

## ISOLATION AND CHARACTERIZATION OF THE 7S COMPONENT OF SOYBEAN GLOBULINS<sup>1</sup>

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### ABSTRACT

A method is described for isolating a portion of the 7S fraction of the acid-precipitable soybean proteins, the globulins, as an approximately 90% ultracentrifugally pure preparation. Evidence is given that the 7S component, so isolated, is representative of the whole 7S fraction of the acid-precipitable protein. The amino acid analysis, carbohydrate content, and the N-terminal amino acids of the 7S fraction are compared with those reported for the whole globulin mixture.

The 7S fraction undergoes a specific and characteristic  $7S \rightleftharpoons 9S$  isomerization under proper environmental conditions, and this reaction is shown by all of the 7S fraction of the total globulins. Molecular-weight determinations of the 7S and of the 9S forms verify that this isomerization is a dimerization. The pH and ionic strength dependence of the reaction implies that spatially specific electrostatic forces are involved.

The sedimentation behaviors of the 7S entity in acidic, urea, and detergent solutions are presented. The retention of the ability of the treated protein to dimerize is taken as a criterion of reversibility and lack of denaturation during the treatment. Evidence for the existence of subunits of the 7S is presented.

The globulin fraction of soybean protein contains four components resolvable in the ultracentrifuge at pH 7.6, 0.5 ionic strength (1). This fraction has also been called glycinin, the acid-precipitable protein, or soybean casein, and its components have come to be known simply

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as the 2S, 7S, 11S, and 15S. The 11S and 7S are the major components. The 11S has been isolated and studied extensively (2-6). Naismith (1) partially purified the 7S component through ammonium sulfate fractionation. He noted that the 7S in his preparation underwent an isomerization reaction to a 9S form<sup>2</sup> when the ionic strength was lowered from 0.5 to 0.1 at pH 8.0. Wolf (2) also partially purified a 7S fraction by differential extraction of isoelectrically precipitated globulins. He noted that this preparation also underwent the  $7S \rightleftharpoons 9S$  isomerization reported by Naismith. Wolf further observed that the 7S could be converted to a ~ 2S component by the action of sodium n-octyl benzene sulfonate (SOBS). He concluded that the 7S moiety was composed of noncovalently linked subunits.

A method has been developed, using ammonium sulfate fractionation and gel filtration, which yields the 7S in approximately 90% purity. The remaining 10% has a sedimentation coefficient in the 11S region. The present report is concerned with the characteristic properties of the 7S of this preparation.

### Materials and Methods

*Soybean Meal.* Hexane-defatted soybean meal (2,3) was extracted with 10 volumes of water at pH 7.5. The globulin fraction was obtained by acidifying the filtered extract to pH 5.0 with acetic acid (7). The precipitated globulin was redissolved at pH 7.5, and the acid precipitation was repeated.

*Standard Buffers.* The various fractions obtained during the isolation were assayed for 7S protein by ultracentrifugal analysis in a standard phosphate buffer of the composition 0.0325M dipotassium hydrogen phosphate, 0.0026M potassium dihydrogen phosphate, 0.40M sodium chloride, and 0.01M 2-mercaptoethanol. This gave a pH of 7.6 and an ionic strength of 0.5. Wolf (2) has shown that the 7S and 11S components are, to a certain extent, present in the extract as randomly aggregated disulfide polymers. These aggregates are readily dissociated by 0.01M mercaptoethanol. For this reason, mercaptoethanol has been routinely used in the buffers employed in this study.

A second phosphate buffer was used routinely to obtain the 7S protein in its 9S form. This was identical to the one described above, except that it contained no sodium chloride. The ionic strength of this buffer was 0.1. These buffers are referred to hereafter as the 0.5

<sup>2</sup>The sedimentation constants of all of the soy fractions vary with protein concentration. With some this variation is quite marked. Naismith referred to the faster-sedimenting form which is derived from the 7S entity under conditions of 0.1 ionic strength as the 10.5S. This is a correct designation at the approximately 0.5% protein concentration with which he worked. At a 1% concentration of the protein, the value of its sedimentation constant is close to 9S. In the interest of consistency in designation and in the absence of a more meaningful basis for identification, we have chosen to refer to this entity as the 9S form of the 7S component.

and the 0.1 ionic strength standard buffers.

All salts and reagents were of analytical grade, and were used without further purification except for the SOBS, which was prepared according to the method of Hill (8).

*Ultracentrifugal Analysis.* (a) *Sedimentation Velocity.* Routine assay runs were made with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics, at 56,100 r.p.m., at room temperature, in a single-sector 12-mm. cell. Sedimentation coefficients were calculated from measurements made directly from the schlieren patterns (9) and were corrected to  $S_{20,w}$  by the method of Svedberg and Pedersen (10).

(b) *Molecular Weight Determinations.* The molecular weights (MW's) of the 7S and 9S forms of the protein were determined from Trautman Plots (11), an approach to equilibrium method using the Archibald principle (12). For the calculations, the modified terminology of Erlander and Foster was used (13). This method gives a parameter  $X^*$ , which is a function of the area under the schlieren pattern between the cell boundary position and the plateau region in the cell and is related to the protein concentration at the cell boundary, and a parameter  $Y^*$ , which is a function of the concentration gradient at the cell boundary. A plot of  $X^*$  vs.  $Y^*$ , the so-called Trautman Plot, will give a straight line for a monodisperse system, the slope of which is equal to  $S/D$ . The apparent MW can then be calculated by the Svedberg equation

$$M_{app} = \frac{RT}{(1 - V\rho)} \left( \frac{S}{D} \right)$$

where  $M_{app}$  = apparent MW,  $R$  = gas constant,  $T$  = absolute temperature,  $S$  = sedimentation coefficient,  $D$  = the diffusion coefficient,  $V$  = the partial specific volume of the protein, and  $\rho$  = the density of the solution. The  $M_{app}$  is concentration-dependent and is related to the true MW by the equation

$$M_{app} = \frac{M}{1 + C \left( \frac{\delta \ln y}{\delta C} \right)_{T,P}}$$

where  $M$  is true molecular weight,  $C$  is protein concentration, and  $y$  is the activity coefficient of the protein. The true MW is found by plotting  $M_{app}$  vs. concentration and extrapolating to zero protein concentration (14).

The MW's were determined from measurements made at the meniscus only. Preliminary experiments with ribonuclease indicated that the true meniscus position was two-thirds of the distance between the

first optically recorded interference fringes of the meniscus image, measured in the centrifugal direction (15). Most of the measurements were made from patterns developed at 12,590 r.p.m.; however, enough measurements were made from patterns obtained at higher speeds to show that the Trautman Plots were linear from the  $X^*$  intercept.

The partial specific volume of the 7S protein was determined pycnometrically to be 0.729 at 20°C. Its variation with temperature was assumed to be the same as that shown by the average protein (10).

*Amino Acid Analysis.* Amino acid analyses were carried out on 12-, 24-, and 48-hr. hydrolysates in the Spinco Model 120 Amino Acid Analyzer by the two-column system of Moore and Stein (16). Hydrolyses were done in evacuated, sealed tubes in 6N HCl at 110°C. The values reported are (a) averages for those amino acids showing small erratic differences between hydrolysis times, or (b) extrapolated zero time values for those amino acids showing a decrease with time of hydrolysis, or (c) the 48-hr. hydrolysis value for those amino acids which increased in amount with time of hydrolysis.

*N-Terminal Analysis.* N-terminal analysis was carried out qualitatively by the dinitrophenol method of Sanger (17,18). The dinitrophenylation was done in 6M guanidine HCl according to a method described by Phillips (19). The DNP-protein was hydrolyzed with glass-distilled constant-boiling HCl for 16 hr. at 110°C. in an evacuated, sealed tube. The ether-soluble DNP-amino acids were paper-chromatographed in the first dimension by the "toluene" system of Beserte *et al.* (18), then in the second dimension with 1.5M phosphate of pH 6. The water-soluble fraction was paper-chromatographed in one dimension with butanol:acetic acid:water, 450:50:125. Identification of the DNP amino acids was made by hydrolyzing them in concentrated  $\text{NH}_4\text{OH}$ , then identifying the resulting free amino acids on the amino acid analyzer (20).

N-terminal amino acids were determined quantitatively by the cyanate method of Stark and Smyth (21), in which  $\text{NH}_2$  groups are carbamylated by reacting the protein with potassium cyanate in 6M urea. The hydantoin of the N-terminal amino acids are then formed and hydrolyzed from the peptide chain by 1-hr. treatment with hot 6M HCl. The hydantoin of the N-terminal amino acids are separated from the amino acids and peptides on appropriate ion-exchange columns. The hydantoin is hydrolyzed with 0.2M NaOH; the resulting amino acids are identified and their amounts estimated by means of the amino acid analyzer.

*Carbohydrate Analysis.* The carbohydrate contents of the various protein fractions were determined by the phenol-sulfuric acid method

of Dubois *et al.* (22). Glucose was used to prepare a standard curve.

*Determination of Protein Concentration.* Nitrogen determinations on the 7S preparation were carried out by a semimicro-Kjeldahl method. The digestion procedure of Pepkowitz and Shive (23) and the titration method of Ma and Zuazaga (24) were used. The nitrogen content of the 7S preparation was 15.5%. Protein concentrations were routinely determined by light absorption at 280  $m\mu$  in a Beckman DU spectrophotometer. The  $E_{280}^{1\%}$  value for the 7S protein was 6.4, and for the 9S form, 6.3.

## Results

*Isolation Procedure.* The 7S preparation was isolated as shown in Fig. 1. Ammonium sulfate fractionation was carried out on a 2%

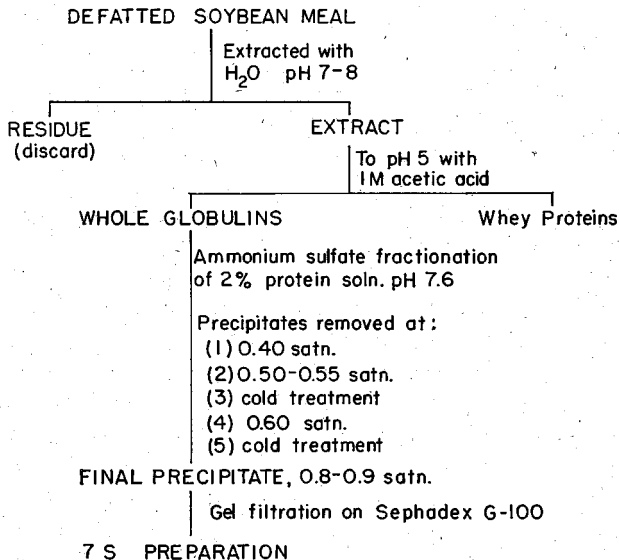


Fig. 1. Isolation of 7S soy protein.

solution of the globulin fraction originally dissolved in the 0.5 ionic strength buffer. The protein components found in each of the fractions follow roughly those reported by Naismith (1). The 2S, the least soluble component in the ammonium sulfate solutions, appeared in high proportion in precipitate 1. Precipitate 2 contained all four components, with the 11S predominating; most of the 15S was removed in this precipitate. An additional precipitate containing mostly 11S was obtained by cooling the solution at this stage. Precipitates 4 and

5 each contained roughly 70% 7S and 30% 11S. The final precipitate, 6, comprised 85% 7S, 5% 2S, and 10% 11S.

The 2S component was removed completely by gel filtration on a column of Sephadex G-100 (45 × 1.8 cm.). Loads of 100 mg. of precipitate 6 were placed on this column. The elution was carried out with the 0.5 ionic strength standard buffer.

The sedimentation patterns of the whole globulin fraction and of the final 7S preparation are shown in Fig. 2. The preparations were

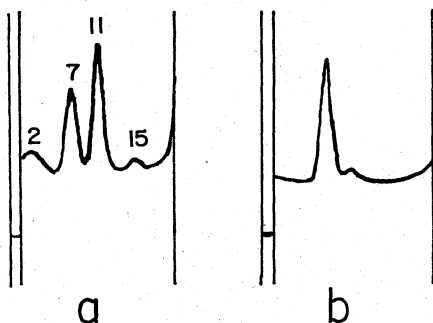


Fig. 2. Sedimentation patterns: a, 2% whole globulin fraction; b, final 7S preparation. Patterns obtained at 48 min., 50° bar angle. Speed, 56,100 r.p.m. Solvent 0.5 ionic strength standard buffer.

dialyzed against water until the protein started to precipitate, then freeze-dried and stored at  $-10^{\circ}$  until used.

In a large-scale preparation, 7.5 g. of precipitate 6 was obtained from 1,300 g. of defatted meal. Since the original defatted meal contained approximately 50% protein, of which the 7S component makes up approximately 30% of the total protein, the total weight of 7S in the starting material would be about 215 g. This means that the 7S preparation represents only about 4% of the total 7S present initially. The low yield is due to losses that occurred in each of the fractionation steps, the purpose of which was to sacrifice yield to gain purity in the 7S preparation finally achieved.

*The 7S  $\rightleftharpoons$  9S Isomerization.* If the electrolyte concentration of a solution of 7S in the pH range 7 to 9 is lowered from 0.5 to 0.1 ionic strength, the 7S is entirely converted to a new sedimenting species having a sedimentation coefficient between 9S and 10S when the protein concentration approximates 1%. At intermediate ionic strengths it is possible to observe varying amounts of the two sedimenting species. Naismith (1) proposed that the observed isomerization was a dimerization.

The apparent molecular weights of the 7S and 9S forms were determined at various protein concentrations. The plots of  $M_{app}$  vs. protein concentration for the 7S and 9S forms are shown in Fig. 3.

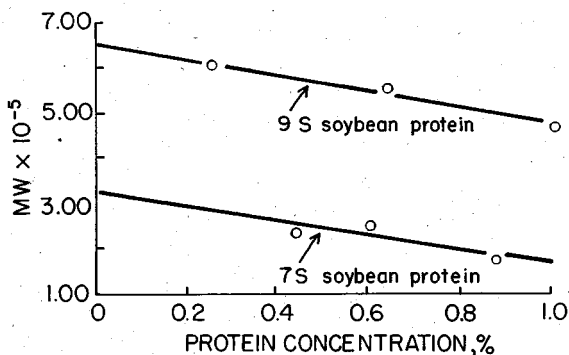


Fig. 3. Dependence of molecular weight on protein concentration for the 7S and 9S forms of the 7S protein.

Extrapolation to zero concentration gave  $M$  values of approximately 650,000 for the 9S form and 330,000 for the 7S. These MW's verify that the 9S form is a dimer of the 7S.

Figure 4 shows the concentration dependence of sedimentation coefficients of the 7S and 9S forms of the 7S and the 11S proteins. This figure clearly illustrates that the sedimentation coefficient of the 9S

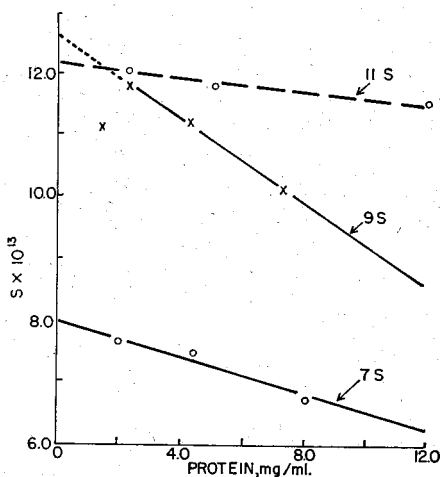


Fig. 4. Dependence of sedimentation coefficients on protein concentration for the 7S and 9S forms of the 7S protein and the 11S protein. Data for 11S protein taken from reference 2.

is more strongly dependent on protein concentrations than are those of the 7S or the 11S. The extrapolated values of the sedimentation constant at zero concentration,  $S_{20,w}^0$ , for the 7S, 9S, and 11S entities are 7.95S, 12.7S, and 12.2S, respectively. These figures explain why, at the concentration of protein usually studied, the 9S has commonly been observed to sediment at a slower rate than does the 11S, which is the entity of lower MW; the MW of the 11S is 360,000, as reported by Wolf (2).

The deviation (Fig. 4) of the S value observed with the 9S entity, at the lowest concentration of protein studies, from the linear relationship obtained at higher concentrations is probably of significance with regard to the  $7S \rightleftharpoons 9S$  relationship. The accuracy of measurement at this concentration (1.5 mg./ml.) is less than at the higher concentrations. Nevertheless, the low value obtained may indicate that the 9S form is actually partially dissociated at this low concentration. Sedimentation properties in the lower concentration range should be investigated, with an optical system more sensitive than the schlieren optics, to explore this possibility further.

Frictional coefficients,  $f/f_0$ , as calculated from M values of molecular weight and  $S_{20,w}^0$  using the formula (10)

$$f/f_0 = 1.19 \times 10^{-15} \frac{M^{2/3} (1 - VP)}{S_{20,w}^0 V^{1/3}}$$

yielded values of 2.17 and 2.14 for the 7S and the 9S entities, respectively.

The dimerization reaction does not occur at a pH value acid to the insoluble isoelectric range of the 7S preparation. At pH values of 3.0 and 2.0, 0.1 ionic strength, the 7S at 1% concentration showed a single peak with an S value of 5.6.

The dimerization reaction can be prevented by blocking 72% of the  $\epsilon$ -amino groups by carbamylation to form homocitrulline residues. This reaction was carried out by allowing a 1% protein solution to stand overnight in 1M potassium cyanate at room temperature. The excess cyanate was removed by dialysis in the cold. The extent of carbamylation was estimated by amino acid analysis (21). A 1% solution of the carbamylated 7S in the 0.1 ionic strength phosphate buffer had a  $S_{20,w}$  of 6.1. The pattern also showed a small amount of 3.7S material, but no 9S.

*Sedimentation Behavior of 7S in the Presence of Various "Denaturing" Agents.* Wolf (2) reported that the 7S component of his preparation, when treated with SOBS at pH 9.0, 0.01 ionic strength, could be converted to a slower-sedimenting material. The conversion was com-



plete when a detergent concentration of  $0.015M$  was reached. The action of detergent on the 7S protein appeared to be reversible.

The actions of a variety of chemical agents on the sedimentation properties of the 7S protein have been studied with the objective of finding conditions under which the slower-sedimenting species could be obtained alone, so that its MW could be determined unequivocally. Abundant evidence has been accumulated which substantiates the conclusion that 7S can indeed be converted into a form showing a slower sedimentation constant than that of 7S itself. However, the search for conditions under which the slower form alone is present has not yet been successful.

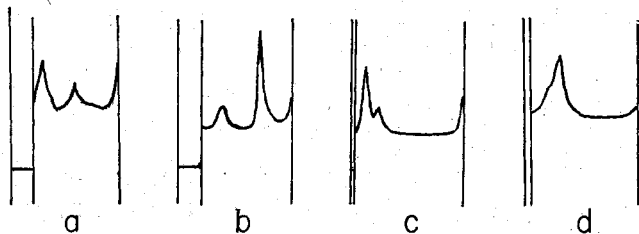


Fig. 5. Sedimentation patterns: a, 1% 7S solution in  $5 \times 10^{-3}M$  SOBS in  $0.01M$  glycine buffer pH 9.2,  $40^\circ$  bar angle; b, 1% 7S in  $1 \times 10^{-3}M$  SOBS,  $55^\circ$  bar angle,  $0.1 \mu$  standard buffer; c, 1% 7S in  $0.01N$  HCl,  $50^\circ$  bar angle; d, same as c after addition of  $0.1N$  NaCl,  $40^\circ$  bar angle. All patterns obtained at 56,100 r.p.m.; a, b, and d were taken at 48 min., c at 56 min.

*Action of Detergent.* Figure 5, a, shows the sedimentation pattern obtained with a 1% solution of 7S protein in  $5 \times 10^{-3}M$  SOBS in  $0.01M$  glycine buffer, pH 9.2. The sedimentation coefficients of the two boundaries observed are 9.4 and 2.6. This pattern is similar to that observed by Wolf (2) for his 7S preparation, under the same conditions.

The uncertainty regarding the influence of the primary charge effects on the sedimentation rates prompted examination of the 7S in solutions at 0.1 ionic strength in various concentrations of SOBS. The detergent-protein solutions were prepared by dialyzing the detergent into the protein solutions. At pH 7.6, 0.1 ionic strength, the conversion of 7S protein to a 3.3S component was found to be optimal at a concentration of  $10^{-3}M$  SOBS (Fig. 5, b). Higher concentrations of detergent promoted nonspecific aggregation, and the proportion of the 3S material was reduced.

*Action of Acid.* The action of acid on the whole globulin fraction (25) and on the 11S component alone (5,6) has been described. Determinations of the average particle weight have shown that the 11S component dissociates in dilute acid solutions.

*Amino Acid Analysis.* A partial amino acid analysis of the 7S preparation is compared in Table I with that by Van Etten *et al.* (26) of the whole globulin fraction. The amino acid composition of the 7S preparation is, in general, similar to that of the whole globulin. However, there are some significant differences, as illustrated by its lower content of threonine, serine, glycine, alanine, and methionine. The arginine content was somewhat higher in the 7S preparation. It is of particular interest that the 7S contained only one-seventh of the relative amount of methionine found in the whole globulin fraction; this is the only essential amino acid in which the whole globulin is deficient. The 7S component thus contributes strongly to this nutritional characteristic.

*Carbohydrate Content.* The carbohydrate content of the 7S preparation was 5.89%, that of the cold-insoluble fraction (2) (approximately 80% 11S) was 1.51%, and that of a pure 2S preparation<sup>3</sup> was 2.79%. The carbohydrate content of the whole globulin fraction was 2.78%. Thus, the 7S preparation contains a major proportion of the carbohydrate associated with the whole globulin fraction.

*N-Terminal Analysis.* The results of the N-terminal analyses of the 7S preparation are given in Table II, along with results reported by

TABLE II  
N-TERMINAL ANALYSIS: COMPARISON OF 7S FRACTION WITH WHOLE GLOBULIN AND COLD-INSOLUBLE PROTEIN

| AMINO ACIDS                | 7S PREPARATION |                          | WHOLE GLOBULINS <sup>a</sup> | COLD-INSOLUBLE <sup>a</sup> FRACTION | N-TERMINALS <sup>b</sup> OF 7S |
|----------------------------|----------------|--------------------------|------------------------------|--------------------------------------|--------------------------------|
|                            | DNP-Method     | Hydantion Method         |                              |                                      |                                |
|                            |                | moles/10 <sup>5</sup> g. |                              |                                      |                                |
| Serine                     | Found          | 0.64                     | 0.41                         | 0.12                                 | 1.92                           |
| Glutamic acid              | Found          | 0.80                     | 0.84                         | 0.08                                 | 2.40                           |
| Glycine                    | Found          | 0.29                     | 1.02                         | 1.51                                 | 0.87                           |
| Valine                     | Found          | 0.34                     | 0.15                         | 0.06                                 | 1.02                           |
| Leucine                    |                |                          |                              |                                      |                                |
| (isoleucine <sup>a</sup> ) | Found          | 0.28                     | 0.68                         | 0.87                                 | 0.84                           |
| Aspartic acid              | Found          | 0.18                     | 0.23                         | 0.02                                 | 0.54                           |
| Alanine                    | Found          | 0.12                     | 0.10                         | ...                                  | 0.36                           |
| Tyrosine                   | Found          | 0.12                     | ...                          | ...                                  | 0.36                           |
| Phenylalanine              | Not found      | ...                      | 0.29                         | 0.39                                 | ...                            |

<sup>a</sup> See reference 27.

<sup>b</sup> Calculated as if all of the preparation was 7S and the 7S had a MW of 300,000.

Weintraub (27) (using the DNP method) for the whole globulin fraction and the cold-insoluble fraction. Good qualitative agreement between the DNP method and the cyanate method was found for the 7S preparation.

The last column in Table II shows the moles of amino acid per

<sup>3</sup> Unpublished results, this laboratory.

mole of 7S protein, calculated on the basis of a MW of 300,000 and on the assumption that the preparation was pure 7S. Two moles each of serine and glutamic acid and one mole each of glycine, valine, and leucine per mole of 7S are indicated. Fractional moles of alanine, tyrosine, and aspartic acid were also found. A minimum of seven peptide chains, differing in N-terminal amino acids, are thus indicated in the 7S preparation. The fractional amounts of aspartic acid, alanine, and tyrosine must be taken to indicate that a heterogeneity exists in this protein preparation. Ultracentrifuge analysis of this preparation detects about 10% of an 11S impurity. The fact that Weintraub found none of these three amino acids as N-terminals in the cold-insoluble fractions seems to indicate that the heterogeneity evidenced by these fractional amounts does not arise from the 11S entity which is the predominant component in the cold-insoluble fraction. These anomalous findings about the N-terminal groups present in the 7S preparation, though dependably reproducible, were not yet explainable. The 7S entity must be further purified, and its subunit composition determined, before reliable conclusions can be drawn.

### Discussion

Since our 7S preparation represents less than 4% of the 7S initially present in the soybean meal, the question arises as to whether the 7S of our preparation is truly representative of the 7S of the whole globulins. The following facts indicate that it is: 1) the 7S present in this preparation undergoes, quantitatively, the dimerization to 9S; 2) the action of SOBS at pH 9 on the sedimentation behavior of our preparation compares closely with that of the 7S in other preparations wherein it has been obtained in partially purified condition by widely different procedures; and 3) the 7S preparation behaves on a calcium phosphate column in a manner identical with that of the total 7S fraction in the whole globulin<sup>4</sup>.

The values of the MW's obtained for the 7S and 9S forms show that this characteristic transformation is a dimerization. The conditions chosen for determining the MW of each form were such that the equilibrium strongly favored the presence of only one form in each case. The concentration dependence of the sedimentation coefficients for each varied in a manner which is to be expected for monodisperse solutions.

The absence of the dimerization reaction on the acid side of the isoelectric point, and the fact that the dimer is stable at low ionic strengths and dissociates to the monomer as the ionic strength is

<sup>4</sup>J. W. Wolf, personal communication.

raised, indicate that there is an electrostatic bonding involved in the formation of the dimer. The prevention of the dimerization reaction as a result of blocking of some of the free amino groups by mild carbamylation reaction further implicates electrostatic bonds as being involved in the formation of the dimer. The bonding must be spatially specific and limited to the possibility of dimer formation. This property of dimerization is a most characteristic property of the 7S protein and may be used as a means of assaying for the 7S protein, in a manner somewhat analogous to the use of a biological activity. The 7S protein acts as a homogeneous entity in that all the 7S molecules are capable of undergoing this specific dimerization. This must mean that all the 7S molecules possess a common and specifically characteristic region of conformation that (a) allows them to dimerize through secondary linkages, (b) is not possessed by any other molecules in the whole soy globulin fraction, and (c) is lost to the 7S component upon denaturation or modification of its charge structure.

The actions of urea, detergent, and acid on the 7S protein have in common the production of a species having sedimenting characteristics that are slower than that of the 7S. Such behavior can arise either from a dissociation of the 7S molecule into subunits or from a large increase in the frictional coefficient of the protein through unfolding or swelling of the molecule. The reversibility of the action of urea and of acid in the presence of 2-mercaptoethanol rules out the involvement of disulfide bonds. While this slower-sedimenting entity is probably a subunit of the 7S, final confirmation of the existence of such a non-covalently linked subunit of the 7S must await the determination of its molecular weight.

The comparisons of the amino acid analyses, carbohydrate contents, and N-terminal analyses of the various fractions of the whole globulins of soybean add further evidence that the ultracentrifugal components observed at pH 7.6, 0.5 ionic strength, represent individual protein components and are not simply different aggregates of the same building units, as suggested by Kretovich *et al.* (28).

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