation of precipitate to that illustrated in Fig. 2. This rate is independent of the initial protein concentration, but falls off more rapidly with time than required by first-order kinetics. Both the reaction and the decrease in rate continue for as long as we have followed the reaction. The rate becomes so low after 200 hours that a plateau appears to be reached at which about 10% of the protein remains soluble. The high salt stopping conditions thus detect a species which is formed several hundred times as rapidly as that detected by the low salt conditions and transform the reaction to a complex first-order type. These conditions therefore meet the criteria previously suggested for studying denaturation by solubility methods.[4] The nature of the kinetics and their dependence on initial protein concentration and on the salt concentration of the stopping conditions have been examined in detail at several *p*H's between 4 and 10, with results similar to those shown in Figs. 1 and 2.

Because of the impossibility of characterizing an entire rate curve of the type in Fig. 2 by a single parameter, we have employed initial first-order rates to demonstrate the variation of rate with *p*H. These are not very accurately defined in the case of fast reactions since when $\log k > -2$ they may depend on one or two early points. The logs of these constants are plotted against *p*H in Fig. 3. The points at *p*H's lower than the *p*H of maximum rate near 4 are taken from our previous paper.[4] The change from simple to complex first-order kinetics occurs abruptly as the *p*H is increased through the maximum.

Several additional characteristics of the reaction have been determined which may be noted here. (1) No indication of reversibility could be found. When reaction mixtures were heated and then cooled to 25 or 2°, no change in the amount of precipitable protein occurred on standing at the lower temperature. When cooled reaction mixtures were heated again, the reaction proceeded again from the point at which it was stopped and at the same rate. (2) There was no change in the amount of precipitate with time of standing in the stopping buffer. This type of instability of the system was checked carefully because of its occurrence under similar circumstances with conalbumin.[9] (3) The rate was not changed by deionizing the protein on anion, cation or mixed bed ion exchange resins or by extraction with hexane. (4) Several fractions were prepared by fractional crystallization of BPA with $(NH_4)_2SO_4$ or with Hg and ethanol. No difference in the rate of denaturation of these fractions and the original BPA could be demonstrated.

It may be concluded that aggregation is an important part of the processes occurring during denaturation, but that proper choice of solubility conditions permit the study of a rate-controlling

(9) A. Wishnia and R. C. Warner, unpublished observations.

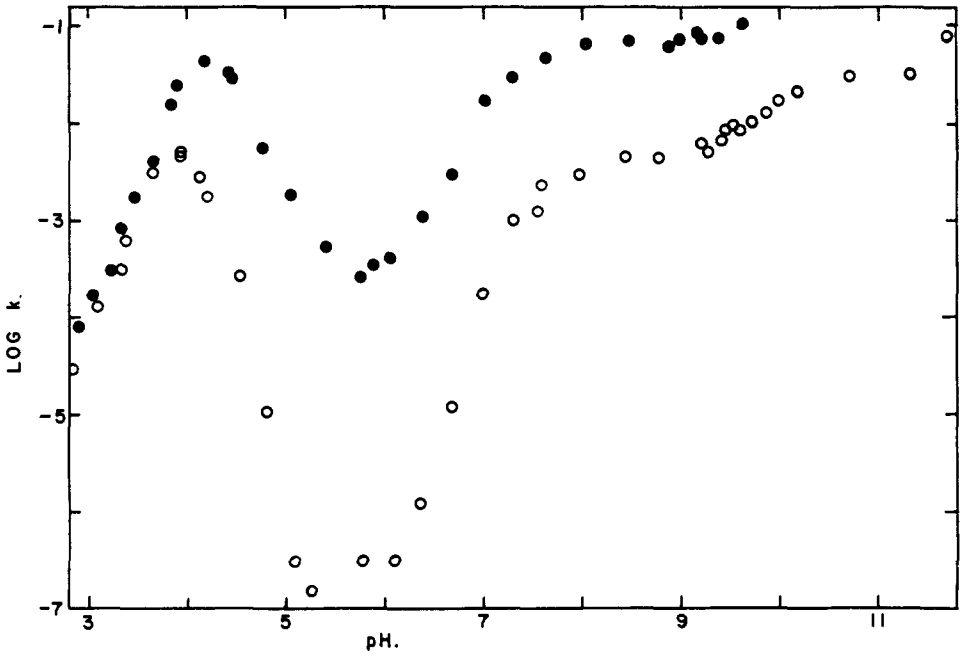

Fig. 3.—Rate of denaturation of BPA as a function of *p*H. *k* is the first-order rate constant for the initial phase of the reaction; ●, 65.7°, and ○, 56.1°, determined by the use of the high salt stopping conditions.

intramolecular reaction. However, it is evident that a more discriminating study of the reaction mixture is necessary to understand the complexity of this kinetic system. Our further work has consisted of studies of the solubility and ultracentrifugal behavior of the components of the reaction mixture and of experiments on the relation of sulfhydryl groups to the reaction process.

Sedimentation.—Reaction mixtures after heating in sealed tubes were examined in the ultracentrifuge. The progress of the reaction in each tube was followed using both the low and high salt stopping conditions. Some typical patterns obtained under the same conditions of heating used for the experiments in Fig. 2 are shown in Fig. 4, and a summary of one experiment is given in Table I. The initial pattern consisted of a component with a sedimentation coefficient of 4.31*S*[11] and a trace of a faster component (6.5*S*) which

TABLE I

SEDIMENTATION OF BPA HEATED AT 65.7° AND *p*H 7

Expt.	Time of heating, hr.	Fraction pptd.[a] Low salt	Fraction pptd.[a] High salt	Slow component $s_{20,w}$	Slow component Fract.[b]	Fast component $s_{20,w}$	Fast component Fract.[b]	Other $s_{20,w}$	Other Fract.[b]
1	0.0	0	0	4.31	0.97	..	..	6.8	0.03
2	.033	0	0.58	4.2	.93	..	..	5.8	.07
3	.167	0	.73	4.3	.49	16	0.42	6.1	.09
4	1.0	0	.78	4.4	.34	14	.66		
5	3.0	0	.82	4.3	.27	16	.73		
6	4.8	0	.83	4.2	.19	19	.82		
7	25.5	0.22	.85	4.6	.22	22	.78		
8	123.3	.83	.90	4.4	.17	31	.83		
9	238.6	.83	.89	4.6	.13	33	.87		
10	310.6	.83	.89	4.2	.14	39	.86		
11[c]	0.033			4.3	.25			6.5	.75
12[d]	3.0			4.2	1.0				
13[e]	3.0					12	1.0		
14[f]	3.0	0	0.77	4.1	0.25	15	0.75		
15[g]	92.7	0.34	.49	4.3	.70	24	.30		
16[h]	2.3	0	.73	4.5	.41	20	.59		
17[i]	3.0	0	.72	4.2	.36	18	.64		

[a] Fraction of total protein precipitated at the low salt and high salt stopping conditions, respectively. In replicate experiments the range of variation is ±5% of the fraction precipitated. [b] Fraction of the area of the pattern. These values have been corrected for radial dilution, but not for the Johnston–Ogston[10] effect. Such a correction would improve the agreement of the fraction of the fast component with the fraction precipitated at the high salt stopping conditions. In replicate experiments the range of variation of these fractions is about ±3%. [c] Fraction insoluble at the high salt stopping conditions (D + B). [d] Fraction P_3 (E); see text. [e] Fraction P_1 (C); see text. [f] Heated with 1.7×10^{-3} *M* cysteine. [g] Heated with 10^{-3} *M* caprylate. [h] Mercaptalbumin. [i] Anaerobic.

(10) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

(11) *S* = svedberg unit = 10^{-13} sec.

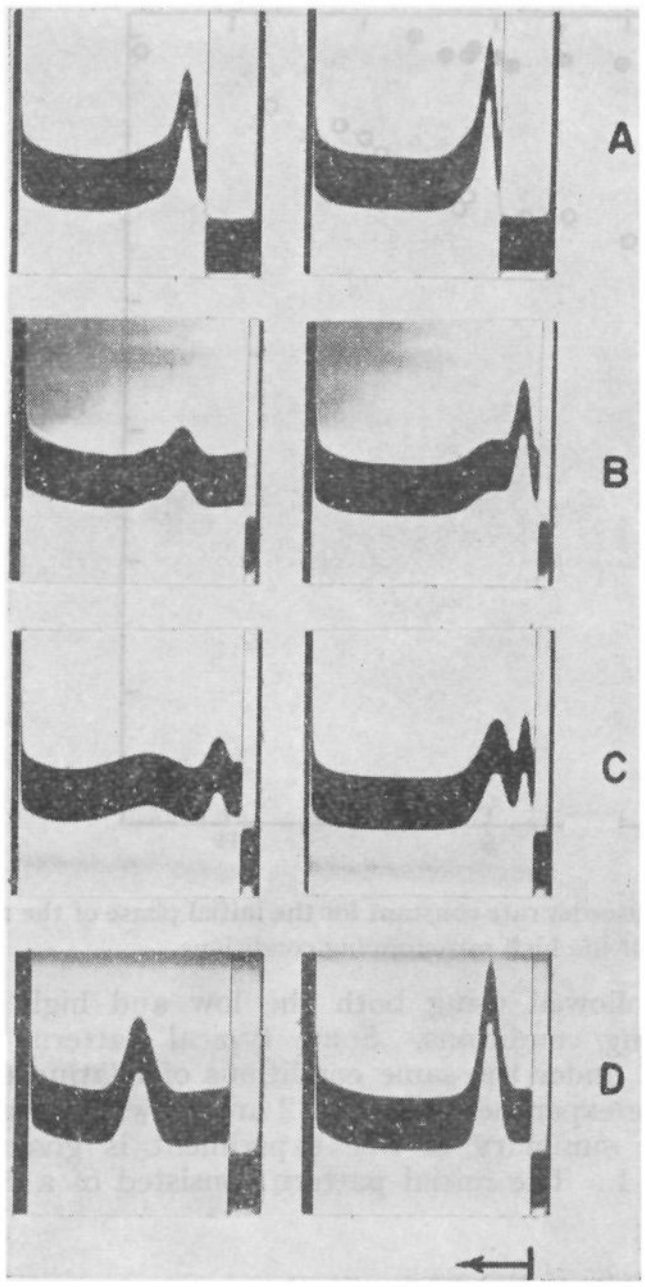

Fig. 4.—Sedimentation patterns of solutions of BPA heated at 65.7°, *p*H 7.00, ionic strength 0.2, and of a fraction prepared from the heated solution, 59,780 r.p.m.: A, heated 0.033 hr., centrifuged 12 and 20 min.; B, heated 0.167 hr., centrifuged 20 and 36 min.; C, heated 3 hr., centrifuged 10 and 26 min.; D, fraction P_3 (E) centrifuged 44 and 108 min.

may be a dimer.[12] As the reaction progressed the 4.3*S* peak decreased in area and faster components appeared. By one hour the faster material was all present in a diffuse boundary with a coefficient of about 15*S*. That the average coefficient of this boundary and the fraction of the pattern that it represented increased with further heating is indicated in Table I. In the intermediate patterns at 0.033 and 0.167 hour a significant increase in the 6.5*S* peak also can be noted, indicating the stepwise nature of the aggregation process. The other important feature of the patterns is that some of the 4.3*S* component persisted even after the longest periods of heating.

Comparison of the sedimentation results with solubility under the two conditions of precipitation shows that a high degree of aggregation is required to produce insolubility at the low salt concentrations. No precipitate was formed in this and other experiments until the fast component had a coefficient of greater than 20*S*. After further heating when the coefficient had risen to 35 to 55*S* almost all of the aggregated protein became insoluble. This is consistent with the concentration-dependent kinetics determined by insolubility under these stopping conditions (Fig. 1). At the high salt stopping conditions the opposite prevailed and the fraction of protein precipitated was greater than the quantity of fast component for samples heated 3 hours or less. This is most clearly demonstrated by the results after 0.033 hour of heating. No fast component was present at this time and there was only a small increase in the 6.5*S* component so that 93% of the protein retained the original sedimentation coefficient although 58% was precipitated. This result provides direct evidence for the existence of an insoluble form with no substantial change in sedimentation properties and conforms to the conclusion drawn from the kinetics that an intramolecular change is rate controlling under these conditions. In the early stages of the reaction the 4.3*S* component is thus a mixture of the native protein and an unaggregated denatured species. The two forms differ in solubility, but whether differences exist in other physical properties will have to await further examination of the isolated fractions. After 4 hours of heating the 4.3*S* component did not contain any protein that was insoluble and good correspondence was found between the fraction of the pattern in the fast peak and the fraction of the protein precipitated by the high salt stopping buffer (Table I).

A consideration of the sedimentation and solubility data in Table I thus enable us to distinguish five different species: A, native protein, 4.3*S;* B, dimer or other small aggregate, 6.5*S;* *C* aggregated fraction, 14 to 39*S;* D, denatured, unaggregated protein, insoluble at high salt stopping conditions, 4.3*S;* E, unaggregated protein, soluble at the high salt conditions which persists as a 4.3*S* component after long periods of heating. The individuality of some of these species was confirmed by experiments on fractionated reaction mixtures described below.

A similar experiment to that in Table I was performed on bovine mercaptalbumin.[8] The results showed that the same components were formed as with BPA. However, the initial rate as measured with the high salt stopping buffer was slightly less ($k = 0.016$ as compared with $k = 0.019$) for BPA at the same *p*H, and there was less of the fast component and more of the slow. The latter may be seen by comparing expt. 16, Table I, with similar heating times for BPA. In addition the sedimentation coefficient of the fast component was higher and rose to 43*S* after 143 hours and to 63*S* after 262 hours of heating.

Less extensive sedimentation data were obtained on BPA heated in formate buffer, *p*H 3.3, ionic strength 0.2, at 65.7°. These experiments provided evidence for species corresponding to A, C and D, but did not show the presence of a component with the properties of E. Fraction D was isolated by collecting the protein that was soluble in the low salt and insoluble at the high salt stopping conditions. This material yielded a single component sedimentation pattern (4.7*S*).

(12) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, **60**, 1633 (1956).

Because of the well established stabilization of BPA by fatty acid and other organic anions,[13] the effect of caprylate was tried in our system. When potassium caprylate was added to the reaction mixture (conditions as in Fig. 2) at a concentration of 0.005 *M*, the denaturation as detected by the high salt stopping conditions proceeded so slowly that no precipitate was formed in 12 hours and only a very small amount in 24 hours. At a caprylate concentration of 0.001 *M* the initial first-order rate constant was 0.00011 as compared with 0.019 when no caprylate was present. This rate decreased with time in the same manner as in Fig. 2. Several reaction mixtures were sedimented and one set of values is included in Table I. Just as in the experiments without caprylate, a fast component accumulated which represented a smaller fraction of the protein than was precipitated by the high salt stopping buffer.

Salting-out Curves.—In order to provide additional evidence for the existence of the species indicated to be present by sedimentation and to define the conditions for the preparation of these species, a more complete examination of the solubility relations in heated reaction mixtures was made by the method of Derrien and Roche.[5] Solubility diagrams giving the amount of protein in solution as measured by the optical density as a function of the salt concentration are shown in Figs. 5, 6 and 7.

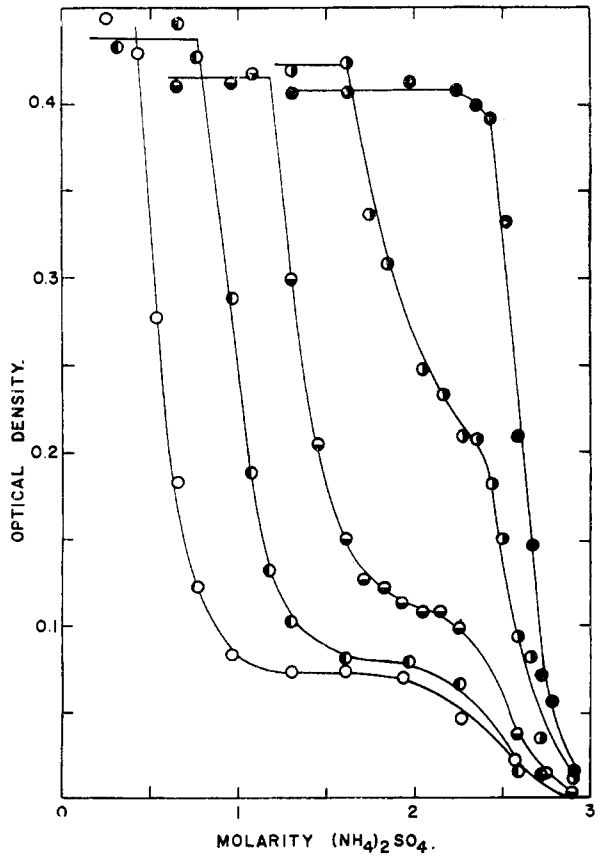

Fig. 5.—Salting out of BPA heated at 65.7°, *p*H 7, by ammonium sulfate at *p*H 5.42. Time of heating: ●, native, not heated; ◑, 0.033 hr.; ◒, 0.167 hr.; ◐, 3 hr.; ○, 28.3 hr.

(13) G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lum, *J. Clin. Invest.*, **23**, 454 (1944); *J. Biol. Chem.*, **153**, 589 (1944).

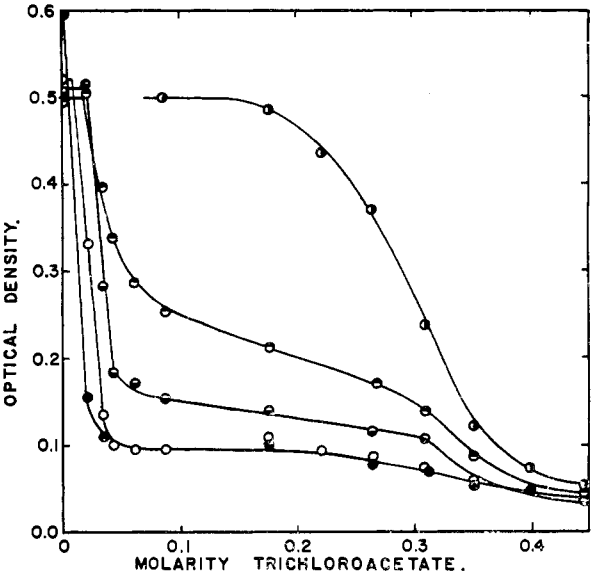

Fig. 6.—Salting out of BPA heated at 65.7°, *p*H 7, by potassium trichloroacetate at *p*H 4.15. Time of heating: ◑, native, not heated; ◒, 0.033 hr.; ◒, 0.167 hr.; ○, 3 hr.; ●, 27.4 hr.

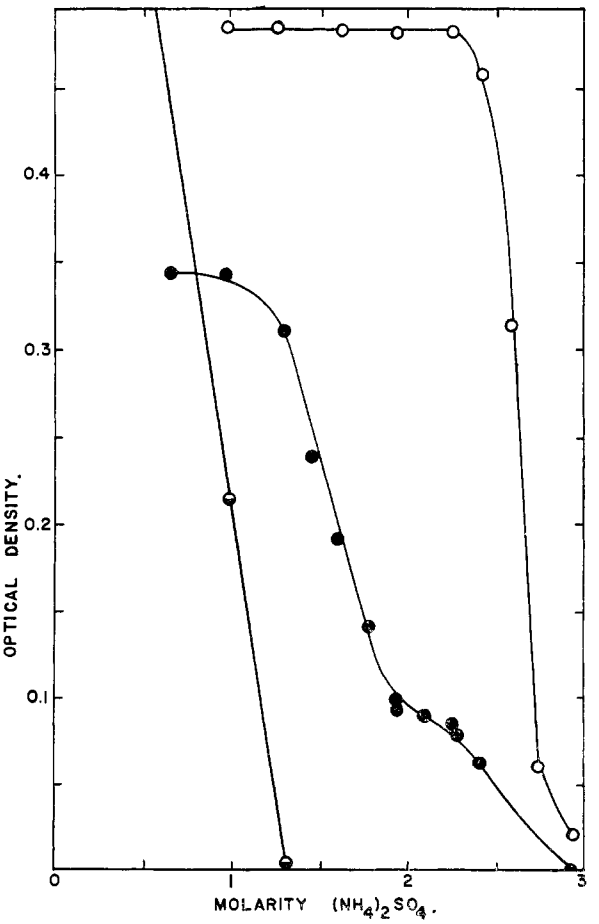

Fig. 7.—Salting out of fractions prepared from BPA heated for 3 hr. at 65.7°, *p*H 7, by ammonium sulfate at *p*H 5.42: ◒, fraction P_1 (C); ●, fraction P_2; ○, fraction P_3 (E).

The approximately monotonic curves for native BPA are transformed into biphasic curves on heating. The more readily precipitated fraction (Fig. 5) was precipitated at lower and lower ammonium sulfate concentration as heating proceeded and increased in amount. This fraction corresponds to species C and D detected ultracentrifugally. No distinction can be made between the two since a decrease in solubility evidently accompanies not only the conversion of D to C, but also the further aggregation of C. The less readily precipitated fraction retained its solubility characteristics and only slowly was converted into the less soluble form. It thus corresponds to E. The ammonium sulfate concentration of the high salt stopping conditions used in the kinetic work was 1.75 *M* and is thus on the plateau between the two phases of the precipitation curve.

Salting out of the same reaction mixtures with ammonium sulfate buffered at *p*H 4.9 produced similar curves. Trichloroacetate was tried as the precipitating salt instead of ammonium sulfate. In this experiment (Fig. 6) the plateau between the two phases of precipitation in the 3 and 24 hour reaction mixtures is much longer and a third intermediate phase of salting out can clearly be seen in the 0.033- and 0.167-hour reaction mixture which may correspond to species D. It is evident that buffered trichloroacetate can be used at low concentrations to make good solubility distinctions in protein mixtures.

Fractionation experiments were carried out under the conditions suggested by Fig. 5. A 3-hour reaction mixture was brought to 1.24 *M* ammonium sulfate at *p*H 5.4. The precipitate was removed, suspended in water and dialyzed. The portion remaining insoluble (P_1) was dissolved by adjusting the *p*H to 7. The small fraction which dissolved in water (P_2) was recovered by salting out with ammonium sulfate. Additional ammonium sulfate was added to the filtrate from the 1.24 *M* separation and the remaining protein which precipitated sharply at 2.4 *M* was removed and dialyzed (P_3). Solubility determinations on these fractions are shown in Fig. 7 and sedimentation data on P_1 and P_3 are given in Table I. A pattern of P_3 is reproduced in Fig. 4. The solubilities of the fractions are those expected from the curve for the 3-hour heated protein in Fig. 5 and a reaction mixture reconstituted from the fractions followed the same curve. Each sedimentation pattern consisted of a single peak which may be identified as E in P_1 and C in P_2. Except for a small reduction in the sedimentation coefficient of C, the isolated fractions have retained the solubility and sedimentation properties found for the components of the original reaction mixture. They are therefore not components of a mobile equilibrium system, but are different forms derived from the original BPA. Fraction P_2 contained some of each component (Fig. 7) but represented only about 5% of the total protein.

Species D could not be detected in the preceding experiment because it is no longer present after 3 hours of heating. It was prepared as the fraction of a 0.033-hour reaction mixture which was insoluble at the high salt stopping conditions. The sedimentation pattern of this fraction (Table I, expt. 11) contained two peaks which may be identified with D and B. The relative amount of D is somewhat less than would be predicted from expt. 2, presumably because of some differential loss or further aggregation during precipitation. However, its presence confirms the existence of a species with the properties assigned to D.

The isolated fraction P_3 (E) also was tested for the rate at which material insoluble at the high salt stopping conditions was formed. Kinetic curves are given in Fig. 8. The low rate of this reaction (compare Fig. 2) provides a means of distinguishing this fraction from native BPA which has a similar solubility and sedimentation coefficient.

Sulfhydryl Relationships.—Aggregation of proteins during denaturation by urea as a result of a sulfhydryl–disulfide exchange reaction was postulated by Huggins, Tapley and Jensen.[14] The significance of this type of exchange in producing intramolecular rearrangements or aggregation during denaturation as well as in other protein reactions has been investigated by several workers.[15–19] The involvement of similar mechanisms in the formation of the various species indicated above was investigated by the determination of the influence of sulfhydryl compounds and combining reagents on the rate of denaturation and by amperometric titration of the sulfhydryl content as a function of time and in various fractions. A summary of the influence of various substances on the rate is given in Figs. 2 and 8. The rate is seen to be reduced by an excess of *p*-chloromercuribenzoate (PCMB), cystine or by Hg in a molar ratio to the sulfhydryl groups of one and is increased by cysteine or by Hg in greater concentration. These changes in rate are consistent with a requirement for free sulfhydryl groups. Mercury at the higher ratio may be expected to increase the hydrolysis of disulfide groups. According to the mechanism discussed by Cecil,[20] this would lead to the formation of more sulfhydryl groups than the Hg involved would combine with. Cysteine not only increased the rate of reaction of BPA but also of fraction P_3 (Fig. 8). It was anticipated that sulfhydryl exchange processes might be restricted to the aggregation reaction. However, these rate effects are upon a reaction that has been demonstrated above to be an initial non-aggregation step. This argues for a mechanism based on intramolecular exchange, but does not exclude a dependence of the aggregation on a similar mechanism.

(14) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951); V. D. Hospelhorn, B. Cross and E. V. Jensen, THIS JOURNAL, **76**, 2827 (1954).

(15) M. Halwer, *ibid.*, **76**, 183 (1954).

(16) R. B. Simpson and W. Kauzman, *ibid.*, **75**, 5139 (1953); J. Schellman, R. B. Simpson and W. Kauzman, *ibid.*, **75**, 5152 (1953); W. Kauzman and R. B. Simpson, *ibid.*, **75**, 5154 (1953); H. K. Frensdorff, M. T. Watson and W. Kauzman, *ibid.*, **75**, 5157 (1953); **75**, 5167 (1953).

(17) W. Kauzman and R. G. Douglas, Jr., *Arch. Biochem. and Biophys.*, **65**, 106 (1956).

(18) G. Markus and F. Karush, THIS JOURNAL, **79**, 134 (1957).

(19) P. Bro, S. J. Singer and J. M. Sturtevant, *ibid.*, **80**, 389 (1958).

(20) R. Cecil, *Biochem. J.*, **47**, 572 (1950).

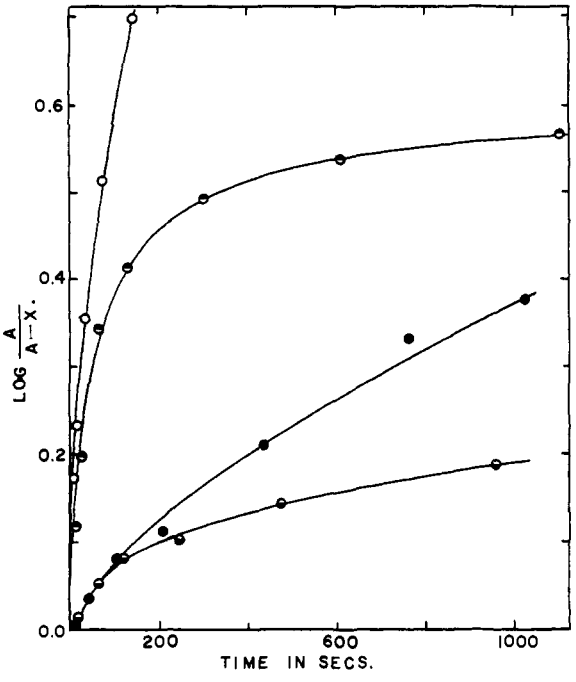

Fig. 8.—Rate of formation of precipitate at the high salt stopping condition from BPA at 65.7°, *p*H 7, in the presence of $HgCl_2$ and from fraction P_3 (E) with and without cysteine; O, molar ratio Hg/BPA = 10; ●, ratio = 0.75; ◒, fraction P_3; ◓, fraction P_3 in 1.4×10^{-3} *M* cysteine. Experiments at several other ratios of Hg/BPA demonstrated that the maximum reduction in rate was obtained at a ratio of 0.75.

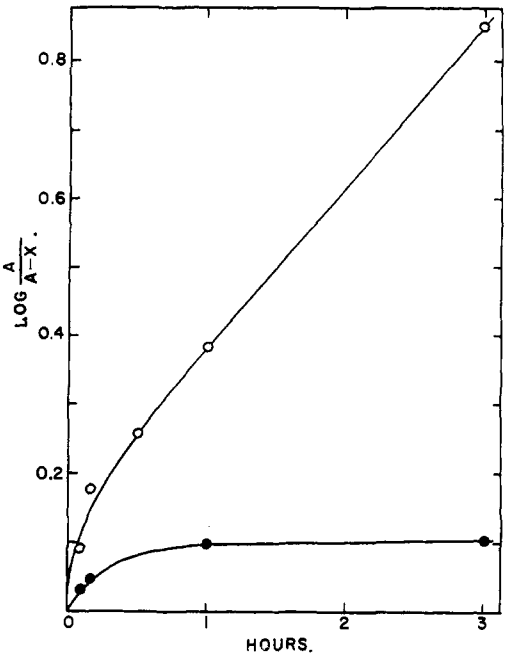

Fig. 9.—Rate of disappearance of sulfhydryl groups of 65.7°, *p*H 7, in air (O) and in N_2 after incubation with glucose, glucose oxidase and peroxidase (●). No further change was produced in the latter experiment by heating overnight.

The amperometric titrations demonstrated that the protein sulfhydryl groups disappeared during heating, although never to zero even after extended periods (Fig. 9).[21] The rate of disappearance was much lower than the rate of denaturation and the sulfhydryl content had dropped to about 70% of its original value by the time the rate of denaturation was reduced 20-fold (*cf.* Fig. 2). These experiments were carried out in tubes with the reaction mixture open to the air. When sealed tubes were employed and the air was replaced by N_2, no difference in the rate of denaturation[22] or of the disappearance of sulfhydryl groups was detected. However, when the method of Benesch and Benesch[7] for the enzymatic removal of the last traces of O_2 was used there was a sharp reduction in the rate of sulfhydryl disappearance, but no comparable effect on the rate of denaturation.[22] The sulfhydryl content under these conditions fell to 60 to 80% of its initial value and then remained constant (Fig. 9). Sedimentation of a reaction mixture heated for 3 hours under anaerobic conditions showed distinctly less of the fast component (Table I, compare expts. 17 and 5). Such reactions mixture were also fractionated and fractions corresponding to P_1 (C) and P_3 (E), (Table I) were prepared and titrated; P_1 did not contain any sulfhydryl groups, whereas the soluble fraction P_3 contained as much as 0.44 mole per mole of protein. In this procedure not all of the sulfhydryl of the heated solution was recovered in the products. This indicates a greater lability of the sulfhydryl in the denatured protein to destruction at room temperature, presumably by oxidation, than in the native protein. A similar observation has been reported by Kolthoff, *et al.*,[23] after urea denaturation. The increased lability in our heated solutions evidently was less marked than in those prepared by urea denaturation since gentle rocking in air of a 3-hour anaerobic reaction mixture reduced the sulfhydryl content only 5%. The possibility that any part of our results were caused by reduction of sulfhydryl content due to the manipulation of sampling is thus eliminated.

In separate experiments the influence of *p*H of the reaction mixture on the rate of disappearance of sulfhydryl groups during heating in air at 65.7° was followed. The rate was first order to at least 50% reduction in sulfhydryl content and, as indicated in Fig. 10, increased continuously from *p*H 2.5 to 8.2. Comparison of Fig. 10 with Fig. 3 shows that a change in the stability of sulfhydryl groups cannot be important in determining the *p*H dependence of the rate of denaturation. The rate at any given *p*H may nevertheless depend on the sulfhydryl concentration.

Kinetic Relationships.—The various species demonstrated by sedimentation and solubility may be assembled in the reaction scheme of Fig. 11. The considerations on which the scheme is based and some of the properties of the reactions may be outlined as follows: (1) The initial rate of formation of insoluble protein depends primarily on reaction

(21) The assistance of Arnold Rubin and John Perz with these experiments is acknowledged.

(22) The sealed tube technique is not adequate for measurement of the rate of the fast initial step of the reaction and the results indicated only that no change of large magnitude has occurred. This point will have to be reinvestigated at a lower temperature.

(23) I. M. Kolthoff, A. Anastasi, W. Stricks, B. H. Tan and G. S. Deshmukh, THIS JOURNAL, 79, 5102 (1947).

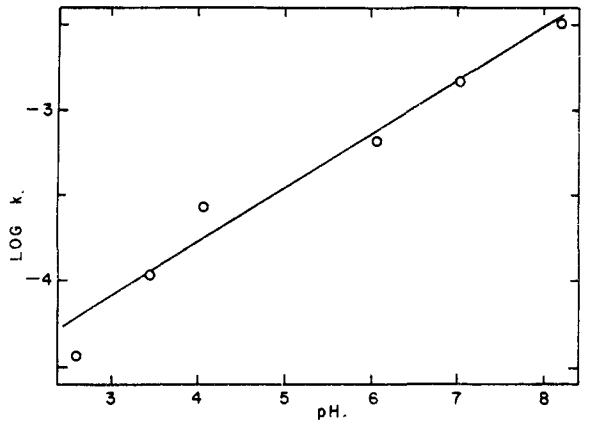

Fig. 10.—Rate of disappearance of sulfhydryl groups of BPA heated at 65.7°, *p*H 7, in air as a function of *p*H. The solid line is a plot of log $k = -5 + 0.32$ *p*H.

A → D. The dependence of this rate on sulfhydryl reagents and compounds and its monomolecular character as indicated by sedimentation and rate data make it probable that the reaction is an intramolecular sulfhydryl–disulfide exchange which permits sufficient rearrangement to yield a product of lowered solubility.

(2) Product D aggregates readily to give B (dimer) and then C which increases in size as the reaction progresses. We have no direct evidence that this reaction is based on an intermolecular sulfhydryl–disulfide exchange. However, this would seem reasonable in view of the occurrence of this exchange in the preceding reaction and the demonstration of this type of aggregation of BPA under other conditions.[14–19] The absence of sulfhydryl groups in isolated C and the dependence of the amount of C formed on the presence of O_2 may indicate that other factors than exchange influence this polymerization.

(3) The possibility of direct aggregation of A or of A + D is eliminated because the rate of the reaction is independent of the initial concentration of protein.

(4) E is assumed to be formed simultaneously with D from A. It is soluble at the high salt stopping condition and its subsequent slow rate of aggregation is primarily responsible for the fall in the rate of denaturation (Fig. 2). The formation of E from A rather than the existence of E as a separate component or stabilized fraction of BPA is indicated by the failure of fractionation procedures or resin column or extraction treatments to alter the rate and by the accumulation of more E in anaerobic than in aerobic reaction mixtures. The behavior of mercaptalbumin and the finding of sulfhydryl groups in isolated samples of E indicate that E is not related to the non-sulfhydryl fraction of BPA. The increased rate of denaturation of BPA in cysteine together with the finding that the distribution of protein among the components of the sedimentation pattern was not altered by cysteine place the reaction A → E in the same category as A → D as an intramolecular sulfhydryl–disulfide exchange reaction. This is further demonstrated by the accumulation of more E during the heating of mercaptalbumin than

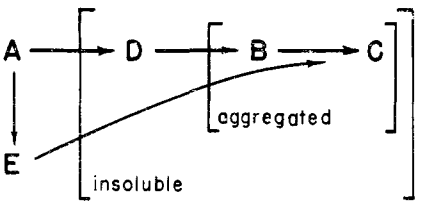

Fig. 11.—Minimal reaction scheme for the denaturation of BPA.

of BPA and the correlation of this with a lower rate of denaturation.

(5) The last points discussed above raise the question of the fate of the non-sulfhydryl fraction of BPA. Since this is only 30% of the protein it would have to proceed along a path involving intermediates of strikingly different properties in order to be detected. Comparison of BPA and mercaptalbumin suggests that it rapidly becomes insoluble.

(6) E proceeds at a very low rate to give insoluble protein as shown in Fig. 8. That the final product is aggregated may be inferred from the sedimentation results (Table I) after long periods of heating. The rate is increased by cysteine. The low sulfhydryl content of E after 3 hours of heating (Fig. 9) may contribute to the low rate. However, the accumulation of E during anaerobic heating and the demonstration that isolated E contains sulfhydryl groups shows that it is not simply a form of the native protein in which these groups have been oxidized.

If both initial paths of reaction A → D and A → E depend on sulfhydryl groups, part of the decrease in rate must be due to their loss by oxidation. Since this is slower than the initial rate at *p*H 7, no striking change in rate occurs when O_2 is excluded. A continuous formation of –SH from –SS– by hydrolysis may provide sufficient –SH to carry the reaction to completion at a low rate. The other factor decreasing the rate is the shunting of protein through the soluble, relatively stable form E. The scheme in Fig. 11, assuming all reactions to be first order, may be integrated to give the rate of formation of insoluble protein as the sum of two exponential terms. The curve of Fig. 2, however, cannot be fitted by less than the sum of three such terms and four are required to account for the extension of the curve to longer time intervals. No derived parameters for such integrated equations are presented because so many are required as to make their unique evaluation uncertain. In addition, the effect on the rate of the oxidation of sulfhydryl groups, their formation by hydrolysis of disulfide links and the behavior of the non-sulfhydryl component of BPA are difficult or impossible to assess. These reactions may account for the additional exponential terms not required by the minimal scheme.

Discussion

Because of the complexity of the denaturation reaction in the *p*H range above 4, the rate constants plotted in Fig. 3 give only a partial description of the kinetics. There is also considerable uncertainty in the determination of some of these

constants since they are derived from the initial rate. For these reasons we have not felt justified in deriving from them an equation for the *p*H dependence of rate based on the postulates previously employed.[4,24] It is apparent that such an equation could be derived readily since the log *k*–*p*H plot consists of a few segments which approach straight lines with integral slopes. It is of interest to note the similarity of this curve to that for ricin[24] in the *p*H range 7 to 9 where both curves level off nearly to zero slopes.

The outstanding feature of the BPA denaturation is the occurrence of the sharp maximum in rate in the neighborhood of *p*H 4. Lowering the *p*H below this value is attended by a decreased rate and by a change in the kinetic character of the reaction whereby the complex sequence of Fig. 11 gives way to a simple first-order reaction. The occurrence of this maximum coincides with the *p*H at which reversible expansion of the BPA molecule takes place at room temperature.[25–27] In discussing the expansion in relation to this maximum, Tanford, *et al.*,[25] have suggested that the greater stability of the expandable form may be due to its greater capacity to absorb thermal energy in a reversible manner. An alternative possibility is that the expansion results in a different spatial arrangement of the sulfhydryl and disulfide groups such that the intramolecular exchange occurs less readily. As formulated above, the reactions A → D and A → E represent two different and presumably specific exchange reactions leading to products with different properties. The irreversibility of these reactions at any *p*H may depend on spatial rearrangements of the polypeptide chain made possible by the exchange reaction. The sedimentation patterns of reaction mixtures at *p*H 3.3, in contrast to those at *p*H 7, did not reveal the presence of a component comparable to E. It may thus be assumed that the expansion results in a decrease in the rate of A → D and an elimination of any significant reaction *via* A → E. The latter change together with the increasingly greater stability of sulfhydryl groups to oxidation (Fig. 10) could account for the transition to simple first-order kinetics. The possibility must also be considered that in acid solution the intermediate identified by sedimentation as having properties similar to D is produced by a different mechanism than at *p*H 7.

This type of suggestion to account for the maximum in the log *k*–*p*H curve may be accommodated by the postulates previously used[4,24] by observing that the transition of BPA to the expanded form is a steep function of *p*H and may depend on a few critical ionizations. According to the electrophoretic studies of Aoki and Foster[29] the related N → F transition is determined by an equilibrium involving three protons.

It is evident that the relation of rate to *p*H exhibited in Fig. 3 in which two minima and a maximum occur cannot be accounted for by the mechanism proposed by Kauzmann.[30] He has suggested that electrostatic repulsions in an expanded activated species determines the variation in rate with *p*H. This theory permits only relatively small alteration in a relation showing a minimum at the isoelectric point and a continuously increasing rate with increase or decrease of *p*H.

In an investigation of the solubility of BPA after treatment with concentrated urea, Kauzmann and Douglas[17] have observed changes that parallel some of those we have found to result from heating. The rate of formation of protein insoluble in low concentrations of salt was dependent on the initial protein concentration while the rate when determined by solubility at high salt concentrations was independent of initial protein concentration. Under the latter conditions, the rate decreased more rapidly with time than predicted by a simple first-order equation. Denaturation in urea differed from that observed under our conditions in that some of the changes in solubility were reversible and the effect of *p*H on the rate was small and did not go through a maximum as in Fig. 3. These authors have discussed their results in terms of various possible rearrangements of the native structure resulting from sulfhydryl–disulfide exchanges. Their form "B" in which intramolecular exchange has taken place is comparable to the species D and E of Fig. 11, except that Kauzmann and Douglas consider form "B" to be a random rearrangement. We have assumed that specific exchanges may lead to specific species because we have independent evidence for the existence of two species and for the absence of aggregation in their formation. They also have considered that polymeric products may result from intermolecular exchange (form "C"), but have suggested the formation of such products directly from native BPA rather than through a monomeric intermediate as in Fig. 11.

The demonstration of intermediate, isolatable species in the denaturation of BPA opens the possibility of studying separately the intramolecular and aggregation steps of the over-all reaction and of investigating the nature of the reactions by examination of the physical and chemical properties of the intermediates. Other cases in which evidence has been presented for intermediates in denaturation reactions include arachin[31] and ovalbumin.[32]

(24) M. Levy and A. E. Benaglia, *J. Biol. Chem.*, **186**, 829 (1950).

(25) C. Tanford, J. G. Buzzell, G. D. Rands and S. A. Swanson, THIS JOURNAL, **77**, 6421 (1955).

(26) J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954).

(27) In connection with some measurements of the time dependence of the titration curve of conalbumin[9] by the method of Steinhardt and Zaiser[28] we have made a similar examination of the titration of BPA over the *p*H range in which expansion has been shown to occur. Within the limits of the apparatus which permitted measurements to be made within 3 sec. of mixing, the proton equilibria of BPA were not time dependent. The *p*H established by rapid mixing of BPA and acid in these experiments varied from 3.2 to 4.0, at an ionic strength of 0.2. We are indebted to Dr. J. S. Steinhardt for the use of his rapid flow apparatus and to Dr. R. J. Gibbs for assistance with the experiments.

(28) J. Steinhardt and E. M. Zaiser, *J. Biol. Chem.*, **190**, 197 (1951).

(29) K. Aoki and J. F. Foster, THIS JOURNAL, **79**, 3385 (1957).

(30) W. Kauzmann, in W. D. McElroy and B. Glass, eds., "The Mechanism of Enzyme Action," The Johns Hopkins Press, Baltimore, Md., 1954, p. 70.

(31) W. E. F. Naismith and R. K. Williams, *J. Appl. Chem. (London)*, **6**, 421 (1956).

(32) A. S. Tsyperovich and A. L. Losera, *Biokhimiya*, **21**, 546 (1956). Quoted from English translation, Consultants Bureau, N. Y., 1957, Vol. 21, p. 566.

Throughout this paper the term denaturation has been used to denote in a general way the phenomenon of loss of various characteristics of the initial structure of a protein by more or less irreversible processes. It is probably not meaningful to attempt to attach a more precise definition since in specific cases the criteria by means of which it can be studied will vary widely and will not always indicate the same changes or extent of change. "Native" and "denatured" are biological terms to which we wish to give chemical meaning by investigation of the isomerizations, conformational changes and other rearrangements of the bonded structures which characterize these reactions of proteins.

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WOODS HOLE, MASS.

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Fluorine-containing Pyrimidines and Purines: Synthesis and Properties of Trifluoromethyl Pyrimidines and Purines[1]

BY ALFREDO GINER-SOROLLA[2,3] AND AARON BENDICH

RECEIVED DECEMBER 7, 1957

Syntheses of the following purines are described: 6- and 8-trifluoromethyl, 2-amino-6-trifluoromethyl, 2,6-diamino-8-trifluoromethyl and 2-amino-6,8-bis-trifluoromethyl. The syntheses of the 2- and 8-trifluoromethyl derivatives both of hypoxanthine and adenine also are described. A number of 6-trifluoromethylpyrimidines were prepared, some of which served as intermediates for the above purines. They include the uracil, 2-thiouracil, isocytosine and some 5-phenylazo derivatives. The 5-phenylazo compounds were converted to the amines by catalytic hydrogenolysis. The dissociation and ultraviolet absorption behavior of many of these compounds were studied and it was found that the trifluoromethyl group exerted a powerful inductive influence which was manifested by base-weakening and acid-strengthening effects on the parent pyrimidines and purines. The electron-withdrawing effect of the trifluoromethyl group was found to be quantitatively greater than the electron-donating effect of the amino group in certain of the purines. Evidence is presented that it probably is the pyrimidine moiety of the purine molecule which accepts the proton in acid solutions.

In 1939, May and Litzka[4] reported that 3-fluorotyrosine inhibited the growth of experimentally induced tumors in mice and rats. Since that time other compounds containing fluorine atoms in place of hydrogen have also shown striking biological effects. A few examples are the extremely toxic fluoroacetate,[5] the enhanced hormonal action of 9-α-fluorohydrocortisone[6] and the anti-microbial activity of 3-fluorophenylalanine.[7] Because of the special interest in the application of purines (such as 6-mercapto-[8] and 6-chloropurine[9]) to the control of neoplastic disease (see ref. 10), a program of synthesis of fluorine-containing purines (and pyrimidines) was begun in 1955 in the hope that other agents could be developed which might be useful in human cancer chemotherapy. A preliminary account of a portion of this work has appeared[11] and this paper presents the synthesis and properties of such compounds. Recently, several tumor-inhibiting fluorine-containing pyrimidines[12,13] and purines[14] have been prepared.

Synthetic Studies.—Because of the anti-tumor activity of purine and 6-chloropurine,[9] it was considered of importance to prepare 6-fluoropurine. Of especial interest was the failure[11] to synthesize this compound by application of the Schiemann reaction to adenine (6-aminopurine) despite the ready conversion of α- and β-aminopyridine (but not the γ-isomer) to the corresponding fluoropyridines by this method.[15]

The conversion of 6-hydrazinopurine[8] to 6-chloropurine upon reaction with ferric chloride in dilute hydrochloric acid[16] prompted the application of this diazotization-type reaction[17] to 6-hydrazinopurine in the presence of ferric fluoride and hydrofluoric or fluoroboric acid, but it was without success. Direct replacement of the halogen in 6-chloro- and 6-iodopurine[18] by means of a variety of fluorides was also unsuccessful. Accordingly, the synthesis of other analogs was undertaken.

6-Methylpurine[19] has been found to be highly toxic to mammals[20] and mouse and human tumor

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(2) Fellow of the Institute of International Education (1954–1956) and the Damon Runyon Memorial Fund (1956–1957).

(3) From the thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Sloan-Kettering Division of Cornell University Medical College.

(4) H. May and G. Litzka, *Z. Krebsforsch.*, **48**, 376 (1939).

(5) M. B. Chenoweth, *Pharmacol. Revs.*, **1**, 383 (1949).

(6) J. Fried and E. F. Sabo, THIS JOURNAL, **76**, 1455 (1954).

(7) H. K. Mitchell and C. Niemann, *ibid.*, **69**, 1232 (1947).

(8) G. B. Elion, E. Burgi and G. H. Hitchings, *ibid.*, **74**, 411 (1952).

(9) A. Bendich, P. J. Russell, Jr., and J. J. Fox, *ibid.*, **76**, 6073 (1954).

(10) G. H. Hitchings and C. P. Rhoads, *Ann. N. Y. Acad. Sci.*, **60**, art. 2, 185 (1954).

(11) A. Bendich, A. Giner-Sorolla and J. J. Fox in "The Chemistry and Biology of Purines," Wolstenholme and O'Connor editors, J. and A. Churchill, London, 1957, p. 3; a Ciba Foundation Symposium, held in London, May, 1956.

(12) C. Heidelberger, N. K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R. J. Schnitzer, E. Pleven and J. Scheiner, *Nature*, **179**, 663 (1957).

(13) R. Duschinsky, E. Pleven and C. Heidelberger, THIS JOURNAL, **79**, 4559 (1957).

(14) J. A. Montgomery and K. Hewson, *ibid.*, **79**, 4559 (1957).

(15) A. Roe and G. F. Hawkins, *ibid.*, **69**, 2443 (1947).

(16) A. Giner-Sorolla and A. Bendich, *ibid.*, **80**, 3932 (1958).

(17) O. A. Seide, S. M. Scherlin and G. V. Bras, *J. prakt. Chem.*, **138**, 55 (1933).

(18) G. B. Elion and G. H. Hitchings, THIS JOURNAL, **78**, 3510 (1956).

(19) S. Gabriel and J. Colman, *Ber.*, **34**, 1234 (1901).

(20) F. S. Philips, S. S. Sternberg, L. Hamilton and D. A. Clarke, *Ann. N. Y. Acad. Sci.*, **60**, art. 2 (1954).