Gelation Phenomena of Soybean Globulins. II. Protein-Water Miscible Solvent Interactions

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ABSTRACT

Dispersions of soybean globulins in water-alcohol and water-glycol mixtures form gels of higher apparent viscosity than do water dispersions. Rheological data indicate that the length of the aliphatic chain of the alcohol and the degree of branching affect the apparent viscosity of the gel significantly. The apparent viscosity increases with the length of the aliphatic chain. A branched-chain alcohol produces lower viscosity gels than does a linear-chain alcohol of the same number of carbon atoms. Glycols are less effective gel inducers, and the resulting apparent viscosities of the gels are much lower than those obtained with alcohols. The effect of the dielectric constant of the medium is very pronounced. However, for the same dielectric increment value, different values of apparent viscosity are produced depending on the alcohol or glycol. This is an additional indication that, besides the dielectric effect of the medium, the abilities of alcohols and glycols to unfold protein molecules may play an important role in the formation of the gel. Mixtures of water with acetone, dioxan, dimethyl formamide, and dimethyl sulfoxide had a marked enhancing effect on gel viscosity. However, only acetone and dioxan affected the progel viscosity, probably as a result of denaturation. Dimethyl formamide and dimethyl sulfoxide increased the gel viscosity probably through hydrogen bonding.

The effect of water-miscible organic solvents on the denaturation of soybean globulins has been studied by several investigators (1-7). Aqueous solutions of alcohols reduce the solubility of soybean globulin components (1,2). The 7S component is denatured very rapidly with ethanol-water mixtures of alcohol concentration above 20% (3). The 11S and 15S components are denatured slowly, and the 2S component is not denatured at all. Similar results were obtained with isopropanol-water mixtures (4). Maximum denaturation occurs with 40% isopropanol, and the 7S component shows the greatest susceptibility to denaturation. Increasing the temperature during alcohol treatment causes all of the components to be denatured at a faster rate including denaturation of the 2S component. Finally, the dependence of the denaturation of the 11S component on the hydrophobicity of the alcohols employed has been demonstrated (5,6). The ability of the alcohols to disrupt the internal structure of the protein molecules was related to the length of the aliphatic chain, the position of branching, and the hydrocarbon content of the alcohol molecule. Fukushima (7) has also shown that the denaturing power of several water-miscible solvents increased with addition of water up to a certain ratio of water to solvent in the mixture.

Work in this laboratory (8,9,10) has indicated that the rheological properties of soybean globulin gels provide a means for the study of soybean protein-protein and protein-lipid interactions at high concentrations and temperatures. The present paper shows the effect of alcohols, glycols, and other water-miscible solvents on such interactions in an attempt to contribute to a better understanding of the mechanism of the gelation phenomena.

MATERIALS AND METHODS

Materials

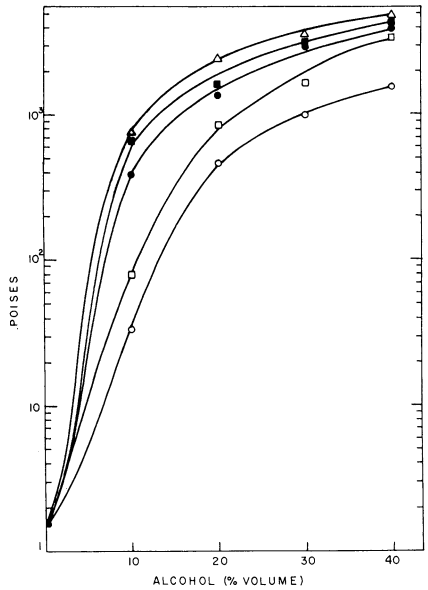
Soybean globulins were prepared by aqueous extraction of defatted soybean flakes. The extracts were centrifuged, and the pH of the supernatant was adjusted to 4.5. The precipitated globulins were washed thoroughly with water, neutralized, and spray-dried (8). The alcohols and glycols were Fisher certified reagent grade.

Gelation

Dispersions of the protein were made up on a weight percentage basis. Water-solvent mixtures were prepared before the addition of protein. A Sorvall Omni-Mixer, operated at full speed and room temperature, was used to ensure complete dispersion, and entrapped air was removed by centrifugation (9). The dispersions were placed in 25 X 150-mm. stoppered test tubes and heated at the specified temperatures in a water bath. After heating, the samples were removed immediately and cooled at 4°C. (9).

Viscosity Measurements

Viscosities were determined with the Brookfield Synchro-Lectric viscometer model LVT. The Helipath stand with the series T spindles was used in all experiments (8).



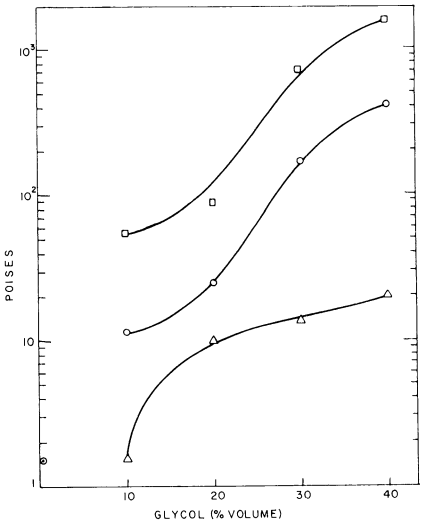


Fig. 1 (left). Effect of alcohol concentration on the apparent viscosity of soybean globulin gels (6% protein) formed by heating at 80°C. for 30 min. and cooled at 4°C. for 1 hr. Key: open circles, methanol; open squares, ethanol, open triangles, 1-propanol; solid circles, 2-propanol; solid squares, *tert*-butanol.

Fig. 2 (right). Effect of glycol concentration on the apparent viscosity of soybean globulin gels (6% protein) formed by heating at 80°C. for 30 min. and cooled at 4°C. for 1 hr. Key: circles, ethylene glycol; squares, propylene glycol; triangles, glycerol.

RESULTS

Effect of Alcohol Concentration

In the present studies the following water-soluble alcohols were used: methanol, ethanol, 1-propanol, 2-propanol, and *tert*-butanol. Results shown in Fig. 1 were obtained with a 6% dispersion of soybean globulins in different concentrations of the alcohols in water. The mixtures were heated at 80°C. for 30 min. and cooled at 4°C. for 1 hr. Apparent viscosity data were plotted as a function of alcohol concentration (% volume). The presence of the alcohols increased the viscosity of the gel in the order 1-propanol > *tert*-butanol > 2-propanol > ethanol > methanol. Also a good correlation was observed between viscosity and alcohol concentration.

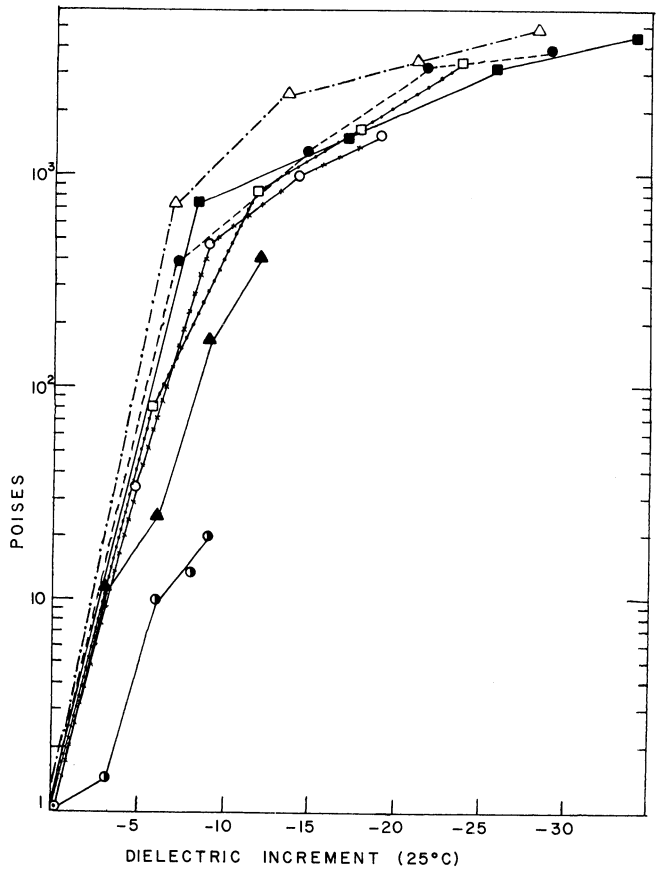


Fig. 3. Effect of the dielectric increment of the solvent on the apparent viscosity of soybean globulin gels (6% protein) formed by heating at 80° C. for 30 min. and cooled at 4° C. for 1 hr. Key: open circles, methanol; open squares, ethanol; solid circles, 2-propanol; solid squares, *tert*-butanol; open triangles, 1-propanol; solid triangles, ethylene glycol; open/solid circles, glycerol.

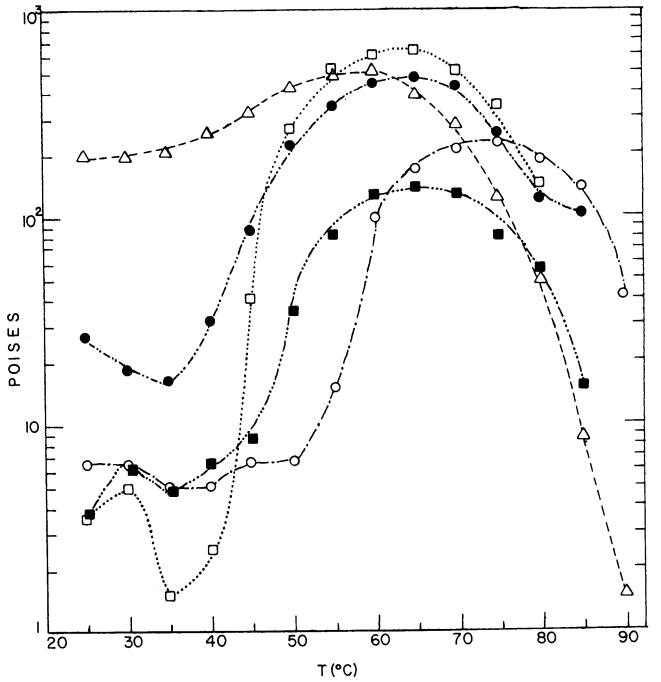


Fig. 4. Effect of temperature on the apparent viscosity of progel (6% protein) in the presence of 4M alcohol. Key: open circles, methanol; open squares, ethanol; open triangles, 1-propanol; solid circles, 2-propanol; solid squares, *tert*-butanol.

Maximum viscosities were obtained at 40% alcohol volume content. About 50% alcohol in water (v./v.) the soy globulins precipitate and no gel is obtained.

Effect of Glycol Concentration

Ethylene glycol, propylene glycol, and glycerol are miscible with water and their effect on gel formation was studied in comparison with the alcohols (Fig. 2). Soybean globulin dispersions (6%) were heated in the presence of different concentrations of glycol at 80°C. for 30 min. and cooled at 4°C. for 1 hr. The viscosities were of the order propylene glycol > ethylene glycol > glycerol. Significantly lower viscosities were obtained with glycols in comparison to alcohols. The effect of propylene glycol was rather similar to that of methanol.

Dielectric Constant Effect

The viscosity data shown in Figs. 1 and 2 were plotted as a function of the dielectric increment due to the presence of solvent (Fig. 3). In general, decrease of the dielectric constant is associated with higher observed viscosities. However, it is significant that for the same dielectric increment, different values of viscosity were obtained depending on the alcohol or glycol. This is an indication that the dielectric effect, although very pronounced, is not the only factor determining the strength of the gel.

Effect of Temperature on the Viscosities of Progel and Gel in the Presence of Alcohols

Soybean globulin dispersions (6%) in the presence of 4M alcohol, pH 7, were heated at the rate of 0.5°C. per min. At each experimental temperature (internal temperature of the progel), a tube was removed from the water bath and the viscosity was determined immediately while a duplicate sample (heated at the same temperature) was stored at 4°C. and cooled for 1 hr. Viscosities measured at the indicated ambient internal temperature were taken as those of the progel state and the viscosities obtained after cooling as representative of the gel state. Figures 4 and 5 show the changes in viscosity of the progel and gel, respectively, as a function of temperature. Maximum viscosities for the progel were observed at different temperatures for each alcohol. Invariably, the viscosity of the progel increased to a maximum value and then dropped significantly on overheating. The maximum viscosities in the presence of ethanol, 1-propanol, and 2-propanol were almost of

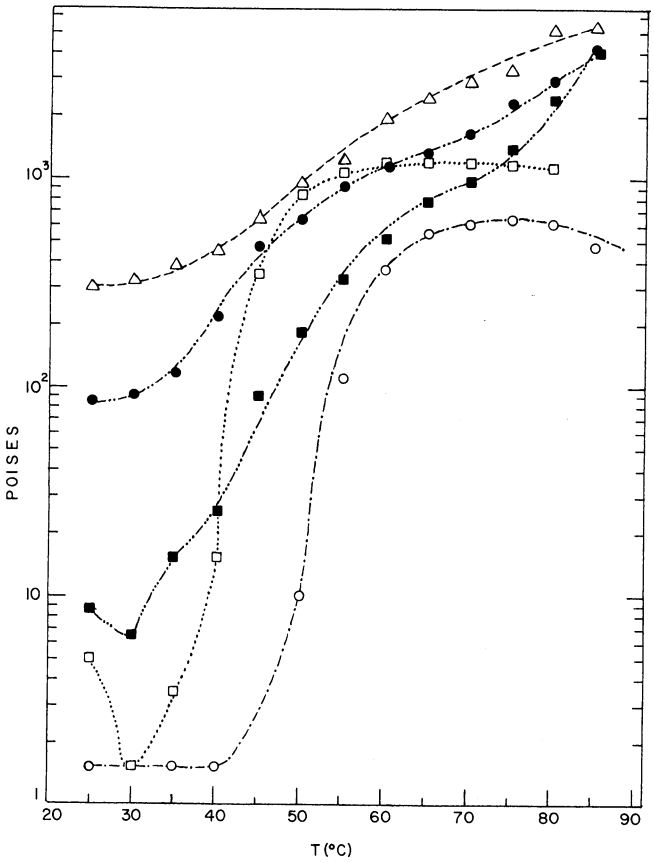


Fig. 5. Effect of temperature of heating of progel on the apparent viscosity of gel (6% protein) in the presence of 4M alcohol. Key: open circles, methanol; open squares, ethanol; open triangles, 1-propanol; solid circles, 2-propanol; solid squares, *tert*-butanol.

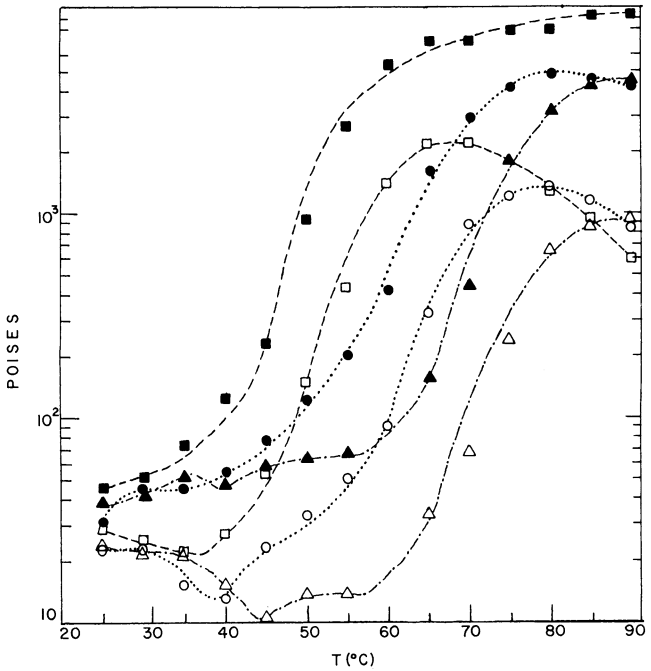


Fig. 6. Effect of temperature on the apparent viscosity of progel and gel (6% protein) in the presence of 4M glycol. Key: open circles, ethylene glycol (progel); open squares, propylene glycol (progel); open triangles, glycerol (progel); solid circles, ethylene glycol (gel); solid squares, propylene glycol (gel); solid triangles, glycerol (gel).

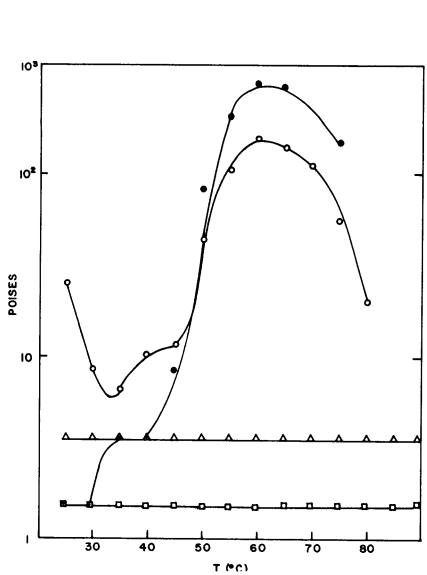
the same magnitude, whereas those in the presence of methanol and *tert*-butanol were much lower. In the gel state, the effect of the length and branching of the hydrophobic chain was very pronounced. The viscosities obtained in the presence of the straight-chain alcohols followed the order 1-propanol > ethanol > methanol. 2-Propanol exhibited higher viscosities than *tert*-butanol but both were less effective than 1-propanol. Thus, increased branching of the hydrophobic chain results in reduction of the gel strength.

Effect of Temperature on the Viscosities of Progel and Gel in the Presence of Glycols

Figure 6 shows the results obtained in a similar experiment as that described above but in the presence of ethylene glycol, propylene glycol, and glycerol. The protein concentration was 6% and that of glycols 4M. The viscosities of the gel were again higher than those of the progel. Maximum viscosities obtained at different temperatures followed the order propylene glycol > ethylene glycol > glycerol.

Effect of Other Water-Miscible Organic Solvents on Gelation

Soybean globulin dispersions (6%) were heated at different temperatures in the presence of 3M concentrations of the following solvents: dioxan, acetone,



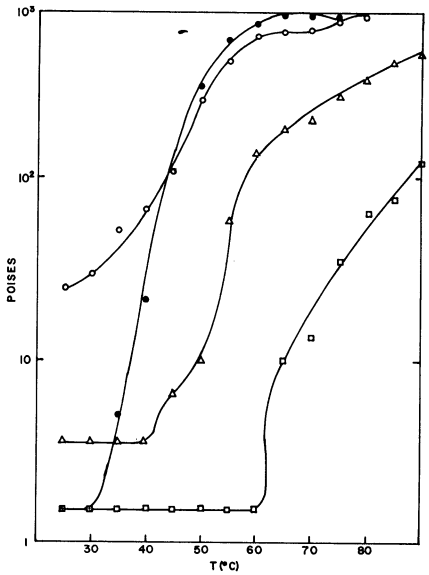


Fig. 7 (left). Effect of temperature on the apparent viscosity of progel (6% protein) in the presence of 3M water-miscible organic solvent. Key: open circles, dioxan; open squares, dimethyl sulfoxide; open triangles, dimethyl formamide; solid circles, acetone.

Fig. 8 (right). Effect of temperature on the apparent viscosity of gel (6% protein) in the presence of 3M water-miscible organic solvent. Key: open circles, dioxan; open squares, dimethyl sulfoxide; open triangles, dimethyl formamide; solid circles, acetone.

dimethyl formamide, and dimethyl sulfoxide. Figures 7 and 8 show the results of the effect of these solvents on progel and gel formation, respectively. It appears that dimethyl formamide and dimethyl sulfoxide inhibit progel formation but enhance gel formation probably through hydrogen bonding. Acetone and dioxan have a marked effect on both progel and gel formation. This may indicate that acetone and dioxan, besides being able to form hydrogen bonds, may also exhibit an unfolding effect on the soybean proteins.

DISCUSSION

It was suggested by Ferry (11) that gelation of globular proteins involves an unfolding of the molecule, exposure of reactive groups, and cross-link association of the random chains in a three-dimensional network. The bonds responsible for the formation and stability of the gel structure could be covalent, noncovalent (hydrogen and hydrophobic), or electrostatic in nature. Furthermore, the total concentration of the protein must be such that the probability of intermolecular contacts will be close to that of intramolecular contacts for an effective overlapping of two associated chains (12).

Previous work in this laboratory (9) demonstrated that soybean globulin dispersions (concentration $> 8\%$) are activated by heat to the progel state which is characterized by an increase in apparent viscosity measured at the ambient

temperature of the progel. Formation of the gel is effected by cooling of the progel and is accompanied by additional increase in viscosity.

In the present study it was considered desirable to investigate the effect of alcohols and glycols on the gelation of soybean globulins for the following reasons: (a) increased concentration of alcohol or glycol in water will lower the dielectric constant of the medium, (b) the aliphatic part of the alcohol and glycol molecules are capable of hydrophobic bonding, (c) increase in the length of the aliphatic chain should increase the hydrophobic site attachments, (d) beyond a certain proportion of alcohol or glycol to water, the hydrophobic contributions will be diminished and intermolecular hydrogen bonds between protein molecules will be favored (13).

Results of the present study indicate that the length of the aliphatic chain of alcohols and the degree of branching affect significantly the macroscopic viscosity of the gel. The viscosity increases with the length of the aliphatic chain. A branched chain is less effective than a linear chain of the same number of carbon atoms. This phenomenon could be explained in terms of hydrophobic interactions of the heat-exposed nonpolar protein groups with the aliphatic part of the alcohol (14). However, since hydrophobic bonds are favored by increase in temperature (15), the most informative experimental data are expected to be those of the progel formation as a function of temperature. The viscosity obtained with *tert*-butanol is lower than that with methanol. In addition, no significant difference was found in the maximum viscosity obtained in the presence of 1-propanol, 2-propanol, and ethanol. Thus it appears that the size of the hydrophobic groups of the alcohols does not affect significantly the viscosity of the progel. However, the formation of the gel (which occurs during cooling) shows a very good relation to the chain length of the alcohol. This might be related to the degree of unfolding that each alcohol exerts on the protein during the heating stage. The exposed polar groups may interact during the cooling of the progel by way of hydrogen bonding to form gels of varying viscosity reflecting the action of the individual alcohol. In support of this consideration are the results obtained with glycols and other water-miscible organic solvents. Glycols are less effective in unfolding globular proteins (16,17,18) and the resulting viscosities of the gels are much lower than those obtained with alcohols. Apparently, acetone and dioxan have a denaturing effect on soybean proteins, whereas dimethyl sulfoxide and dimethyl formamide contribute to the viscosity of the gels by way of hydrogen bonding.

In regard to the dielectric constant of the medium, it is very significant that for the same dielectric increment value, different values of viscosity are obtained depending on the alcohol or glycol. This is an additional indication that besides the dielectric effect, which is very pronounced in general, the unfolding abilities of the alcohols and glycols play an important role in the formation of the gel.

At high concentrations of solvent in water ($> 40\%$), soybean globulins aggregate and no gel is obtained. It appears that the depletion of water from the medium weakens the hydrophobic bonds that stabilize the tertiary structure of globulins (13). The absence of hydrophobic interactions can cause disorientation of the internal organization of the protein molecules. At this stage the predominant forces may be the intra- and intermolecular hydrogen and ionic bonds which cause protein aggregation and precipitation. In the presence of sufficient water, intermolecular hydrogen bonds are preferentially formed between protein and water. But if water

is depleted below a critical level, these bonds may be formed between neighboring protein molecules, thus greatly facilitating the aggregation process. Fukushima (7) has also discussed possible mechanisms of denaturation of soybean proteins by water-miscible organic solvents which are in general agreement with data presented in this work.

Literature Cited

1. MANN, R. L., and BRIGGS, D. R. Effects of solvent and heat-treatments on soybean proteins as evidenced by electrophoretic analysis. Cereal Chem. 27: 258 (1950).
2. SMITH, A. K., JOHNSEN, V. L., and DERGES, R. E. Denaturation of soybean protein with alcohols and with acetone. Cereal Chem. 28: 325 (1951).
3. ROBERTS, R. C., and BRIGGS, D. R. Characteristics of the various soybean globulin components with respect to denaturation by ethanol. Cereal Chem. 40: 450 (1963).
4. WOLF, W. J., SLY, DAYLE ANN, and BABCOCK, G. E. Denaturation of soybean globulins by aqueous isopropanol. Cereal Chem. 41: 328 (1964).
5. FUKUSHIMA, D. Internal structure of soybean protein molecule (11S protein) in aqueous solution. J. Biochem. (Tokyo) 57: 822 (1965).
6. FUKUSHIMA, D. Internal structure of 7S and 11S globulin molecules in soybean proteins. Cereal Chem. 45: 203 (1968).
7. FUKUSHIMA, D. Denaturation of soybean proteins by organic solvents. Cereal Chem. 46: 156 (1969).
8. CIRCLE, S. J., MEYER, E. W., and WHITNEY, R. W. Rheology of soy protein dispersions. Effect of heat and other factors on gelation. Cereal Chem. 41: 157 (1964).
9. CATSIMPOOLAS, N., and MEYER, E. W. Gelation phenomena of soybean globulins. I. Protein-protein interactions. Cereal Chem. 47: 559 (1970).
10. CATSIMPOOLAS, N., and MEYER, E. W. Gelation phenomena of soybean globulins. III. Protein-lipid interactions. Cereal Chem. 48: 1275 (1971).
11. FERRY, J. D. Protein gels. Advan. Protein Chem. 4: 1 (1948).
12. VEIS, A. The macromolecular chemistry of gelatin, p. 359. Academic Press: New York (1964).
13. TANFORD, C. Contribution of hydrophobic interactions to the stability of the globular conformation of proteins. J. Am. Chem. Soc. 84: 4240 (1962).
14. HILL, R. L., and SMITH, E. L. Leucine aminopeptidase. VI. Inhibition by alcohols and other compounds. J. Biol. Chem. 224: 209 (1957).
15. SCHERAGA, H. A. Intramolecular bonds in proteins. II. Noncovalent bonds. In: The proteins, ed. by H. Neurath, second ed., p. 528. Academic Press: New York (1963).
16. TANFORD, C., and DE, P. K. The unfolding of β-lactoglobulin at pH 3 by urea, formamide, and other organic substances. J. Biol. Chem. 236: 1711 (1961).
17. TANFORD, C., BUCKLEY, C. E., III, DE, P. K., and LIVELY, E. P. Effect of ethylene glycol on the conformation of γ-globulin and β-lactoglobulin. J. Biol. Chem. 237: 1168 (1962).
18. VON HIPPEL, P. H., and WONG, K. Y. On the conformational stability of globular proteins. J. Biol. Chem. 240: 3909 (1965).

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