[CONTRIBUTION FROM THE NORTHERN UTILIZATION AND DEVELOPMENT DIVISION[1]]

Behavior of the 11S Protein of Soybeans in Acid Solutions. I. Effects of *p*H, Ionic Strength and Time on Ultracentrifugal and Optical Rotatory Properties[2]

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Ultracentrifugal and optical rotatory measurements were made to determine the effects of *p*H, ionic strength and time on the behavior of the 11S protein of soybeans in acid solutions. Low *p*H and low ionic strength convert the 11S protein into a slowly sedimenting component apparently as the result of dissociation of the protein into subunits. An intermediate dissociation product is observed under certain conditions. Effects of *p*H and ionic strength on the 11S protein suggest that dissociation is due to forces of electrostatic repulsion between the subunits. Changes noted with time in some systems were dissociation, aggregation without precipitation or aggregation with precipitation. Dissociation is accompanied by an increase in levorotation, indicating that configurational changes of the subunits take place. These configurational changes may be responsible for irreversibility of dissociation and for aggregation reactions noted under certain conditions of ionic strength and *p*H.

Ultracentrifugal investigations of water- and salt-extractable soybean proteins in alkaline buffers have been reported by Naismith,[3] Wolf and Briggs[4] and Wolf.[5] Four resolvable fractions having approximate $s_{20,w}$ values of 2, 7, 11 and 15S were found, plus unresolvable material having an $s_{20,w}$ value greater than 15S.[4] The unresolvable material consists partly of disulfide polymers of the 7S and 11S proteins.[5]

The major portion of the proteins in an aqueous extract of soybean meal can be precipitated by adding acid to a *p*H of 4–5. Ultracentrifugal examination of the acid-precipitated protein mixture, dissolved in alkaline buffers at 0.5 ionic strength, shows that it contains the four resolvable fractions observed in unfractionated aqueous extracts[3,5] but that the relative amounts of the different fractions are changed because of incomplete precipitation of the 2S and 7S fractions.[5] Rackis, *et al.*,[6] recently examined the ultracentrifugal behavior of phytate-free acid-precipitated protein in the *p*H range of 2.0–3.8. The behavior of this protein system was found to be very complex. The number of sedimenting fractions and their relative concentrations were dependent upon *p*H and ionic strength, indicating that association–dissociation reactions were occurring.

The present paper describes the effects of *p*H, ionic strength and time on the ultracentrifugal and optical rotatory properties of acid solutions of the 11S protein, which is the major component of the acid-precipitated protein. Previous results suggesting that the 11S protein dissociates in acid solution[7] have been confirmed, and some clarification of the behavior of the acid-precipitated protein in acid solution[8] has been obtained.

Experimental

Preparation of 11S Protein.—The 11S protein was prepared by cooling an aqueous extract of defatted soybean flakes (Adams variety, 1955 crop used earlier)[6] as described elsewhere.[8] Pre-existing disulfide polymers[8] of the 11S protein were eliminated by reduction with mercaptoethanol, and repolymerization was prevented by treating the reduced protein with N-ethyl maleimide in the following manner:

One g. of freeze-dried 11S protein (17.33% N and 0.08% P) was dissolved in 100 ml. of phosphate–sodium chloride buffer (*p*H 7.6, 0.5 ionic strength) containing 0.01 *M* mercaptoethanol. The resulting turbid solution was centrifuged for 60 minutes in a Spinco[9] preparative ultracentrifuge at 40,000 r.p.m. (90 minutes at 21,000 r.p.m. appeared to be equally effective). To the clear supernatant solution was added 138 mg. of N-ethyl maleimide (10% excess) to react with sulfhydryl groups of the protein and the excess mercaptoethanol. When the reaction was complete (negative nitroprusside test), the solution was adjusted to *p*H 5.0 with *N* HCl and the protein was precipitated[10] by diluting the solution with nine volumes of distilled water. Buffer salts were removed from the precipitated protein by slurrying with distilled water[12] and by centrifuging several times in a Servall angle centrifuge. After the washed precipitate was freeze-dried a yield of 0.8 g. of 11S protein containing 17.14% N and 0.06 P (both on dry basis) was obtained.

Preparation of Solutions for Ultracentrifugal Analysis.—All the 11S protein solutions were prepared by adding the weighed protein (moisture-free basis) to a known volume of buffer in the ratio of 0.70 g./100 ml. Phosphate–citrate buffers were used,[13] and sodium chloride was added when necessary to adjust the system to a desired ionic strength. Insoluble protein remained in the majority of the buffer systems and was removed in a Servall angle centrifuge. The amount of soluble protein was determined by semi-micro Kjeldahl analysis of the clarified solutions. The difference between the amount of protein added to the buffer solution and the amount that dissolved was used as a measure of the amount of insoluble protein. The protein solutions were analyzed in the ultracentrifuge and polarimeter at 2, 26 and 261 hours after preparation. Sedimentation analyses were performed so that optimal resolution of the ultracentrifuge pattern occurred at the times indicated. Between measurements all of the solutions (except at 1.0

(1) One of the Divisions of the Agricultural Research Service, U. S. Department of Agriculture.

(2) Presented before the Division of Biological Chemistry, 133rd Meeting, American Chemical Society, San Francisco, California, April 13–18, 1958.

(3) W. E. F. Naismith, *Biochim. et Biophys. Acta*, **16**, 203 (1955).

(4) W. J. Wolf and D. R. Briggs, *Arch. Biochem. Biophys.*, **63**, 40 (1956).

(5) W. J. Wolf, Ph.D. Dissertation, University of Minnesota, Minneapolis, Minnesota, 1956.

(6) J. J. Rackis, A. K. Smith, G. E. Babcock and H. A. Sasame, THIS JOURNAL, **79**, 4655 (1957).

(7) W. J. Wolf and D. R. Briggs, *Arch. Biochem. Biophys.*, in press.

(8) D. R. Briggs and W. J. Wolf, THIS JOURNAL, **72**, 127 (1957).

(9) Mention of trade or company names does not imply endorsement by the U. S. Department of Agriculture over similar products or firms not mentioned.

(10) If the protein was allowed to stand in the presence of excess N-ethyl maleimide for an hour or longer, a pink color developed. This color also formed in the absence of the protein when a 0.01 *M* solution of mercaptoethanol in buffer (*p*H 7.6, 0.5 ionic strength) was made 0.011 *M* with respect to N-ethyl maleimide. The color reaction may be similar to that reported by Benesch, *et al.*,[11] but contrary to their observations the color is stable in the presence of water.

(11) R. Benesch, R. E. Benesch, M. Gutcho and L. Laufer, *Science*, **123**, 981 (1956).

(12) In early experiments it was found that low yields of the washed protein were obtained. Subsequent work showed that the *p*H of the protein–water suspension gradually decreases with each washing and appreciable solubilization of the protein occurs. The loss of protein can be prevented by maintaining the *p*H of the protein–water suspension at 5.0 with 0.1 *N* sodium hydroxide.

(13) P. J. Elving, J. M. Markowitz and I. Rosenthal, *Anal. Chem.*, **28**, 1179 (1956).

TABLE I

EFFECT OF *p*H, IONIC STRENGTH AND TIME ON OPTICAL ROTATORY AND ULTRACENTRIFUGAL PROPERTIES OF THE 11S PROTEIN OF SOYBEANS

					Ultracentrifuge data[a]					
					Distribution of protein as resolvable components, %				% Protein unresolved[b]	Protein insoluble,
*p*H	Ionic strength	Time, hr.	Protein concn., g./100 ml.	$-[\alpha]^{25}D$	3S	7S	11S	>11S	11S	%
7.6	0.5	3	0.660	46.7	4(~2)	6(~7)	56(12.6)	34	..	5
		261	.660	43.0	...	3(~7)	57(12.5)	30	5	5
3.8	1.0	2	.631	42.8	...	2(~7)	48(12.6)	24	15	11
		26	.631	46.1	...	4(~7)	52(13.1)	28	5	11
		261	.631	44.2	4(~3)	4(7.2)	57(12.5)	25	..	11
3.8	0.2	2	.660	50.4	...	27(8.3)	53(13.3)	10	4	6
		26	.637	50.8	...	26(8.2)	49(12.6)	9	17	9
		261	.560	53.5	5(~3)	23(7.5)	37(13.3)	8	7	20
3.8	.1	2	.637	54.1	2(~3)	29(8.5)	48(13.1)	12	..	9
		26	.637	55.5	2(~3)	33(8.4)	40(12.6)	11	5	9
		261	.637	57.4	1(~3)	31(8.0)	40(13.0)	10	9	9
3.8	.01	2	.672	59.8	14(3.0)	56(7.3)	22(11.4)	..	4	4
		26	.672	62.9	...	...	...	..	..	4
		261	.672	68.9	37(3.2)	46(7.2)	12(11.3)	..	1	4
3.0	1.0	2	.526	45.5	...	27(6.9)	35(12.9)	5	8	25
		26	.479	52.0	...	19(6.8)	38(12.6)	6	5	32
		261	.350	53.8	2(~3)	15(6.8)	22(12.8)	..	11	50
3.0	0.2	2	.694	67.0	9(~3)	69(8.3)	13(~14)	..	8	1
		26	.694	70.4	9(~3)	35(8.4)	6	40	9	1
		261	.694	72.1	16(~3)	29(8.3)	...	55	..	1
3.0	.1	2	.678	65.9	75(3.8)	~18(~8)	...	..	3	4
		26	.678	67.4	66(3.9)	...	27[c](~11)	..	4	4
		261	.678	71.3	54(4.1)	...	37[c](10.9)	..	6	4
3.0	.01	2	.700	64.2	27(3.4)	58(7.6)	11(12.0)	..	4	$\ll 1$
		26	.700	67.8	61(2.7)	32(8.5)	...	..	7	$\ll 1$
		261	.700	71.4	70(3.0)	27(7.2)	...	..	3	$\ll 1$
2.2	1.0	2	.364[d]	...	7(0–4)	45(7.9)	...	..	..	48
		26	.140	59.4	...	15(7.5)	...	..	5	80
		261	.053	...	...	...	...	..	..	93
2.2	0.2	2	.684	74.7	54(~5)	...	33[c](~11)	..	11	2
		26	.684	74.7	25(4.9)	...	43[c](13.9)	7	23	2
		261	.684	74.2	15(5.5)	...	48[c](14.9)	15	20	2
2.2	.1	2	.695	77.7	80(3.4)	~12(~8)	...	..	7	1
		26	.695	76.4	66(3.7)	...	33[c](~11)	..	..	1
		261	.695	79.5	54(3.8)	...	44[c](12.4)	..	1	1
2.2	.01	2	.701	80.9	96(2.3)	...	...	..	4	$\ll 1$
		26	.701	80.4	96(2.3)	...	...	..	4	$\ll 1$
		261	.701	82.4	100(2.2)	...	...	..	0	$\ll 1$

[a] $s_{20,w}$ values are given in parentheses. [b] Determined as 100% − (% of protein as resolvable components + % of protein insoluble). [c] Very broad 3S and 11S peaks were observed which overlapped in the 7S region. No resolvable 7S peak was observed. [d] Calculated from the area of the ultracentrifugal pattern. Continuous precipitation of protein with time prevented accurate determination of soluble protein by Kjeldahl analysis and precluded optical rotation measurements.

ionic strength) were stored in the refrigerator. The solutions were allowed to stand at room temperature 1–2 hours prior to re-analysis. Large amounts of protein precipitated irreversibly in the cold from solutions at an ionic strength of 1.0; hence, they were kept at room temperature using toluene as a preservative.

Ultracentrifugal Analysis.—Sedimentation analyses were performed at room temperature with a Spinco, Model E, ultracentrifuge at 35,600 or 47,660 r.p.m. Constant temperature was maintained during ultracentrifugation with a rotor temperature indicating and control unit designed by Spinco Division of Beckman Instruments, Inc. A 30-mm. cell equipped with a plastic double-sector centerpiece was used. The solution was placed in one sector, the solvent was in the other, thereby enabling the schlieren patterns for each to be recorded simultaneously on a single photograph. Sedimentation constants were corrected to the standard state of water at 20° in the usual manner,[14] using 0.719 for the partial specific volume of the protein.[5]

Ultracentrifuge data presented in Table I are apparent compositions because no correction has been made for the Johnston–Ogston effect.[15] The area of each sedimenting peak (corrected for radial dilution) is expressed as the percentage of the total area corresponding to a protein concentration of 0.70 g./100 ml. at zero time of sedimentation. The total area was calculated, as indicated earlier,[4] by assuming the specific refractive index increment to be 0.00185, which is the value obtained at 546 mμ, at *p*H 7.6 and 0.5 ionic strength.[5]

(14) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, New York, N. Y., 1940.

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

Optical Rotation.—Optical rotations were measured in a Bates–Fric saccharimeter at 25°, using a sodium vapor lamp as the light source.

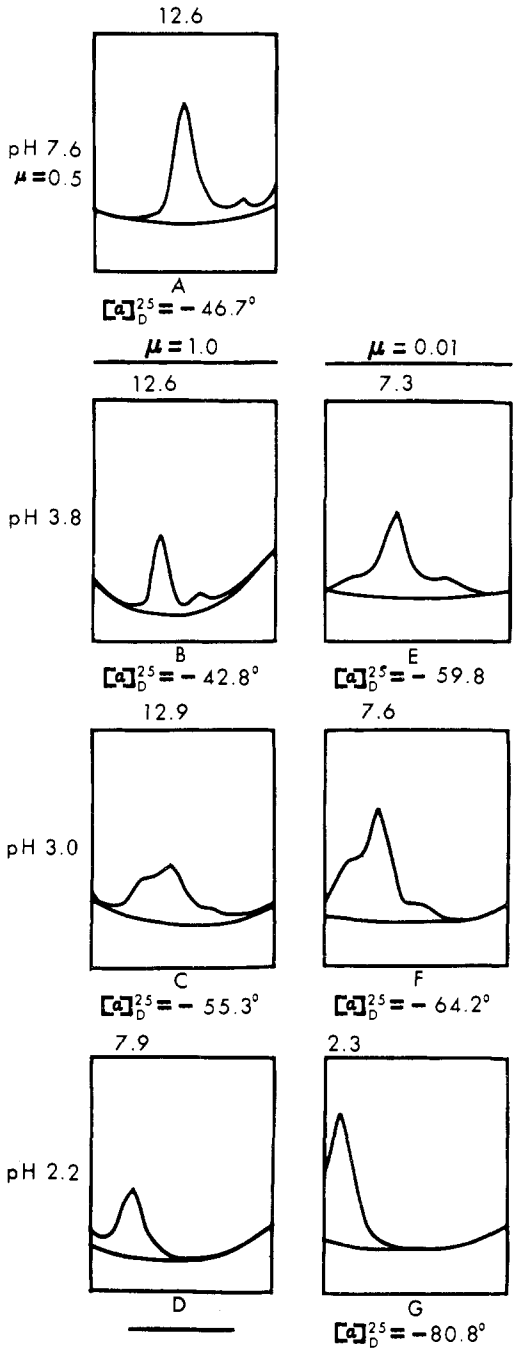

Fig. 1.—Effect of *p*H and ionic strength on the ultracentrifugal pattern and specific rotation of solutions of 11S protein in phosphate–sodium chloride (*p*H 7.6) and phosphate–citrate buffers 2 hours after preparation. Ultracentrifuge patterns were photographed after 93 minutes of centrifugation at 35,600 r.p.m. at a bar angle of 65° with the following exceptions: (b) 45 minutes at 47,660 r.p.m., 70°; (e) 70°; (f) 60°. Direction of sedimentation is from left to right. Area analyses and $s_{20,w}$ values are given in Table I. The number above the major peak of each pattern is the $s_{20,w}$ value in Svedberg units. The solution corresponding to (d) was too turbid for optical rotation measurements.

Results

The 11S protein used in the present study was the cold-insoluble fraction of soybean protein treated with mercaptoethanol and N-ethyl maleimide.[8] This protein gave an ultracentrifuge pattern at *p*H 7.6, 0.5 ionic strength (Fig. 1a), which was similar to that obtained with earlier preparations from another variety of soybeans.[5,7,8] However, in the present preparation the sedimentation constant of the 11S component was about 10% higher[16] and area measurements (Table I) showed that the relative amount of faster sedimenting material was greater. The faster sedimenting material consisted of at least three components having $s_{20,w}$ values of approximately 17S, 20S and 25S. The faster sedimenting components were not affected by 0.01 *M* mercaptoethanol, thus indicating that the preparative reactions involving depolymerization and blocking of the sulfhydryl groups[8] were complete. The 11S component and the faster components are stable in solution at *p*H 7.6, 0.5 ionic strength; no significant change occurred in the ultracentrifuge pattern when a solution of the protein stood at 0–4° for 261 hours (Table I).

Optical rotation measurements indicate that at *p*H 7.6, 0.5 ionic strength, the 11S protein is a globular protein in its native state. Eight different determinations involving a range in protein concentration of 0.7–2.7% and a variation in the age of the solutions of 1–261 hours gave an average value of $[\alpha]^{25}D$ −45.2 ±1.3°. This value is approximately in the middle of the range of the specific rotations found for native globular proteins.[17,18]

Effect of *p*H and Ionic Strength.—The ultracentrifugal properties of the 11S protein were studied in phosphate–citrate buffers in the *p*H range of 2.2–3.8 and ionic strength range of 0.01–1.0. The data in Table I show that the behavior of the 11S protein in acid buffers is a function of *p*H and ionic strength; in certain cases it is also dependent on time. The dependence of the number of sedimenting species on *p*H and ionic strength is illustrated in Fig. 1 which shows the ultracentrifugal patterns for solutions 2 hours after preparation. At *p*H 3.8, 1.0 ionic strength (Fig. 1b), the protein sediments essentially as the native 11S protein because the sedimentation pattern is very similar to that observed at *p*H 7.6, 0.5 ionic strength (Fig. 1a). However, at *p*H 3.8, 0.01 ionic strength (Fig. 1e), two additional components are observed having approximate $s_{20,w}$ values of 3S and 7S[19] and

(16) The authors are indebted to Dr. D. R. Briggs of the University of Minnesota for checking and confirming this observation in an independent experiment. No explanation can be given at present for the difference in sedimentation constants for the two preparations of 11S protein. However, the difference does not appear to be due to treatment of the protein with N-ethyl maleimide since untreated protein also sedimented faster.

(17) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).

(18) A solution of the cold-insoluble fraction of soybean protein (*i.e.*, the 11S protein without treatment with mercaptoethanol and N-ethyl maleimide) had a specific rotation of −47.2° at *p*H 7.6, 0.5 ionic strength. Addition of mercaptoethanol to a concentration of 0.01 *M* had no effect on the specific rotation. It therefore appears that the preparative steps, involving treatment with mercaptoethanol and N-ethyl maleimide, did not cause any large changes in configuration of the 11S protein molecule.

(19) The two slower sedimenting components are henceforth referred to as the 3S and $\overline{7S}$ components, using the designations adopted

there is a corresponding decrease in the areas of the 11S and >11S peaks.

As the *p*H is lowered from 3.8 to 2.2, at an ionic strength of 1.0 (Fig. 1b-1d) there is a gradual conversion of the 11S protein into the $\overline{7S}$ component plus an increase in the amount of insoluble protein (Table I). The same *p*H change at 0.01 ionic strength (Fig. 1e–1g) causes a complete conversion of the 11S and $\overline{7S}$ components into the 3S component. All of the minor components observed at *p*H 7.6, 0.5 ionic strength, and *p*H 3.8, 1.0 ionic strength, are also converted into the slowly sedimenting material at *p*H 2.2, 0.01 ionic strength. Ultracentrifugation for 210 minutes (47,660 r.p.m.) at *p*H 2.2, 0.01 ionic strength, failed to cause resolution into more than one sedimenting peak. Table I shows that varying degrees of conversion of the 11S protein into the 3S and $\overline{7S}$ components occur at ionic strengths of 0.1 and 0.2 in the *p*H range of 2.2–3.8.

Conversion of the 11S protein into slower sedimenting species indicates that a change in shape and/or size of the molecule has occurred. However, the magnitude of the change in sedimentation constant from 11S → $\overline{7S}$ → 3S suggests that a change in size has taken place, *i.e.*, dissociation into subunits. Similar changes in sedimentation properties of arachin, the peanut globulin which is like the 11S protein of soybeans in many respects, have been shown to be caused by dissociation into subunits.[20] Our results are therefore interpreted as indicating that the 11S protein dissociates into subunits under conditions of low *p*H and low ionic strength.

Optical Rotation Studies.—Examination of Table I shows that in all cases where dissociation occurred there is an increase in levorotation as compared to the specific rotation of the protein at *p*H 3.8, 1.0 ionic strength where no dissociation took place. Furthermore, Fig. 1 shows that the increase in levorotation parallels the degree of dissociation of the 11S protein. At *p*H 3.8, 1.0 ionic strength, the specific rotation has a value of −43° (2-hour reading), whereas at *p*H 2.2, 0.01 ionic strength, where the protein is completely dissociated into the 3S state, the specific rotation is −81°. Intermediate values of $[\alpha]^{25}D$ are obtained at conditions between *p*H 3.8, 1.0 ionic strength, and *p*H 2.2, 0.01 ionic strength. A correlation of increase in levorotation with extent of dissociation is also observed at *p*H 3.0, 0.01 ionic strength when the solution is studied as a function of time (Fig. 2a). Interestingly the specific rotation at *p*H 3.8, 1.0 ionic strength (−43°), is the same as the average value (−45°) obtained for the 11S protein at *p*H 7.6, 0.5 ionic strength, where the protein is undissociated and appears to be in an undenatured state.

earlier.[7] The symbol $\overline{7S}$ is used to designate the largest subunit formed on dissociation of the 11S protein in order to differentiate it from the apparently different 7S component observed in the salt- and water-extractable soybean protein mixture at *p*H 7.6, 0.5 ionic strength.[3,4] In the area analyses given in Table I, no correction has been made in the 3S and $\overline{7S}$ areas for the small amounts of 2S and 7S material originally present in the 11S protein preparation at *p*H 7.6, 0.5 ionic strength.

(20) C. W. Cater, W. E. F. Naismith, R. H. K. Thomson and G. R. Ure, *Nature*, **180**, 971 (1957).

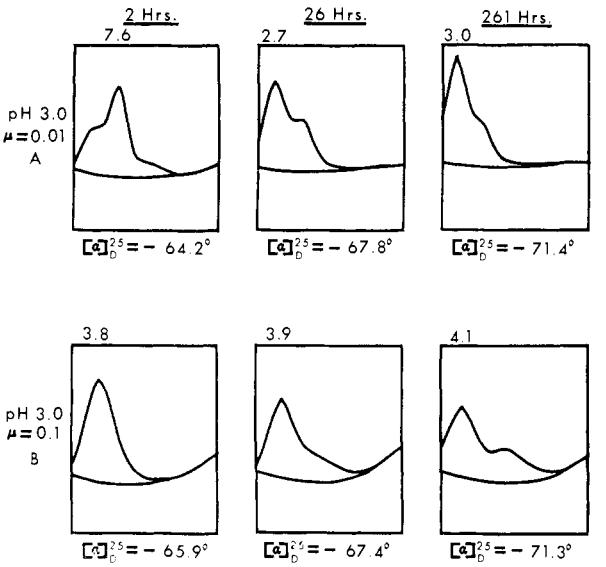

Fig. 2.—Effects of time on the ultracentrifugal pattern and specific rotation of acid solutions of the 11S protein: (a) dissociation, and (b) aggregation without precipitation. Ultracentrifuge patterns were photographed after 93 minutes of centrifugation at 35,600 r.p.m. at 60°.

Effect of Time.—Preliminary experiments indicated that the ultracentrifugal properties of the 11S protein in acid solutions were dependent upon time.[21] The effect of time on the ultracentrifugal pattern and optical rotation was therefore followed for all conditions of *p*H and ionic strength studied (Table I). No appreciable changes with time were noted at the extreme ends of the range of conditions selected, *i.e.*, *p*H 3.8, 1.0 ionic strength, and *p*H 2.2, 0.01 ionic strength, the conditions under which the 11S protein is undissociated and completely dissociated, respectively. However, at intermediate conditions one of the following changes was noted: (1) dissociation, (2) aggregation without precipitation of protein and (3) aggregation with precipitation of protein.

Dissociation with time was noted at 0.01 ionic strength at *p*H 3.0 and 3.8. In both cases the freshly prepared (2-hour solutions analyses) contained 3S, $\overline{7S}$ and 11S components with the $\overline{7S}$ component present to the largest extent. Dissociation of the 11S and $\overline{7S}$ proteins into the 3S state was indicated when the solutions were reanalyzed after 26 and 261 hours. In neither case did the reaction go to completion in 11 days, but the extent of dissociation was greater at *p*H 3.0 than at *p*H 3.8. The changes observed in the ultracentrifuge pattern with time at *p*H 3.0, 0.01 ionic strength are shown in Fig. 2a.

Aggregation of protein without precipitation was noted primarily at *p*H 2.2 and 3.0 at ionic strengths of 0.1 and 0.2. At *p*H 2.2 and 3.0, at 0.1 ionic strength, the freshly dissolved protein is largely in the 3S state; but with time, there is a slow aggregation reaction resulting in the formation of a faster sedimenting peak having an $s_{20,w}$ value of approximately 11S (Fig. 2b). At 0.2 ionic strength, *p*H 2.2 and 3.0, initial dissociation

(21) W. J. Wolf and D. R. Briggs, unpublished experiments.

into the 3S component was less complete but aggregation into a larger particle size was observed. Table I shows that at 0.2 ionic strength after 261 hours 35% (*p*H 2.2) and 55% (*p*H 3.0) of the protein sedimented faster than the 11S protein.

Several of the systems showed aggregation with precipitation. Increases in the amount of insoluble protein with increased time of standing are noted under the last column of Table I. Most extensive precipitation with time occurred at *p*H 2.2, 1.0 ionic strength; only about 7% of the total protein remained soluble after 11 days.

Discussion

Results obtained in this study show that *p*H and ionic strength are important variables in determining the state of the 11S protein in acid solution. By proper selection of these variables it is possible (1) to prevent dissociation, (2) to cause partial dissociation or (3) to effect complete dissociation into subunits. Dissociation of 11S protein at *p*H 3.0 and 0.1 ionic strength[7] is confirmed in the present work, but the dissociation process appears to be different in phosphate–citrate buffer than in sodium chloride solution.[7] After 14 hours in 0.1 *M* sodium chloride at *p*H 3.0, the protein solution contains 3S, $\overline{7S}$ and 11S components,[7] whereas in phosphate–citrate buffer at the same *p*H and ionic strength, the 11S protein is initially completely dissociated and aggregates to an 11S component upon standing (Fig. 2b). This difference indicates that the state of the 11S protein in acid solutions may also be dependent on the ionic composition of the solvent used.

Also, present results are in general agreement with previous work[7] regarding the dissociation of the 11S protein in alkaline solutions. In both studies the dissociation into a 3S subunit appears to proceed *via* a $\overline{7S}$ intermediate. Effect of time on the protein at *p*H 3.0, 0.01 ionic strength (Fig. 2a) is evidence for the $\overline{7S}$ state as an intermediate in acid dissociation to the 3S state. Effects of *p*H and ionic strength on the 11S protein indicate that acid dissociation is caused by electrostatic repulsion forces between the positively charged subunits.[22] The enhancement of dissociation noted with a decrease in *p*H may be due to an increase in electrostatic repulsion forces resulting from an increase in the number of positive charges on the subunit molecules. The suppression of dissociation at high ionic strengths may be ascribed to a shielding of the electrostatic charges with the result that the repulsion forces are no longer able to counteract the attractive forces responsible for holding the subunits together. The attractive forces may involve weak secondary forces such as hydrogen bonds and/or van der Waals type of interactions.

The increase in negative rotation observed on dissociation indicates that configurational changes of the subunits occur during the dissociation process.[17] These changes may be responsible for the irreversibility of acid dissociation reported earlier[7] where solubility and ultracentrifugal behavior at *p*H 7.6, 0.5 ionic strength, were used as criteria of reversibility. Configurational changes may also account for the aggregation of protein noted in the present study. For example, at *p*H 3.0, 0.1 ionic strength, an aggregate is gradually formed with an $s_{20,w}$ value similar to that of the 11S protein (Fig. 2b). However, this reaction does not appear to be a simple re-association of protein subunits into the native 11S state for the following reasons: (1) the freshly prepared solution does not contain any 11S protein, indicating that the native form is unstable under these conditions; (2) the reaction does not involve the formation of a resolvable $\overline{7S}$ peak as noted on dissociation (Fig. 2a) but, rather, a heterogeneous material with $s_{20,w}$ values distributed between 3S and 11S is observed and (3) the levorotation of the solution increases slightly during the reaction (Fig. 2b), but a decrease would be expected on the basis of the specific rotation changes noted during dissociation (Figs. 1 and 2a). The possibility also exists that the aggregate is formed by association of the subunits of 17S, 20S and 25S components which are present as contaminants in the 11S protein preparation and are dissociated in the freshly prepared solution. However, the appearance of the 11S aggregate indicates that regardless of their origin the protein subunits retain to a considerable degree the specificity of interaction characteristic of the subunits in the native proteins. The formation of aggregates larger than the 11S-25S proteins under certain conditions suggests a greater loss of specificity plus the possibility of intermolecular reaction of groups which normally form intramolecular bonds in the native state of the subunits.

From this study the indication is that effects of *p*H and ionic strength on acid-precipitated soybean protein in acid solutions[6] are, at least partly, the result of dissociation of the 11S protein into subunits. The two components observed in a solution of the acid-precipitated protein at *p*H 2.0 and 3.0, 0.06 ionic strength, appear to represent mixtures of the 2S and 7S proteins observed in the acid precipitate at *p*H 7.6, 0.5 ionic strength,[3,5] plus the 3S protein formed by dissociation of the 11S protein. Analysis of a solution of the 11S protein dissolved in glycine–sodium chloride buffer, *p*H 3.8, 0.058 ionic strength, for 26 hr., indicated the following composition: 7% (3S); 46% ($\overline{7S}$); 25% (11S); 12% (>11S) and 10% insoluble. Under these same conditions a solution of the acid-precipitated protein would therefore be expected to consist of a mixture of 2S, 3S, 7S, $\overline{7S}$ and undissociated 11S protein.

Table I shows that considerable variation occurs in the $s_{20,w}$ values for the 11S protein and its dissociation products with variation in conditions. These changes are probably due to charge effects (at 0.01 ionic strength), changes in protein concentration of the various components and configurational changes. The soybeans used in this work were of the same lot as that used earlier,[6] therefore the $s_{20,w}$ values reported here for the 11S protein in alkaline and acid solutions indicate that the 13S component of Rackis, *et al.*,[6] is identical with the

(22) Solubility measurements indicate that the isoelectric point of the 11S protein is approximately *p*H 5[5].

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

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[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]

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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

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

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(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.