DENATURATION OF SOYBEAN GLOBULINS BY AQUEOUS ISOPROPANOL[1]

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ABSTRACT

Soybean globulins were treated with aqueous isopropanol, dried, and dialyzed against buffer at pH 7.6, ionic strength 0.5, containing 0.01*M* mercaptoethanol. Insolubility of the proteins in the buffer was used as a criterion of denaturation. Effects of isopropanol concentration, time, and temperature of isopropanol treatment on solubility of the proteins in buffer were studied. Ultracentrifugal and chromatographic analyses of the proteins soluble in buffer showed the 7S, 11S, and 15S components to be denatured by isopropanol at 25°C., whereas the 2S component was undenatured. Maximum denaturation in 2 hr. at 25°C. occurred with 40% isopropanol, and the 7S component showed the greatest susceptibility to denaturation. Increasing temperature of the alcohol treatment to 50° or 75°C. increased the rate of denaturation and also caused the 2S fraction to be denatured, although at a slower rate than the other three fractions.

Previous studies have demonstrated that treatment of defatted soybean meal with aqueous alcohols insolubilizes the proteins (1,2). Elec-

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trophoretic studies indicated that the globulin fraction of soybean protein was most susceptible to alcohol denaturation (1). The observation that alcohol washing of isolated soybean globulins removes lipid-like materials, which act as foam inhibitors (3), prompted a study of the effect of aqueous alcohols, at selected concentrations, on the physical properties of the proteins (4). Recently Roberts and Briggs (5) made a detailed study of the effects of ethanol on isolated soybean globulins.

Prior to preparing isopropanol-washed soybean proteins for other work, it was decided to obtain more information on the denaturing effects of this alcohol on the isolated proteins. In this paper are reported the effects of isopropanol concentration, time, temperature, and pH on the solubility, ultracentrifugal, and chromatographic properties of isolated soybean globulins.

Materials and Methods

Adams (1960 crop) soybeans stored at 5°C. were cracked, dehulled, and flaked before being defatted with hexane at room temperature. Soybean globulins were isolated by water extraction of defatted meal at 25°C. and isoelectric precipitation at pH 4.5 with hydrochloric acid (3). Four globulin samples designated I–IV were prepared. Samples I, II, and III were used to determine the effects of isoelectric precipitation and drying on protein solubility and ultracentrifugal composition. Experimental details for preparation of samples I–III are given below ("Results"). Sample IV was a single batch of globulins separated from the whey solution by centrifugation and dried in a Stokes[2] Model 2003F-2 freeze-dryer. Sample IV had been stored at room temperature for approximately 1 year and was used for all of the isopropanol denaturation studies.

Samples of isolated soybean globulins (375 mg. of protein) were treated with 25 ml. of isopropanol solution in 50-ml. screw-capped test tubes or 50-ml. round-bottomed flasks fitted with condensers (at temperatures above 25°C.). At the end of the treatment, isopropanol was removed by filtration through a sintered-glass funnel; this was followed by thorough washing of the proteins with 50 ml. of diethyl ether to remove rapidly the residual isopropanol and to dry the proteins. Unless specified otherwise, all studies were made at pH 4.5. All alcoholic concentrations are expressed as percentages by weight.

Protein solubilities were determined by suspending 375 mg. of protein in 25 ml. of potassium phosphate-sodium chloride buffer (0.0325*M* K_2HPO_4, 0.0026*M* KH_2PO_4, 0.4*M* NaCl), pH 7.6, ionic strength 0.5, 0.01*M* mercaptoethanol (henceforth also referred to as

[2]Reference to specific equipment or organizations does not necessarily constitute endorsement by the U.S. Department of Agriculture.

buffer), and by dialyzing against buffer at 4°C. for 48 hr. or longer. After dialysis the volume (usually about 23 ml.) was adjusted to 25 ml., and insoluble protein was removed by centrifuging (12,000 × *g*) for 10 min. at 25°C. Results are expressed as percentages of the total nitrogen (as determined by the Kjeldahl method) remaining soluble after dialysis.

Protein solutions obtained in the solubility experiments were analyzed at room temperature in a Spinco Model E ultracentrifuge at 47,660 r.p.m. A 30-mm. cell with a plastic double-sector center piece was used.

Chromatography on hydroxylapatite (Hypatite C, Lot 6273, Clarkson Chemical Co., Williamsport, Pa.) was performed with columns 1 × 25 cm. equilibrated with 0.03*M* potassium phosphate,[3] pH 7.6, at room temperature (6). The protein solutions obtained in the solubility experiments were equilibrated by dialysis against 0.03*M* potassium phosphate, pH 7.6, and 20-ml. portions were applied to the columns. Elution was begun with 270 ml. of the same buffer in a mixing chamber. The mixing chamber was connected to a reservoir of *M* potassium phosphate, pH 7.6, after 20 ml. of effluent was collected to permit the first fraction to be eluted before onset of the linear potassium phosphate gradient. Fractions of 1 ml. were collected and diluted to 4 ml. for absorbancy measurements in a 1-cm. cuvet at 280 mμ. After chromatography, the tubes for each peak in the elution diagram were pooled, pervaporated to concentrate the protein, and equilibrated against the pH 7.6, 0.5 ionic strength buffer containing 0.01*M* mercaptoethanol before ultracentrifugal analysis. Chromatographic recoveries based on areas under the elution diagrams were 90–92%.

Results

Proteins Insolubilized by Isolation Procedure. In the previous study (4) only 80% of the nonalcohol-treated soybean globulins were soluble in buffer (pH 7.6, ionic strength 0.5, containing 0.01*M* mercaptoethanol). In the present study the ultracentrifugal identity of the insoluble proteins was determined.

A water extract of defatted meal (20 g. meal/200 ml. water) and a soybean whey solution, prepared from a portion of the water extract by adjusting to pH 4.5 and centrifuging, were both dialyzed against buffer for 2 days, adjusted to a standard volume, and analyzed in the ultracentrifuge. Areas under the ultracentrifuge patterns were measured and the areas for the whey protein peaks were subtracted from the

[3]Prepared by titrating potassium dihydrogen phosphate to pH 7.6 with potassium hydroxide solution.

TABLE I

ULTRACENTRIFUGAL COMPOSITION OF WATER-EXTRACTABLE PROTEINS, WHEY PROTEINS, AND GLOBULINS OF SOYBEAN MEAL

PROTEIN FRACTION AND TREATMENT	NITROGEN CONTENT [a]	ULTRACENTRIFUGAL AREA [b]				
		2S	7S	11S	15S	Total Area
	mg./ml.					
Water-extractable	3.12	8.4	10.3	10.6	1.8	31.1
Whey	0.45	2.7	2.0	00.0	00.0	4.7
Globulins (by difference)	2.67	5.7	8.3	10.6	1.8	26.4
Globulins, undried (I)	2.21	3.8	7.1	9.6	1.1	21.6
Globulins, freeze-dried, laboratory (II)	2.07	3.4	7.1	9.6	1.4	21.7
Globulins, freeze-dried, Stokes (III)	2.18	3.6	7.0	10.1	1.7	22.4

[a] Kjeldahl nitrogen of solutions adjusted to standard volume.
[b] Areas are corrected for radial dilution and are expressed in arbitrary units.

corresponding peaks of the pattern for water-extractable proteins to yield the composition of the globulin fractions in area units (Table I). The resulting calculated composition of the globulin fraction in Table I is compared with observed compositions for three different globulin preparations (I, II, and III), all isolated from aliquots of the same water extract.

The first globulin sample (I) was not dried and was made by adjusting the water extract to pH 4.5, centrifuging, dispersing the precipitate in buffer, dialyzing, adjusting to standard volume, and centrifuging before analysis. The second (II) was isolated like the first, but then frozen in a carbon dioxide-alcohol bath, and dried in a small laboratory freeze-dryer. The third (III) was also isolated like the first, but was frozen in a deep-freeze, and dried in a Stokes freeze-dryer. Both freeze-dried preparations were dispersed in buffer, dialyzed, adjusted to standard volume, and centrifuged before analysis. On the basis of nitrogen content and total area of the ultracentrifuge pattern, 17.2 and 18.2%, respectively, of the globulins in preparation I are insolubilized by merely precipitating them at pH 4.5. Further changes that occur upon subsequent freeze-drying (samples II and III) are small. The major fractions insolubilized by the acidification step are the 2S and 7S; approximately 34 and 15% of these, respectively, are sensitive to this treatment. Additional evidence for sensitivity of these two fractions to pH 4.5 treatment was obtained by comparing the ultracentrifuge patterns for a water extract of the meal and for an aliquot of the same water extract that was titrated to pH 4.5 with hydrochloric acid before equilibrating against buffer. In the latter approximately 13% of the water-extractable protein remained insoluble after equi-

libration against the buffer, and areas of the 2S and 7S peaks were decreased.

Effect of Isopropanol Concentration on Denaturation. Earlier studies (4,5) demonstrated that alcohol treatment of soybean globulins renders a portion of the proteins insoluble in buffer (pH 7.6, ionic strength 0.5, 0.01*M* mercaptoethanol). The proteins insoluble in buffer are presumed to be denatured, and this criterion is used as a measure of denaturation in this work. A single batch of globulins (sample IV) was used in all of the isopropanol denaturation studies. Sample IV had a solubility in buffer of 70% as compared to 82% calculated for sample III (Table I) which was prepared under similar conditions. However, sample IV had been stored at room temperature for about a year; thus some insolubilization may have occurred during storage. The percentage of sample IV remaining soluble in buffer after a 2-hr. exposure to isopropanol concentrations ranging from 0 to 100% is shown in Fig. 1. As the isopropanol concentration is increased to

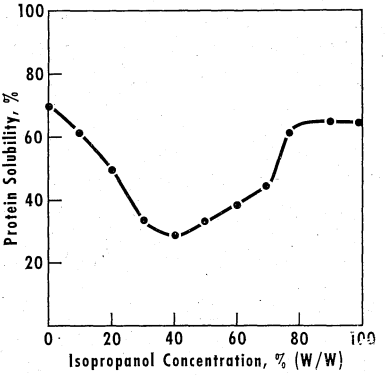

Fig. 1. Solubility of soybean globulins in buffer as a function of isopropanol concentration after 2-hr. treatment at 25°C.

40%, protein solubility decreases to a minimum and then increases again to a nearly constant value of 65% at 80–100% isopropanol. Figure 2 shows ultracentrifuge patterns obtained for the globulins remaining soluble after the 2-hr. treatment at 25°C. with several isopropanol concentrations. Ultracentrifugal compositions for the soluble portion of globulin sample IV before and after treatment with 40% and with 77% isopropanol are given in Table II.

Figure 3 shows solubilities of the ultracentrifugal components of the globulin mixture in buffer after 2-hr. treatment with 0–100% isopropanol at 25°C. The solubilities in Fig. 3 are expressed as the percent of each component remaining soluble after treatment with

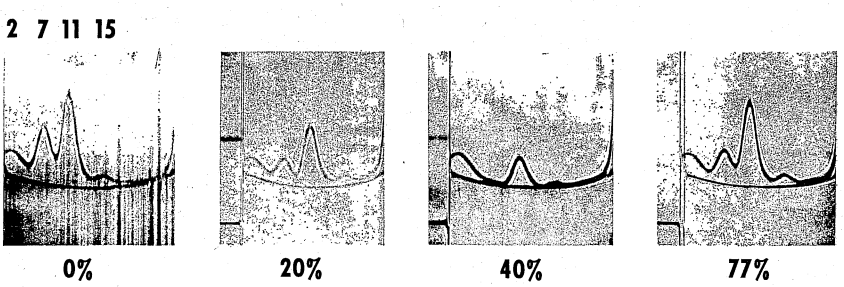

Fig. 2. Ultracentrifuge patterns for proteins remaining soluble after the 2-hr. treatment at 25°C. with 0, 20, 40, and 77% isopropanol. Ultracentrifugal compositions for the soluble proteins after treatment with 0, 40, and 77% isopropanol are given in Table II.

TABLE II

ULTRACENTRIFUGAL COMPOSITION OF HYDROXYLAPATITE CHROMATOGRAPHY FRACTIONS

Concentration of Isopropanol Used	Chromatography Data			Ultracentrifugal Composition			
	Fraction	Area [a]	Percent of Total	2S	7S	11S	15S
%			%	%	%	%	%
0	Total	47.8	100	16	29	44	11
	A	5.2	11	100	..	..	..
	B	10.4	22	76	24	..	..
	C	16.7	35	4	22	63	11
	D	15.5	32	..	51	36	13
40	Total	22.2	100	44	7	42	7
	A	5.2	23	100		..	..
	B	7.1	32	70	30	..	..
	C	9.9	45	8	4	73	15
77	Total	37.5	100	20	21	46	13
	A	4.9	13	100	..	..	..
	B	8.4	22	78	22	..	..
	C	16.1	43	..	12	67	21
	D	8.1	22	..	42	34	24

[a] Area under chromatography elution diagram in arbitrary units. These areas are directly comparable for the three experiments.

isopropanol as compared to the total amount of each component present in starting globulin preparation. These values were obtained by measuring the total area under the ultracentrifuge pattern for the untreated globulin preparation having a solubility of 70%, and calculating the total area corresponding to 100% solubility. The area each component would have in the completely soluble sample was then calculated, assuming the following composition (based on the area measurements by difference in Table I): 22%, 2S; 31%, 7S; 40%, 11S; and 7%, 15S. The percent solubility of each component was obtained by comparison of measured areas with the areas calculated for 100% solubility of the globulin preparation. The values for the un-

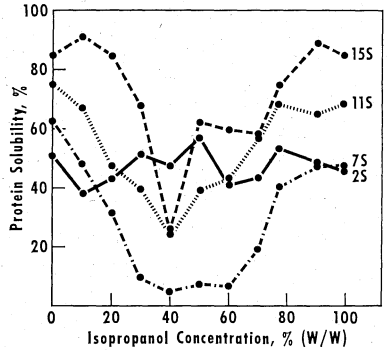

Fig. 3. Solubility of ultracentrifugal components in buffer as a function of isopropanol concentration after the 2-hr. treatment at 25°C.

treated sample (zero isopropanol concentration in Fig. 3) again show that the 2S and 7S fractions were insolubilized to the greatest extent during isolation and storage of the proteins. Significant amounts of the 11S and 15S fractions were also insolubilized. As isopropanol concentration increases from 0 to 40%, the 7S, 11S, and 15S fractions show a marked decrease in solubility. At higher isopropanol concentrations denaturation becomes progressively less, but the 7S fraction shows a broad minimum in its solubility curve from 30 to 60% alcohol. The 11S and 15S fractions show a sharp minimum at 40% isopropanol, whereas the 2S fraction fluctuates around a median value near 50% solubility over the entire range of alcohol concentrations. Area measurements of the 2S fraction are difficult to make, since this peak is broad and is poorly resolved from the 7S fraction. The fluctuation observed in solubility of the 2S fraction may be caused by random errors in measurement.

Figure 4 shows chromatographic behavior on hydroxylapatite columns for the globulins remaining soluble in buffer before and after isopropanol treatment (2 hr. at 25°C.). The untreated globulins consist of four major fractions, A to D, whose ultracentrifugal compositions are shown in Table II. Since fraction D has a tendency to tail excessively, complete elution is effected by adding *M* potassium phosphate, pH 7.6, after collecting 200 ml. of effluent. Treatment of the globulins with 40% isopropanol causes a decrease in fractions B and C and completely eliminates fraction D, which consists primarily of a 7S component. Treatment with 77% isopropanol causes only minor changes, but fractions B and D are decreased (Table II). Fraction A, which contains only 2S component, is not affected by isopropanol (Table II) in agreement with Fig. 3, which shows that the 2S component is undenatured at 25°C.

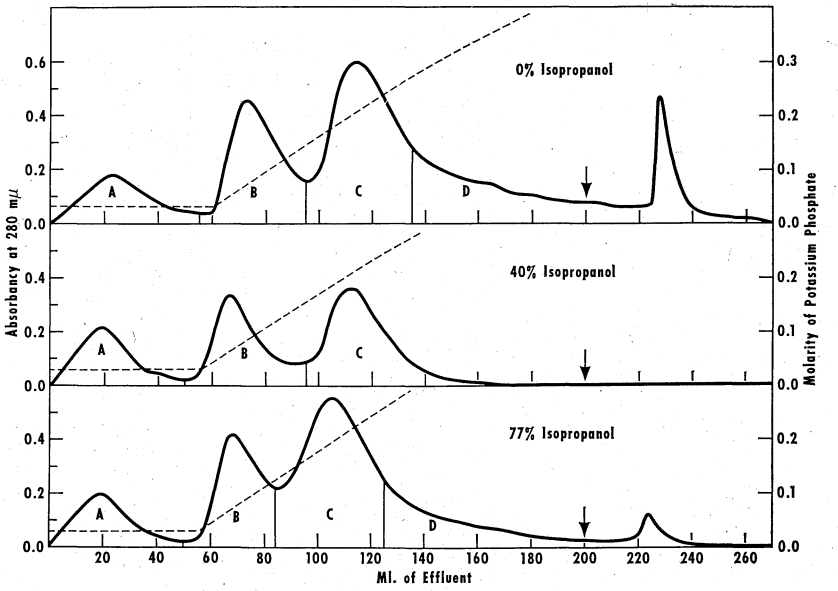

Fig. 4. Hydroxylapatite chromatography elution diagrams for soluble soybean globulins after treatment with 0, 40, and 77% isopropanol for 2 hr. at 25°C. Respective column loads of protein (N × 6.25) were 193, 87, and 171 mg. Solid curve is absorbancy, dashed curve is concentration of eluting buffer in the effluent, and vertical arrows are points at which *M* potassium phosphate was added.

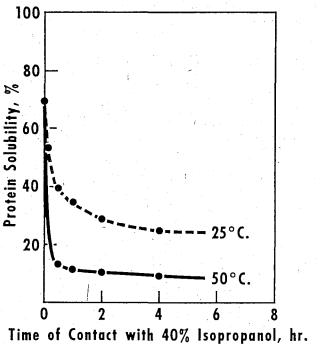

Fig. 5. Protein solubility as a function of time of treatment with 40% isopropanol at two temperatures.

Effects of Time and Temperature. Figures 5 and 6 show the effects of time and temperature of isopropanol treatment at concentrations of 40 and 77% on the solubility of the entire globulin mixture in buffer. Under all conditions studied, there is a rapid decrease in solubility during the first hour of treatment, followed by a very slow decrease in solubility up to times as long as 16–24 hr. The curves obtained upon

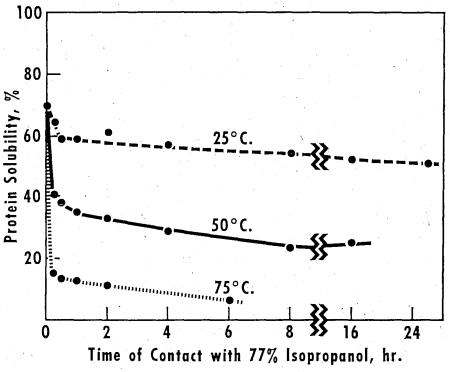

Fig. 6. Protein solubility as a function of time of treatment with 77% isopropanol at three temperatures.

treatment at 50° and 75°C. with 77% isopropanol are nearly the same as the curves obtained with 40% isopropanol treatment at 25° and 50°C. Use of 40% isopropanol thus is approximately equivalent to the use of 77% isopropanol at a temperature 25° higher.

Figure 7 shows changes in the solubilities of the individual ultra-

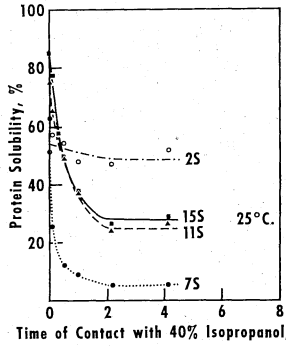

Fig. 7. Solubility of ultracentrifugal components as a function of time of contact with 40% isopropanol at 25°C.

centrifugal components in buffer as a function of time of treatment with 40% isopropanol at 25°C. The method of calculation of solubilities was similar to that described for Fig. 3. The 7S fraction decreases rapidly in solubility to a value of about 5% of its original concentration. The 11S and 15S are also denatured extensively but at a slower rate than the 7S fraction, and they level off at higher solubility values. By contrast, the 2S fraction shows little, if any, decrease in solubility

in agreement with Fig. 3. However, the 2S fraction is sensitive to alcohol at higher temperatures. For example, 0.5-hr. treatment with 40% isopropanol at 50°C. insolubilizes all the proteins except the 2S fraction, which is slowly insolubilized on continued time of treatment (Fig. 5). With 77% isopropanol at 75°C., approximately 2 hr. of contact with the alcohol is required to denature completely all the proteins except the 2S fraction; the 2S fraction is also slowly insolubilized, as indicated by the portion of the 75°C. curve between 2 and 6 hr. in Fig. 6.

Effect of pH and Other Factors. In all the experiments described, the proteins were at pH 4.5 when treated with isopropanol. Several experiments were performed by adding an amount of concentrated ammonium hydroxide to the isopropanol solution such that when the dry, alcohol-treated proteins were suspended in water, a pH of 7.6 was obtained. A protein sample treated in this manner at pH 7.6 for 2 hr. at 25°C. with 77% isopropanol was 58% soluble in buffer, which is essentially the same as obtained at pH 4.5 (61%, Fig. 1), and there were no significant changes in the ultracentrifugal composition as compared with a sample treated at pH 4.5.

Treatment at pH 7.6 with 40% isopropanol was more complex. Under these conditions part of the protein dissolved in the alcohol, and the insoluble portion became highly hydrated, necessitating centrifugation to remove the alcohol (2-hr. treatment at 25.C). The results are shown in schematic outline in Fig. 8. Approximately 24%

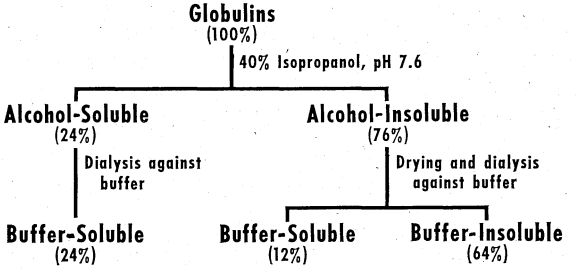

Fig. 8. Solubility of soybean globulins denatured with 40% isopropanol at pH 7.6.

of the total globulin fraction dissolved in 40% isopropanol remained soluble upon dialysis of the alcohol solution against buffer, and consisted of 70% 2S component and 30% of material sedimenting as a very diffuse boundary through the 7S and 11S range. Only a portion of the protein insoluble in 40% isopropanol dissolved in buffer; it represented 12% of the total globulins and consisted of 25%, 2S; 57%, 11S; and 18%, 15S components. The ultracentrifugal composition of

the total buffer-soluble protein (sum of alcohol-soluble plus alcohol-insoluble protein) was approximately the same as the buffer-soluble protein obtained after 2-hr. denaturation with 40% isopropanol at pH 4.5. The sum of the solubilities of the alcohol-soluble plus alcohol-insoluble proteins in buffer after denaturation at pH 7.6 was 36% compared to 29% at pH 4.5 where none of the proteins dissolved in alcohol.

The effect of sulfhydryl and disulfide reagents was investigated briefly. In one experiment a globulin sample was titrated to pH 7.0 with dilute alkali, adjusted to 0.01*M* with N-ethylmaleimide, and then poured into isopropanol (25°C.) to give a final concentration of 77% alcohol. Time of contact with alcohol was approximately 4 hr. Comparison of the N-ethylmaleimide-treated protein with a control treated identically except for addition of N-ethylmaleimide showed no significant differences in solubility in buffer or ultracentrifugal composition. Treatment of soybean globulins with either 40 or 77% isopropanol containing 0.01*M* mercaptoethanol (pH 4.5, for 2 hr. at 25°C.) likewise had no significant effect on either protein solubility or ultracentrifugal composition of the buffer-soluble portion of the globulins.

Discussion

The partial insolubility of freeze-dried soybean globulins, observed earlier (4), can now be attributed primarily to a sensitivity of the 2S and 7S fractions to precipitation at pH 4.5 (Table I). Subsequent freeze-drying has relatively little effect. Sensitivity of the 2S fraction to precipitation at pH 4.5 is surprising, because the 2S fraction remaining soluble after the isolation procedure resists isopropanol denaturation at 25°C. (Figs. 3 and 7). This resistance suggests the presence of two 2S components: (a) one which is sensitive to precipitation at pH 4.5 and (b) one which is stable to precipitation at pH 4.5 and also stable to isopropanol treatment at room temperature.

Roberts and Briggs (5) did not report insoluble protein in their globulin preparation. However, they redissolved their protein sample at pH 7.6 and clarified by centrifuging before freeze-drying. The clarification step probably eliminated the insoluble protein encountered in our studies. The composition of the globulin fraction reported by Roberts and Briggs is similar to compositions calculated from area measurements of the soluble portion of our preparations in Table I.

The effect of isopropanol concentration upon protein solubility (Fig. 1) is similar to that observed by Smith *et al.* (2) on extractability of the proteins from isopropanol-treated soybean meal. The nonprotein meal constituents and the differences in pH between soybean

meal (~pH 6.5) and the isoelectric-precipitated proteins appear to be minor factors in denaturation of the globulins by isopropanol. Results of ultracentrifugal analyses (Figs. 2, 3, and 7) clearly show that the 7S fraction is the most readily alcohol-denatured fraction of soybean globulins in agreement with the ethanol denaturation studies of Roberts and Briggs (5) and our earlier studies (4).

The greater susceptibility of the 7S fraction to denaturation by isopropanol is also demonstrated by hydroxylapatite chromatography of the proteins remaining soluble in buffer after alcohol treatment (Fig. 4 and Table II). The untreated protein contains 7S components eluted in fractions B, C, and D with a high concentration in the last fraction. When the globulins are treated with 40% isopropanol, fraction D is completely denatured, and the residual 7S component (approximately 5% of the original 7S fraction, Fig. 3) elutes mainly (84% based on data in Table II) in fraction B.

The mechanism involved in alcohol denaturation of soybean globulins is obscure. Our limited experiments with sulfhydryl and disulfide reagents indicate that aggregation through a sulfhydryl-disulfide interchange reaction is not an important factor. Preliminary experiments indicate that the isopropanol-denatured globulins are soluble in 4*M* guanidine hydrochloride or 8*M* urea; these results suggest the absence of extensive covalent cross-linkages, such as disulfide bonds in the denatured proteins.

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