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Heat Denaturation of Soybean 11S Protein[1]

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ABSTRACT

Factors influencing heat denaturation of soybean 11S protein were studied. Effects of time of heating at 100°C. in pH 7.6 solution were determined at different ionic strengths with varying levels of mercaptoethanol. Quantity of precipitate formed and composition of the fraction remaining soluble were determined by ultracentrifugal analysis. Heating at 0.5 ionic strength rapidly converted the 11S protein into a fast sedimenting aggregate and a slow sedimenting fraction of 4S. On continued heating, the soluble aggregate increased in size and precipitated. Heating the 11S protein in 0.01 and 0.5M mercaptoethanol accelerated the precipitation reaction. Lowering the ionic strength from 0.5 to 0.1 and to 0.01 in 0.01M mercaptoethanol accelerated disappearance of the 11S protein and formation of the precipitate. When the 11S protein was heated in 0.5 ionic strength buffer containing 0.01M N-ethylmaleimide, a 3S fraction and a soluble aggregate formed. The aggregate did not precipitate on continued heating. Heating apparently disrupts the quaternary structure of the 11S protein with subsequent separation of the subunits into two distinct fractions. One fraction consists of soluble subunits of 3-4S, which are stable to heating for 30 min. or more. The other fraction of subunits has a pronounced tendency to interact to form a soluble aggregate which, in turn, rapidly converts to an insoluble state on continued heating when sulfhydryl groups are present.

Although the need for moist heat to gain optimal nutritional value of soybean meal has been recognized for more than 50 years (1), little is known about the physical and chemical changes that occur in the proteins as a result of such treatment. One of the most obvious changes resulting from heating soybean meal is insolubilization of major proteins in aqueous solvents (2,3). Changes in solubility of proteins are a measure of the extent of denaturation but give no information about the protein components involved or the nature of the changes in proteins at the molecular level. Mann and Briggs (4) sought such information by heating solutions of soybean proteins and analyzing them electrophoretically. Their results indicated that nonglobulin proteins were most sensitive to heating in aqueous solutions. When heated for 2 hr. at 75°C. these proteins were converted from components with different electrophoretic mobilities into an aggregate migrating as a single electro-

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phoretic peak. When heated longer, the cold-precipitable protein, since shown to be primarily the 11S ultracentrifugal component (5), also appeared to be incorporated into the aggregate formed by nonglobulins.

Watanabe and Nakayama (6) confirmed the formation of soluble aggregates during heating of water extracts of defatted meal. Their ultracentrifugal analyses also indicated that the 11S and 15S fractions, and a portion of the 7S fraction, disappeared after 10 min. at 80°C. or higher temperatures.

Circle et al. (7) showed that heating solutions of commercial preparations of soybean globulins at high concentrations caused gelation. Since cysteine and sodium sulfite inhibited gelation, disulfide crosslinkages were possibly formed during heating and contributed to stabilization of the gel structure.

Because of the complexity of soybean proteins it is difficult to interpret studies on the unfractionated protein mixture; more meaningful conclusions should result from studies of purified components.

Preliminary studies[3] on solutions of crude 11S component indicated that heating at 100°C. caused two changes: about one-half of the protein aggregated and precipitated while the remaining protein sedimented much more slowly than the 11S component. In the presence of a reducing agent the precipitation reaction was accelerated. When sulfhydryl groups were blocked with N-ethylmaleimide, precipitation did not occur, but instead an aggregate of about 67S was formed. We have now re-examined these preliminary observations and studied them in more detail. Here we describe the effects of time of heating, ionic strength, and concentration of mercaptoethanol on solubility and ultracentrifugal behavior of the 11S component. The effect of heating the 11S component in the presence of N-ethylmaleimide was also studied.

MATERIALS AND METHODS

Buffers

Buffers of pH 7.6 with the following molar compositions were used:

Buffer	Ionic Strength	KH_2PO_4	K_2HPO_4	NaCl	Mercaptoethanol
A	0.5	0.0026	0.0325	0.4	0
B[4]	0.5	0.0026	0.0325	0.4	0.01
C	0.5	0.0026	0.0325	0.4	0.5
D	0.1	0.0062	0.0316	0	0.01
E	0.01	0.0009	0.0031	0	0.01

Preparation of 11S Protein

Cold-insoluble fraction prepared earlier (9) served as the source for crude 11S protein. The 11S protein was purified by fractional precipitation at pH 4.6 (9) except that the ratio of cold-insoluble fraction to pH 4.6, 0.5 ionic strength buffer was decreased from 10 mg. per ml. to 4.3 mg. per ml. This change increased the yield of 11S protein. Ultracentrifugal compositions in buffer B of the starting material and of the two preparations of 11S protein obtained were as follows:

[3] W.J. Wolf. Physical and chemical studies on soybean proteins. Ph. D. Dissertation. University of Minnesota. (1956).

Preparation	2S %	7S %	11S %	15S %	Yield %
Cold-insoluble fraction	7	10	65	18	100
I	...	4	89	7	27
II	...	2	91	7	34

Yield is expressed as percentage of the recovered cold-insoluble fraction (uncorrected for moisture in starting material). Further purification of either hydroxylapatite chromatography or gel filtration was omitted, because these procedures gave low yields (9) and because large quantities of protein were needed for our studies. The purified 11S protein was stored at 0° to 4°C. as a 2 to 3% stock solution in buffer B. Such stock solutions of 11S protein can be kept for 6 to 12 months without significant changes if the solutions are dialyzed against buffer B with buffer changes every 1 to 2 weeks. Maintaining the concentration of mercaptoethanol at 0.01M apparently prevents bacterial growth.

Heating Experiments

Stock solution of 11S protein in buffer B was transferred to the desired solvent by dialysis for 48 hr. or longer at 0° to 4°C. The protein solution was then diluted to 5 mg. per ml., as determined spectrophotometrically at 280 mμ (8).

In experiments with N-ethylmaleimide, a portion of stock protein solution was made 0.011M with N-ethylmaleimide by adding the solid reagent. After being stirred gently until the reagent dissolved, the solution was immediately dialyzed against buffer A (without mercaptoethanol) to remove the reaction product between N-ethylmaleimide and mercaptoethanol. After dialysis, the protein solution was diluted to 0.5%, and N-ethylmaleimide was added to make the solution 0.01M before heating studies were conducted. This procedure prevents formation of disulfide polymers, which occur when mercaptoethanol is removed by dialysis against buffer A.

Heating studies were carried out in a boiling water bath with 2-ml. portions of 0.5% protein solution in 18 × 150 mm. test tubes which were loosely stoppered with glass marbles to reduce evaporation of water; control experiments with buffer A indicated a water loss of 0.2% per min. of heating. Corrections for changes in protein concentration caused by evaporation were therefore applied to all samples heated for 10 min. or longer. After initial immersion in the boiling water bath, the tubes were agitated for 1 min. to hasten temperature equilibration. At the end of the desired heating time, the test tubes were removed and quickly cooled in an ice bath to stop the denaturation reaction as rapidly as possible.

The cooled tubes were stoppered, inverted several times to ensure remixing of condensed water with the solutions, and centrifuged for 10 min. at 12,000 r.p.m. (17,300 × g) if a precipitate was present. The resulting solutions were then analyzed in the ultracentrifuge and by the Kjeldahl method.

Ultracentrifugal Analyses

Protein solutions were analyzed at 47,660 r.p.m. and 22°-27°C. in the solvents used for the heating experiments. A 30-mm. plastic double-sector cell was used. A portion of the unheated solution in each series of experiments was also analyzed to determine initial protein concentration in area units on the ultracentrifuge patterns.

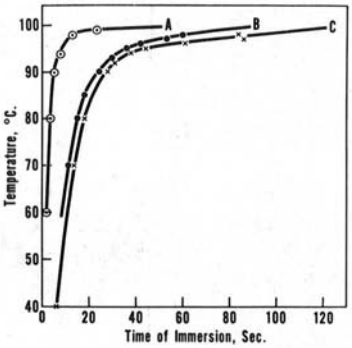

Fig. 1. Reaction temperature of test tube contents as a function of heating time showing time lag in temperature equilibration. Curve A, thermometer immersed in bath at zero time; B, buffer added to pre-equilibrated test tube; and C, buffer and test tube immersed in bath simultaneously.

Changes in protein composition were calculated by expressing the area for each sedimenting fraction as a percentage of the total area for the unheated sample. The amount of protein that precipitated as a result of heating was obtained by subtracting the sum of the areas of the fractions remaining soluble from the total area in the unheated solution. The amount of protein precipitated was also determined by Kjeldahl analysis.

RESULTS

Preliminary Studies

Initial studies showed that denaturation of 11S protein was very rapid at 100°C. The changes observed were usually complete in 5 min. or less. Because of the speed of the reactions involved and because of the time lag in temperature equilibration of the sample, the rate curves, shown later, do not represent rates of denaturation at 100°C., but rather the rates of denaturation as a function of heating from ~25°C. to a final temperature of 100°C. The time lag in attaining the final temperature was minimized by using small samples (2-ml.). Figure 1 indicates the magnitude of the time lag involved; in experiments where denaturation was most rapid, the lag time was a significant portion of the total time involved. A control experiment, in which a test tube containing 2 ml. of buffer was immersed in the water bath, showed that the temperature rose to 90°C. in about 30 sec. followed by a slower increase in temperature to 100°C. in the next 90 sec. Adding the buffer at zero time to a preheated test tube did not eliminate the temperature lag, although the final temperature was reached 20 to 30 sec. sooner (Fig. 1). The simpler procedure of adding the protein to the test tubes initially and then immersing the tubes was therefore adopted. Immersion of thethermometerdirectly into the water bath showed that the results obtained with buffer were not caused by a lag in thermometer response.

The initial condition chosen for study was pH 7.6, 0.5 ionic strength buffer in the absence of mercaptoethanol (buffer A). Ultracentrifugal analysis of the unheated protein indicated that only 67% of the sample was 11S component as compared to a value of 91% for the same sample in buffer B. Decrease in 11S content was accompanied by an increase in amount of faster-sedimenting proteins (Fig. 2) which apparently were disulfide polymers formed during the removal of

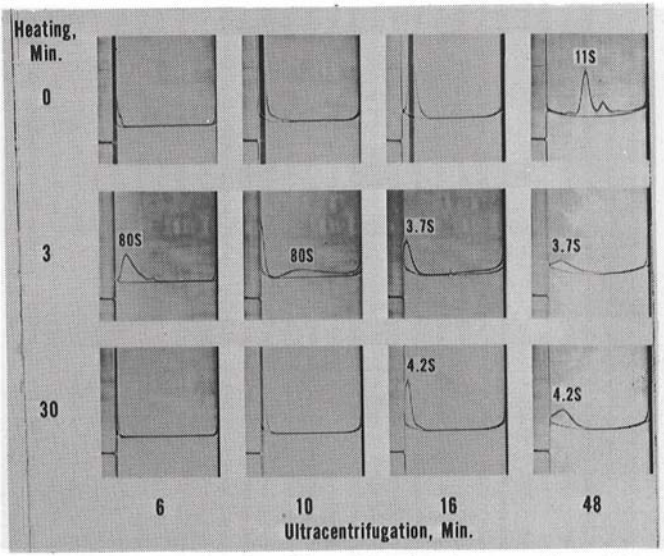

Fig. 2. Ultracentrifuge patterns showing effect of heating time on sedimentation properties of 11S protein in buffer A. Major components are identified by $s_{20,w}$ values. Direction of sedimentation is from left to right.

mercaptoethanol by dialysis. When heated, the solution became opalescent in 30 sec. (~90°C., Fig. 1); on continued heating the solution became turbid and protein began to precipitate.

Ultracentrifugal analysis of samples heated for varying times showed several changes. The 11S component disappeared rapidly while a slowly sedimenting fraction of about 4S and an aggregate of 80-100S (Table I) formed. When the sample was heated longer, the soluble aggregate disappeared and a precipitate formed; only about 40% of the original protein in solution was left as the 4S fraction.

TABLE I. SEDIMENTATION COEFFICIENTS FOR FRACTIONS OBTAINED BY HEATING 11S PROTEIN IN VARIOUS BUFFERS

Buffer	Ionic Strength	Concentration of Mercapto-ethanol	$S_{20,w}$ Values of Fractions: Slow Sedimenting Range[a]	Slow Sedimenting Mean	Soluble Aggregate Range[a]	Soluble Aggregate Mean
			Svedbergs	Svedbergs		
A	0.5	0	3.6-4.2	3.9	79-101	87
B	0.5	0.01	3.3-4.5	3.4	...	...
C	0.5	0.5	3.1-3.6	3.4	...	...
D	0.1	0.01	2.9-3.2	3.0	...	...
E	0.01	0.01	2.1-3.6	2.8	...	...
A plus N-ethyl maleimide			2.4-4.4	3.4	58-67	63

[a]Each range includes values calculated from three or more samples heated for varying times. All samples were at a protein concentration of 0.5% before heating. Variations in sedimentation rates for the slow fraction may reflect changes in protein concentration resulting from precipitation of protein and effects of the wide range of ionic strengths used.

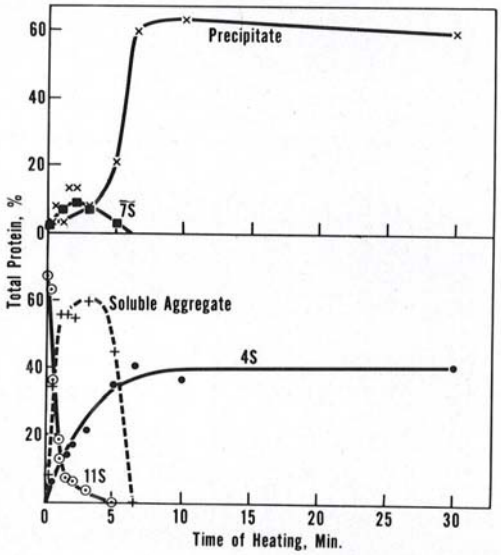

Fig. 3. Effect of heating time on ultracentrifugal composition of 11S protein when heated in buffer A.

Sedimentation of the soluble aggregate was observed before the ultracentrifuge was up to full speed (47,600 r.p.m.). Figure 2 shows ultracentrifuge patterns for solutions heated 0, 3, and 30 min. Pictures taken at 6 (rotor speed ~40,000 r.p.m.), 10, 16, and 48 min. from the start of the runs reveal aggregate in solution heated 3 min. and none in solution heated for 30 min.

Changes in composition of solution as a function of heating time are shown in Fig. 3. Loss of the soluble aggregate coincides with formation of the precipitate. In contrast, formation of the 4S fraction appears to be independent of conversion of the aggregate from soluble to insoluble form. Maximum amount of 4S fraction is observed after about 5 min. of heating when the 11S component has disappeared completely but precipitation has reached only about one-third of its final value (60% of total protein). An increase of protein in the 7S region was also noted in the ultracentrifuge patterns of samples heated for shorter periods. This fraction is referred to as 7S to distinguish it from the 7S component(s) occurring in the native soybean protein mixture.[10] The 7S fraction reached a maximum of 9% after 2 min. of heating and decreased to nearly zero after 5 min. of heating.

In a repetition of this experiment, less soluble aggregate was formed, and the aggregate disappeared completely in less than 2 min. The precipitate and 4S fraction also appeared more rapidly, and each represented about 50% of the total protein as compared to the 40:60 ratio indicated in Fig. 3.

Effect of Mercaptoethanol

The experiments in buffer A were repeated in buffer B (containing 0.01M mercaptoethanol) which is used extensively as a solvent for soybean proteins (8,9,11). In contrast to the results of previous experiments, the solution became

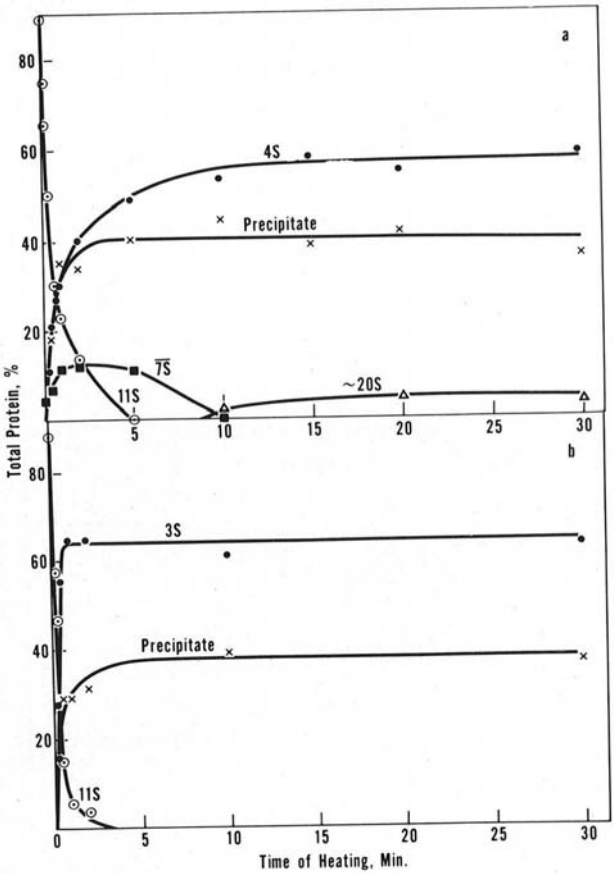

Fig. 4. Influence of heating time on ultracentrifugal composition of 11S protein when heated in (a) 0.01M mercaptoethanol, buffer B, and (b) 0.5M mercaptoethanol, buffer C.

turbid after heating for only 15 sec. (~75°C., Fig. 1), and a definite precipitate formed in 20 sec. of heating (~80°C.). Analysis of the solutions in the ultracentrifuge revealed another difference: no soluble aggregate was noted in any of the samples examined. A small amount of fast-sedimenting material (~20S) appeared, however, after 10 min. of heating. Results of the experiment are summarized in Fig. 4a. Comparison of Figs. 3 and 4a shows that the 11S protein disappeared completely in 5 min. in both samples, but that in the presence of mercaptoethanol, precipitation was complete in about 3 to 4 min., and only 40% of the total protein precipitated instead of 60% as noted in Fig. 3. A transient appearance of 7S material was also observed in buffer B.

Repetition of the experiment with buffer C, which contained 0.5M mercaptoethanol, resulted in a more rapid disappearance of 11S (Fig. 4b) and appearance of 3S fraction (Table I) than in the first two experiments. Rate of

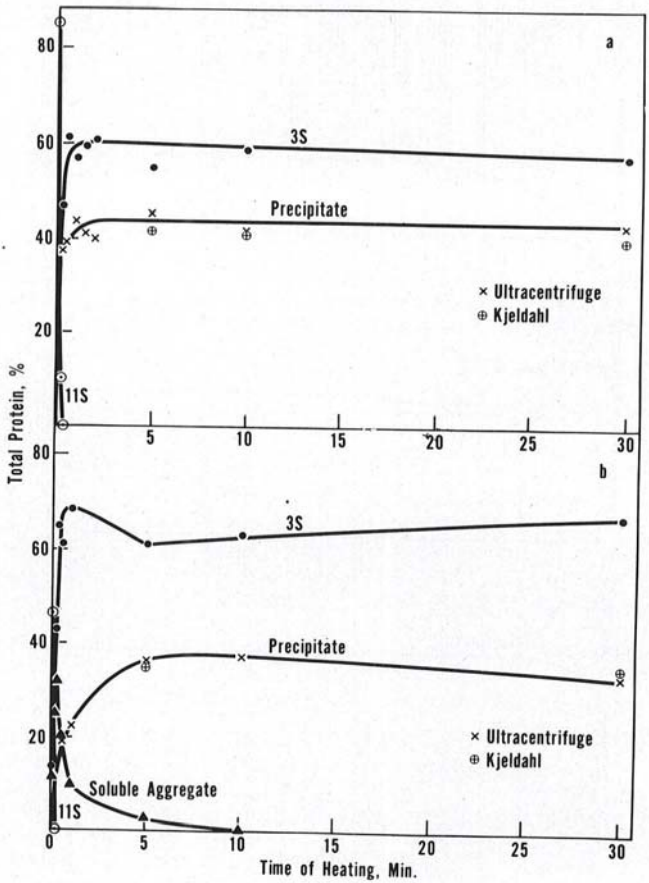

Fig. 5. Effect of heating time on ultracentrifugal composition of 11S protein when heated at (a) 0.1 ionic strength, buffer D, and (b) 0.01 ionic strength, buffer E.

precipitation was similar to that noted with 0.01M mercaptoethanol, and again, no soluble aggregate was noted in any of the solutions. Although the 11S protein was unstable in 0.5M mercaptoethanol when the solution was heated, the unheated solution (protein preparation I) contained 88% 11S component, which percentage indicated that the unheated protein was stable at this concentration of reducing agent.

Influence of Ionic Strength

The effects of lowering the ionic strength from 0.5 to 0.1 and to 0.01 at pH 7.6 in the presence of 0.01M mercaptoethanol were also examined. We selected these buffers because they had been used in previous studies of soybean proteins (10,12).

Results of heating experiments at 0.1 ionic strength (buffer D) are summarized in Fig. 5a. The unheated solution (protein preparation II) contained only 85% 11S component, apparently as a result of partial association into a faster sedimenting form (13). On heating, the 11S component decreased from its initial content of

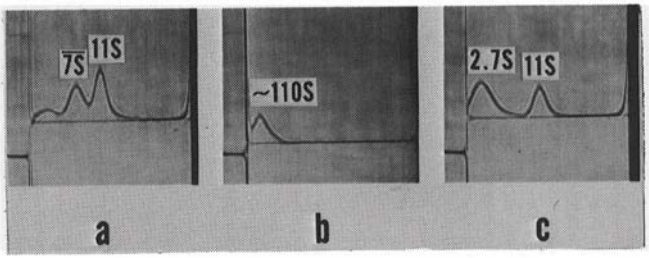

Fig. 6. Ultracentrifuge patterns for 11S protein in buffer E heated for (a) 0 sec.; and (b) and (c) 10 sec. Picture a was taken after 48 min. of ultracentrifugation, whereas pictures b and c were taken after 4 and 48 min., respectively. Direction of sedimentation is from left to right.

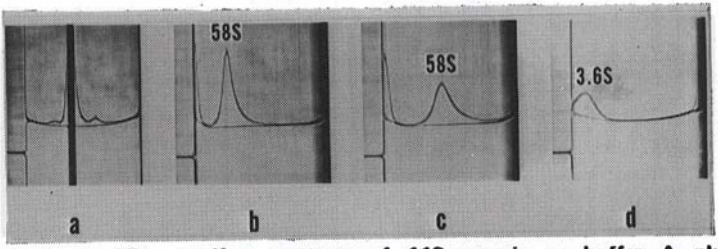

Fig. 7. Ultracentrifuge patterns of 11S protein in buffer A plus 0.01M N-ethylmaleimide after heating for (a) 0 sec.; and (b-d), 30 min. Picture a taken after 48 min. and pictures b-d were taken after 10, 14, and 48 min. of ultracentrifugation, respectively. Direction of sedimentation is from left to right.

85% to only 10% in 15 sec. (~75°C., Fig. 1) and was no longer detectable after heating for 30 sec. (~90°C.). Disappearance of the 11S component was accompanied by an equally rapid appearance of precipitate and 3S fraction. Comparison of Fig. 5a with Fig. 4a shows that the reactions were more rapid at 0.1 ionic strength than at 0.5.

Lowering the ionic strength to 0.01 (buffer E) introduced a complicating factor not present at an ionic strength of either 0.1 or 0.5. At the lowest ionic strength, the 11S component dissociated partially into smaller units (10) before heating as shown in Fig. 6a. Results of heating for various times are shown in Fig. 5b. The 11S component was undetectable after 15 sec. of heating,and the amount of 3S fraction (Table I) reached a maximum in 15 to 60 sec. Also accompanying the loss of 11S component was the formation of a soluble aggregate (Fig. 6b) which disappeared on further heating with concomitant formation of the precipitate. The $\overline{7S}$ fraction present in the unheated solution disappeared almost completely after heating for 10 sec. (Fig. 6c).

The amount of precipitate formed in the various experiments was also determined by Kjeldahl analysis of the solutions before and after heating. Values from Kjeldahl analysis were usually in agreement with the results from ultracentrifugation (Figs. 5a,b). Discrepancies between results of the two methods occurred, however, in samples containing soluble aggregates. These discrepancies apparently arose from loss of part of the soluble aggregate during ultracentrifugation in the form of an insoluble gel-like layer in the bottom of the

[4]Also referred to as standard buffer in previous work (8).

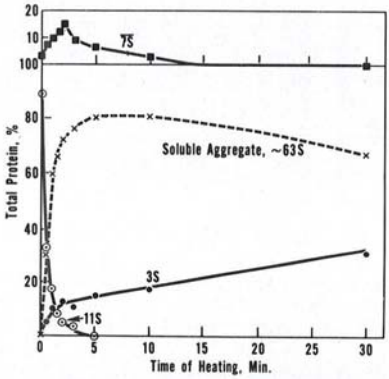

Fig. 8. Relationship between heating time and ultracentrifugal composition of 11S protein when heated in buffer A plus 0.01M N-ethylmaleimide.

ultracentrifuge cell. Insufficient sample required that Kjeldahl analyses be carried out on the solutions after recovery from the ultracentrifuge cell.

Studies with N-Ethylmaleimide

Because mercaptoethanol accelerated the conversion of 11S protein into 3-4S fraction and precipitate, it was desirable to learn what effect a sulfhydryl-blocking agent would have on these reactions. N-Ethylmaleimide (0.01M) was used as the blocking agent in order to confirm and extend previous studies[3]. The unheated solution gave an ultracentrifuge pattern (Fig. 7a) typical of that obtained in buffer B; therefore, the procedure used to remove the mercaptoethanol (Materials and Methods) prevented formation of disulfide polymers as occurred during removal of the mercaptan by dialysis (Fig. 2). Heating the solution for 30 sec. caused it to become opalescent, but no precipitation occurred on continued heating for 30 min. Ultracentrifugal analysis showed the presence of a soluble aggregate and a slow-sedimenting fraction (Fig. 7b-d and Table I). Changes in protein solution as a function of heating time are summarized in Fig. 8. The 11S component disappeared in less than 5 min. of heating. The soluble aggregate reached a maximum concentration in 5 to 10 min. followed by a decrease on continued heating, and the 3S fraction continued to increase slowly apparently at the expense of the soluble aggregate. A transient appearance of 7S material was also noted.

Sedimentation rates of the soluble aggregate were calculated for the six samples heated from 1.5 to 30 min. (Fig. 8). The values ranged from 58 to 67S with an average value of 63S (Table I) which agrees well with 67S the previous value[3]. In contrast to the soluble aggregates described earlier (Figs. 2 and 6b), the aggregate from N-ethylmaleimide treatment sppeared more uniform in particle size (Fig. 7). Apparently aggregation occurs to a definite stage and then stops.

DISCUSSION

Our results confirm and extend previous information[3] (6) about the behavior of 11S protein on heating. The overall conclusion is that heating disrupts the quaternary structure of the 11S protein and separates the subunits into two fractions. Denaturation of 11S protein at 100°C. proceeds through at least three steps designated a to c in the following reaction scheme:

$$11S \xrightarrow{(a)} \text{A-subunits} + [\text{B-subunits}] \quad (1)$$
$$\downarrow (b)$$
$$\text{Soluble aggregates}$$
$$\downarrow (c)$$
$$\text{Insoluble aggregates}$$

In this scheme A-subunits represent the 3-4S fraction formed on heating while B-subunits represent that portion of the 11S molecule which is converted into aggregates through reactions b and c. Transient appearance of $\overline{7S}$ fraction indicates that it is an intermediate in the simplified reaction proposed in scheme 1. It is not clear, however, whether the $\overline{7S}$ fraction observed here is identical to the $\overline{7S}$ detected in previous studies and postulated to be half-molecules of the 11S protein (10,14). If the half-molecules are identical[5], the A-subunits, and B-subunits may arise from the $\overline{7S}$ fraction. The results shown in Fig. 3 support this mechanism; the $\overline{7S}$ fraction disappears just before the A-subunits and B-subunits (as a precipitate) reach a maximum concentration. In contrast, Fig. 4a suggests that disappearance of the $\overline{7S}$ material occurs after the B-subunits reach a maximum concentration (as a precipitate) but before the A-subunits attain their final concentration. These results imply that the $\overline{7S}$ fraction is a precursor for only the A-subunits. Clarification of this point must await finding conditions where accumulation of a larger quantity of $\overline{7S}$ will enable its disappearance to be followed more accurately.

The B-subunits in scheme 1 are shown in brackets, since evidence for their formation in an unaggregated state by reaction a is lacking. Failure to detect the B-subunits in a slow-sedimenting form (unaggregated state) can be explained if reaction b is very rapid as compared to a. This explanation seems reasonable since the soluble aggregate always appears simultaneously with the disappearance of the 11S molecule (Figs. 3, 5b, and 8). The results with N-ethylmaleimide (Figs. 7 and 8) indicate that a well-defined intermediate in the aggregation process can be isolated; thus aggregation involves at least two reactions, b and c. Since mercaptoethanol markedly accelerates appearance of the insoluble aggregate state, it must exert its effect primarily in reaction c. In the absence of mercaptoethanol, reaction c may be catalyzed by protein sulfhydryl groups[6], which can be blocked by N-ethylmaleimide with subsequent inhibition of the reaction. The well-known sulfhydryl-disulfide interchange reaction of proteins (16) is immediately suggested by the effects observed with 0.01M mercaptoethanol.

The mechanism for disulfide exchange predicts that interchange to produce high-molecular-weight aggregates will not occur at high concentrations of sulfhydryl, thus it is surprising that heating the 11S protein in 0.5M mercaptoethanol (Fig. 4b) yields almost the same amount of precipitate as with 0.01M mercaptoethanol (Fig. 4a). Shvarts and Vaintraub (17) report that the 11S protein contains 48 moles of half-cystine per mole based on a molecular weight of 360,000. Because of the

[5]Recent studies revealed eight glycine, two phenylalanine, and two leucine or isoleucine N-terminal residues in the 11S molecule (15). An even number of polypeptide chains supports but does not prove the assumption of identical half-molecules.

[6]Variability in sulfhydryl content may account for the differences observed in rate of denaturation of the 11S protein in buffer A as noted earlier. Removal of mercaptoethanol by dialysis against buffer conceivably could result in formation of variable amounts of mixed disulfides of the protein and mercaptoethanol.

known ability of the 11S protein to form disulfide polymers (5), at least two of the half-cystine residues must be present on the surface of the molecule as cysteine. Thus there is a maximum of 46 half-cystines that could form 23 disulfide linkages in the protein. A 0.5% solution of 11S (of 90% purity) corresponds to a protein concentration of 1.3×10^{-5}M, which is equivalent to a disulfide concentration of 3.0×10^{-4}M. When the protein is in 0.01M mercaptoethanol, the ratio of sulfhydryl:disulfide is 33:1; whereas in 0.5M mercaptoethanol, the ratio is 1,667:1. The combination of high temperature and high concentration of mercaptoethanol would thus be expected to result in a protein completely reduced rather than crosslinked by intermolecular disulfide bonds[7]. If the protein were completely reduced, the action of mercaptoethanol must be attributed to its ability to cleave disulfide crosslinkages which, when broken, permit the molecules to expose reactive groups capable of more favorable interactions to form the insoluble aggregates. Because precipitation occurs at high ionic strength and at high temperature, ionic and hydrogen bonds seem unlikely to be involved in reaction c. Possibly hydrophobic interactions are responsible for conversion of the soluble aggregates to an insoluble state.

With the exception of the experiment with N-ethylmaleimide the changes in the physical properties are complete after heating for about five minutes. Because of the short heating times involved, it is doubtful that chemical changes other than those involving sulfhydryl and disulfide groups play an important part in the physical changes observed.

Since separation into two distinct fractions occurs on heating the 11S protein, one must also consider the possibility that two forms of molecules are present in the original protein sample as noted with ovalbumin (19). If one of these proteins is designated 11S-A and the other 11S-B, then the following alternative reaction scheme can be proposed:

$$\begin{array}{l} \text{11S-A} \xrightarrow{\text{(a)}} \text{A-subunits} \\ \text{11S-B} \xrightarrow{\text{(b)}} [\text{B-subunits}] \xrightarrow{\text{(c)}} \text{Soluble aggregates} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \downarrow \text{(d)} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{Insoluble aggregates} \end{array} \qquad (2)$$

In this scheme A-subunits and B-subunits are formed in two separate reactions, a and b. The B-subunits undergo further reaction through steps c and d to form the insoluble aggregates. Reaction scheme 2 predicts that a constant ratio of A-subunits to aggregates should be obtained based only on the ratio of 11S-A to 11S-B in the starting protein solution. The ratio of A-subunits to B-subunits (as insoluble aggregates), however, varies with reaction conditions (Figs. 3-5,7). Furthermore, various kinetic studies indicate that formation of the A-subunits is closely related to disappearance of all 11S protein rather than to only a fraction of it as required by reaction a of scheme 2. When 11S protein disappears slowly (Figs. 3 and 4a) A-subunits reach a maximum concentration slowly, whereas rapid disappearance of 11S is accompanied by more rapid accumulation of A-subunits (Figs. 4b, 5a, and 5b). Reaction scheme 2 is also inconsistent with available physical and immuno-

[7]Davidson and Hird (18) report that at 37°C. ribonuclease is stable to reduction at a glutathione:protein disulfide ratio of 0.9, but at 55°C. reduction is rapid and nearly complete.

chemical data concerning homogeneity of 11S protein (8,9,15,17,20). The objections to scheme 2 also apply to a modification of scheme 2 in which 11S-B goes directly to soluble aggregate, i.e., no B-subunit intermediate is formed.

Our studies agree with the results of Watanabe and Nakayama (6) concerning heat lability of the 11S fraction and its conversion into aggregates. Their studies did not indicate the simultaneous formation of a slow-sedimenting fraction possibly because of reactions of the A-subunits with other components in the water-extractable proteins which they studied. Nevertheless, one cannot rule out simultaneous formation of A-subunits from the 11S protein and aggregation of some of the proteins in the 2S fraction of the water-extractable proteins. Aggregation of the nonglobulin proteins (i.e., part of the 2S and 7S fractions (8) has been reported by Mann and Briggs (4). The net result would be a failure to detect formation of A-subunits. This possibility emphasizes the difficulty of interpreting studies made on the complex mixture of proteins in a water-extract of defatted soybean meal. Previous work at the Northern Laboratory, however, indicates larger than normal amounts of slow-sedimenting fractions in certain commercial preparations of soybean globulins (21) presumably as a result of heat-treatment. It is therefore possible that A-subunits will remain soluble when the globulin fraction is heated under certain conditions.

Saio et al. (22) recently reported the effects of heating on gel filtration of water-extractable, acid-precipitated, and calcium-precipitated soybean proteins. Heating caused aggregation of protein in all of the samples. Evidence for formation of a low-molecular-weight material analogous to our 3-4S fraction was found only with the acid-precipitated protein.

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