CHAPTER 4

Purification and Properties of the Proteins

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INTRODUCTION

Work done in the past 20 yr shows that proteins prepared by earlier workers were mixtures and that several of the proteins undergo complex reactions. At least seven soybean proteins now appear to be made up of subunits, which may be disrupted under a variety of conditions. Because of their subunit structure the major soybean proteins have molecular weights ranging from about 200,000 to 600,000. In the native state, these large molecules can form still higher particle sizes either through association-dissociation reactions or by forming disulfide-linked polymers. Because of this complexity it is necessary to fractionate soybean proteins before detailed studies are made on them.

This chapter describes progress made in the isolation and characterization of soybean proteins in the past two decades and points out where further work is needed. Coverage is mainly on "storage proteins," but biologically active proteins, such as trypsin inhibitors and hemagglutinins that are also discussed in Chap. 6, are included for completeness. A detailed summary of the chemistry of soybean proteins up to 1948–1949 was compiled by Circle (1950). Brief reviews since 1948–1949 are also available (Wolf and Smith 1961; Bain et al. 1961; Wolf 1969A, 1970A).

NOMENCLATURE

At present there is no nomenclature system generally accepted for soybean proteins. However, this problem is currently under study and a detailed discussion of past terminology, as well as proposals under consideration, can be found elsewhere (Wolf 1969B). Some of the names being considered have recently been introduced (Catsimpoolas 1969A; Catsimpoolas and Ekenstam 1969), but a final decision on terminology has not been reached.

The nomenclature system based on approximate sedimentation coefficients as introduced by Naismith (1955) has been used extensively in the past decade. Because of its adoption by many workers this terminology, as exemplified in Fig. 4.1 is used here. Figure 4.1 also illustrates a shortcoming of the ultracentrifuge terminology: Sedimentation properties of soybean proteins depend on conditions of buffer composition, pH, and other factors. For example, a portion of the 7S fraction observed at pH 7.6, 0.5 ionic strength, dimerizes at 0.1 ionic strength to form a 9S peak, as indicated at the bottom of Fig. 4.1.
FIG. 4.1. EFFECTS OF IONIC STRENGTH ON THE ULTRACENTRIFUGE PATTERN FOR WATER-EXTRACTABLE SOYBEAN PROTEINS AT pH 7.6

SOYBEANS: CHEMISTRY AND TECHNOLOGY

SUBCELLULAR STRUCTURE

Many seeds, particularly those rich in protein and oil, contain numerous subcellular inclusions that are the storage sites for proteins, lipids, and other constituents. The protein storage particles are called protein bodies or aleurone grains while lipid deposits are called spherosomes. Protein bodies and spherosomes have been identified in soybean cotyledons by electron microscopy (Bils and Howell 1963; Saio and Watanabe 1966, 1968; Tombs 1967). Figure 4.2 shows an electron micrograph of a soybean cotyledon in which these structural elements are identified. The protein bodies vary from 2 to 20 μ in diameter (Tombs 1967), but many fall into the narrower range of about 5–8 μ (Fig. 4.2). The spherosomes are interspersed between the protein bodies and are about 0.2–0.5 μ in diameter.

Soybean protein bodies have been isolated by three procedures. Saio and Watanabe (1966) homogenized soybeans in cottonseed oil and then separated the protein bodies by differential centrifugation in cottonseed oil-carbon tetrachloride mixtures. Composition of their protein bodies is given in Table 4.1.

Tombs isolated protein bodies from hexane-extracted soybean flour (350 mesh) by sucrose density gradient centrifugation at pH 5, the pH of minimum solubility of the major proteins, to prevent disruption of the protein bodies. The protein bodies often sediment as 2 bands; a light fraction (density less than 1.30) and a heavy fraction (density less than 1.32). Analyses of the total protein bodies and the two fractions are given in Table 4.1, plus a partial analysis of the starting soybean flour for comparative purposes. Figure 4.3 shows scanning electron micrographs of protein bodies in soybean flour and after isolation by Tombs' procedure.
FIG. 4.2. ELECTRON MICROGRAPH OF A SECTION OF MATURE SOYBEAN COTYLEDON

Seed was soaked in water overnight, fixed with osmium tetroxide, and stained with uranyl acetate and lead citrate. Protein bodies (PB), spherosomes (S), and cell wall (CW) are identified.

TABLE 4.1

COMPOSITION OF SOYBEAN PROTEIN BODIES

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Total Preparation</th>
<th>Preparation</th>
<th>Defatted Soybean</th>
<th>Preparation No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Prep (%)</td>
<td>Light (%)</td>
<td>Heavy (%)</td>
<td>Soybean (%)</td>
</tr>
<tr>
<td>Protein (N x 5.8)</td>
<td>65.0</td>
<td>82.5</td>
<td>97.5</td>
<td>78.5</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>0.94</td>
<td>0.48</td>
<td>0.84</td>
<td>0.90</td>
</tr>
<tr>
<td>Ribonucleic acid</td>
<td>0.53</td>
<td>1.29</td>
<td>0.43</td>
<td>2.04</td>
</tr>
<tr>
<td>Phospholipid</td>
<td></td>
<td>1.0</td>
<td></td>
<td>2.25</td>
</tr>
<tr>
<td>Total lipid</td>
<td></td>
<td>11.3</td>
<td>1.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Phytoic acid</td>
<td></td>
<td>1.35</td>
<td>2.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>8.5</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>7.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>82.7</td>
<td>99.3</td>
<td>100.8</td>
<td>87.4</td>
</tr>
</tbody>
</table>

1 Calculated on a moisture-free basis.
2 Nitrogen-to-protein conversion factor based on amino acid composition data (Tombs 1967).
3 Saio and Watanabe (1966).
4 Tombs (1967).
5 Komoda et al. (1968).
The third method for preparation of protein bodies was reported by Komoda et al. (1968). They soaked soybeans overnight in water, homogenized them in 0.5 M sucrose, and centrifuged to obtain a pellet fraction believed to be protein bodies.

The protein bodies isolated by the three different procedures differ widely in protein content (Table 4.1). The preparations of Saio and Watanabe approach the heavy fraction of Tombs in protein content. The pellet fractions of Komoda et al., however, are considerably lower in protein content than the protein bodies obtained by the other two methods. Tombs' heavy fraction contained cell wall fragments and cytoplasmic attachments while his light fraction appeared to be almost completely protein. The preparations reported by Komoda et al. appear low in protein because of the high lipid contents (23–60%). Since no electron microscopic data are given, it is not clear whether the lipids represent contamination by spherosomes or are associated with the protein bodies. The latter might be removed from the preparations of Saio and Watanabe during the carbon tetrachloride centrifugation, whereas Tombs used hexane-defatted meal for his studies. Attempts to avoid defatting are reported, but intact soybean cotyledons are difficult to homogenize and intractable emulsions of the protein bodies and spherosomes form (Tombs 1967).

Proteins from the protein bodies and the water-extractable proteins from defatted meal differ little by gel filtration and polyacrylamide gel electrophoresis (Saio and Watanabe 1966). Apparently, isolation of the protein bodies does not result in a pronounced fractionation on the basis of differences in distribution of proteins in the protein bodies and the cytoplasm. This conclusion is supported by Tombs' data, which indicate that at least 60–70% of the total protein is...
stored in the protein bodies. Although polyacrylamide gel electrophoresis suggested that only the 11S ultracentrifugal component was located in the protein bodies (Tombs 1967), Catsimpoolas et al. (1968A) observed at least six components in protein bodies by disc immunoelectrophoresis. Disc electrophoretic patterns for the cotyledon proteins and the proteins of isolated protein bodies differed slightly.

Ultracentrifuge patterns of the protein body proteins likewise are similar to patterns of water-extractable proteins from defatted meal; 2S, 7S, 11S, and 15S fractions are present, but there is a reduced concentration of 2S fraction in the protein bodies (Wolf 1970B). The latter result supports Tombs' studies showing that trypsin inhibitor (a protein of the 2S fraction of the water-extractable proteins) is located in the cytoplasm rather than in the protein bodies.

Properties of soybean proteins known at present are largely based on preparations isolated by extracting defatted soybean meal or flakes with aqueous solvents. For example, globulins prepared by acid-precipitation contain low molecular weight compounds that may not be associated with the proteins in vivo but that may have interacted with the proteins during the initial water extraction of the meal (Nash et al. 1967). Such interactions might be prevented by isolating the protein bodies before extracting the proteins. Information about the merits of isolating the proteins by extraction of protein bodies versus the classical procedure of extracting defatted meal is needed, and also knowledge of the subcellular location of nonprotein constituents would be helpful.

The “prepackaging” of the majority of the proteins in soybeans suggests the possibility of developing methods of milling and separation whereby the protein bodies could be isolated in relatively pure form on an industrial scale. Such a process preferably would not use water so as to eliminate a waste disposal problem (soybean whey) associated with present methods for processing soybean protein isolates and certain protein concentrates.

PROTEIN EXTRACTION

Extractability of soybean proteins is influenced by a variety of factors including moist heat treatment (toasting) of the meal, method of oil extraction, particle size, meal age, temperature, solvent-to-meal ratio, pH, and salt concentration. Because these factors are reviewed in detail by Circle (1950), emphasis here will be only on extraction conditions used in basic studies. Work reported since 1950 is included where appropriate.

Preparation of Flakes and Meal

Soybean hulls contain water-soluble pigments, including anthocyanins, which may interact with proteins during their extraction from the meal (Smiley and Smith 1946). Soybeans should therefore be cracked and dehulled if the

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1 Also see previously unpublished data by O. A. Krober cited in Chap. 2.
required equipment is available. After dehulling, cracked beans are ground or preferably flaked and extracted with hexane or diethyl ether at or near room temperature. Moist heat and solvents like alcohols and acetone should be avoided if maximum extractability of the proteins is desired (Belter and Smith 1952; Smith et al. 1951). The defatted flakes may be used directly or may be ground in a hammer mill before aqueous extraction of the proteins.

Recent studies by Smith et al. (1966) should be consulted for details about preparation of undenatured meal. They extracted 90–95% of the total nitrogenous constituents with water (pH 6.5–6.8) or dilute alkali (pH 7.2) from carefully prepared meals. Defatted meals can be stored at room temperature, but protein extractability slowly decreases as the meals age (Smith and Circle 1938; Nash et al. 1971). Methods for preventing or minimizing the decrease in protein extractability on aging are still unknown.

Extraction of Meal

Extraction Solvents.—A large variety of aqueous solvents has been used to extract proteins from defatted soybean meal (Circle 1950). In many studies only the percentage of the total meal protein extracted was reported; few characterization studies are available on the proteins extracted with different solvents. Of all the solvents tried, water, water plus dilute alkali (pH 7–9), and aqueous solutions of sodium chloride (0.5–2 M) are among the most efficient for extracting proteins (Smith and Circle 1938; Smith et al. 1938, 1966). Because these solvents are also mild, they should yield the proteins in an undenatured state. Ultracentrifugal studies failed to show significant differences between water and 1 M sodium chloride extracts of defatted meal (Wolf and Briggs 1956). Likewise, tris-citrate buffer and water extracts of defatted meal were similar by starch gel electrophoresis (Shibasaki and Okubo 1966).

Meal-to-Solvent Ratio.—For many laboratory studies a 1:10 meal:solvent extraction is adequate with or without a second extraction at a 1:5 ratio. A 1:20 or 1:40 ratio may yield a greater amount of the total protein but also results in more dilute solutions for subsequent fractionation steps. An exception to the 1:10 meal:solvent ratio is the 1:5 meal:water ratio recommended for isolation of crude 11S component (cold-insoluble fraction) by cryoprecipitation (Briggs and Wolf 1957; Wolf and Sly 1967).

Extraction Temperature.—Aqueous extracts of defatted meal are generally prepared at room temperature. In a few studies extractions were made at refrigerator temperatures (Danielsson 1949), but no advantages are known for working at low temperatures except for enzyme isolation (Wang and Anderson 1969). In cryoprecipitation of crude 13 protein, a 1:5 meal:water extract at room temperature (25° C) is saturated with respect to 11S component. If the extract is prepared at 40° C, more of the 11S component dissolves; hence, greater yields of 11S protein are obtained when the extract is cooled to 0–4° C (Wolf and Sly 1967).
Effect of pH.—Distilled water extracts of defatted soybean meal have a pH of about 6.4–6.6. Extraction at higher pH by adding alkali increases the amount of protein extracted by 5–10%, but lowering the pH drastically reduces the amount of extractable protein (Fig. 4.4). A minimum in protein solubility exists between pH 4 and 5, the isoelectric region for the major proteins. Extracts can also be made at pH values below the isoelectric region, but irreversible changes in the 11S protein, the major protein, have been noted at pH 2–3 (Wolf and Briggs 1958; Catsimpoolas et al. 1969A). Accordingly, extractions in the pH range of 6.5–9.0 are recommended.

FRACTIONATION METHODS

Quaternary structures of the major soybean proteins limit the rigor of fractionation procedures that can be applied. If conditions are used that dissociate the quaternary structures into subunits, one is faced with separating a larger number of different molecules whose relationship to the parent proteins (subunit assemblies) is unknown. Many of the conditions required to disrupt quaternary structures also cause irreversible conformation changes in the subunits. Fractionation methods, therefore, must be sufficiently mild to maintain the quaternary structures intact. A variety of fractionation techniques have been applied to soybean proteins, but most give only partial separations. Consequently, purification of a given protein requires a combination of two or more methods based on different properties of the protein.
Fractional Precipitation

Isoelectric Precipitation.—Precipitation of soybean proteins from aqueous or alkaline extracts by acidification was placed on a firm basis by Smith and Circle (1938) in their studies on the relationship between pH and solubility of the proteins (Fig. 4.4). The minimum in solubility at about pH 4.2 corresponds to the apparent isoelectric point of the major proteins. Adjustment of water or dilute sodium hydroxide extracts of defatted meal to pH 4.0-4.2 precipitates about 90% of the extracted protein. Isoelectric precipitation is therefore useful for concentrating globulins and for separating them from such minor constituents as sugars and salts, which are extracted from the meal together with the proteins. Acid precipitation also separates globulins from minor proteins found in the pH 4.2-soluble fraction or whey (Smith et al. 1955). Disadvantages of isoelectric precipitation of the proteins particularly from an aqueous extract of defatted meal include: (a) little, if any, fractionation of the globulins; (b) combination of phytate and possibly other low molecular-weight compounds with the globulins (McKinney et al. 1949; Smith and Rackis 1957); and (c) insolubilization of some of the proteins (Wolf et al. 1964; Nash and Wolf 1967; Nash et al. 1971). Because soybeans contain proteolytic enzymes with pH optima of 5.0-5.4 (Pinsky and Grossman 1969), exposure of the proteins to this pH region may also result in their modification through proteolysis.

Moving boundary electrophoresis showed that isoelectrically precipitated protein is a mixture of at least 4 to 5 components (Briggs and Mann 1950). In the ultracentrifuge, acid-precipitated proteins separate into 4 distinct fractions with sedimentation coefficients of 2, 7, 11, and 15S; the sedimentation pattern differs only slightly from the pattern for the unfractioanted proteins obtained by extraction of meal with 10% sodium chloride (Naismith 1955). Whey proteins consist of a portion of the 2S and 7S fractions observed in a water extract of defatted meal (Wolf and Briggs 1959; Eldridge et al. 1966). Acid-precipitated proteins are also heterogeneous by hydroxylapatite chromatography (Wolf and Sly 1965) and by starch gel electrophoresis (Puski and Melnychyn 1968; Shibasaki and Okubo 1966).

Use of Metal Cations.—For centuries metal cations, particularly calcium and magnesium, have been used as precipitants for heated soybean proteins in making tofu in the Orient. Smith et al. (1938) found that 0.0175 N calcium chloride precipitated about 80% of the proteins in a water extract of defatted meal but they did not characterize the proteins which precipitated. Briggs and Mann (1950) found that addition of calcium chloride to aqueous meal extracts after removal of the cold-precipitable protein (also referred to as cold-insoluble fraction and now known to be mainly 11S ultracentrifugal component) caused additional protein to precipitate. Wolf and Briggs (1959) showed that the protein precipitated by calcium ion is mainly 11S component. A more detailed study revealed that the 11S and 15S fractions are quantitatively precipitated by
adding calcium chloride to 0.1 \( N \) and then cooling (Wolf and Sly 1967). However under these conditions, about 1/3 of the 2S and 1/2 of the 7S fraction are also precipitated. This method is therefore attractive if the remaining 2S and 7S fractions are desired. Following this technique Koshiyama (1965) purified a 7S globulin. Closely related to precipitation of soybean proteins by calcium ions is the use of calcium chloride to fractionally extract the proteins from defatted meal (see Fractional Extraction).

Cryoprecipitation.—One of the simplest and mildest methods for partially purifying the 11S protein consists of making a concentrated water extract (with a high meal:water extraction ratio) of defatted soybean meal at 25-40° C and then cooling the extract to near 0° C. The extract clouds up and a precipitate forms that can be removed by centrifuging in the cold (Briggs and Mann 1950; Briggs and Wolf 1957). This process, called cryoprecipitation, yields a protein fraction (cold-precipitable protein or cold-insoluble fraction) that appears homogeneous by moving boundary electrophoresis but comprises up to four fractions by ultracentrifugation (Naismith 1955; Wolf and Briggs 1959). The 11S ultracentrifuge component, however, may make up 69-88% of the cryoprecipitate (Wolf and Sly 1967). Cryoprecipitation is the method of choice for initial purification of the 11S protein.

High concentrations of sodium chloride or sucrose inhibit cryoprecipitation. The 11S component can be precipitated almost completely from a water extract by adjusting the extract to 0.1 \( N \) with calcium chloride or to pH 5.4 before cooling. Under these conditions the cryoprecipitate includes increased amounts of the 2S, 7S, and 15S fractions as compared to the precipitate obtained in the absence of calcium chloride at pH 6.4-6.6 (Wolf and Sly 1967).

Although the 15S fraction is a minor component in the water-extractable proteins, it invariably occurs with the 11S protein in the cryoprecipitate. This behavior indicates that the 15S fraction is also a cryoprotein. If the cold-insoluble fraction is dissolved at 25° C in pH 4.6 acetate buffer, 0.5 ionic strength, and then cooled to 0°-2° C, the 15S fraction precipitates quantitatively along with a part of the 11S protein. This procedure, with some additional steps, can be used to purify the 11S protein as obtained by cryoprecipitation from a water extract. Preparations of 11S with purities ranging from 89 to 94% result (Eldridge and Wolf 1967; Wolf and Tamura 1969).

Ammonium Sulfate Precipitation.—Naismith (1955) fractionated a 10% sodium chloride extract of defatted soybean meal by ammonium sulfate precipitation. He obtained a small fraction at 50% saturation and 5 additional fractions between 50 and 85% saturation. All six fractions were mixtures when analyzed by ultracentrifugation. Part of the heterogeneity may be caused by disulfide polymerization of the 7S and 11S components since the analysis buffer did not contain mercaptoethanol. Naismith studied the sedimentation behavior of partially purified 7S and 11S fractions at different ionic strengths and pH values.
Fractionation of crude 11S protein (cold-insoluble fraction) between 51 and 66% saturation with ammonium sulfate at pH 7.6, followed by precipitation at pH 4.0 between 26 and 40% saturation, yields 11S preparations of about 90% purity (Wolf et al. 1962).

Shvarts and Vaintraub (1967) purified 11S protein by zone precipitation on a Sephadex G-100 column with an ammonium sulfate gradient from 40 to 73% saturation.

Acid-precipitated globulins can be fractionated by ammonium sulfate precipitation to give a 7S globulin of 85% purity with 2S and 11S contaminants. The 2S impurity is removed by gel filtration (Roberts and Briggs 1965).

A number of minor proteins from soybean whey (fraction soluble at pH 4.5) that have been purified by ammonium sulfate precipitation are listed in Table 4.2.

Fractionation With Organic Solvents.—Descriptions of soybean proteins fractionated by precipitation with organic solvents, such as alcohols and acetone, appear in only a few instances. These include a crude trypsin inhibitor (Bowman 1946; Birk et al. 1963), lipoxygenase (Mitsuda et al. 1967) and hemagglutinin (Liener 1953). Because soybean proteins, in common with many other proteins, are sensitive to organic solvents, such precipitants must be used with caution (Smith et al. 1951; Roberts and Briggs 1963; Wolf et al. 1964; Fukushima 1969A).

Fractional Extraction

Neutral Salts.—Soybean meal proteins exhibit unusual solubilities in neutral salt solutions (Smith et al. 1938). Such solutions extract less protein from meal than does water, and in dilute salt solutions the solubility curve shows a sharp minimum at a salt concentration that varies with the salt used. Ultracentrifugal

**TABLE 4.2**

SOYBEAN WHEY PROTEINS PURIFIED BY AMMONIUM SULFATE PRECIPITATION

<table>
<thead>
<tr>
<th>Protein</th>
<th>Saturation Limits</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>0.40-0.65</td>
<td>Mayer et al. (1961)</td>
</tr>
<tr>
<td>β-Amylase</td>
<td>33-50</td>
<td>Gertler and Birk (1965)</td>
</tr>
<tr>
<td>Hemagglutinin</td>
<td>0.40-0.70</td>
<td>Lis et al. (1966B)</td>
</tr>
<tr>
<td>Lipoxygenase</td>
<td>40-60</td>
<td>Mitsuda et al. (1967)</td>
</tr>
<tr>
<td>Chalcone-flavanone isomerase</td>
<td>45-60</td>
<td>Moustafa and Wong (1967)</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.40-0.76</td>
<td>Fridman et al. (1968)</td>
</tr>
<tr>
<td>Allantoicase</td>
<td>35-70</td>
<td>Wang and Anderson (1969)</td>
</tr>
</tbody>
</table>

1 Numbers expressed as decimals are fractional saturation values, whereas others are percentage saturations.
analyses of the proteins extracted by different concentrations of sodium chloride and calcium chloride showed that lowered extractability of protein results primarily from decreased solubility of the 11S and 15S fractions (Wolf and Briggs 1956). Reduction in extractability of these fractions was more pronounced with calcium chloride than with sodium chloride. It is therefore possible to fractionate soybean meal proteins by selective extraction with salt solutions, but this approach has not been studied further.

Salts at pH 4.5.—When soybean meal is dispersed in water with sufficient hydrochloric acid to reach pH 4.5, only about 10% of the nitrogenous compounds will dissolve. If either sodium chloride or calcium chloride is added to the dilute acid, the amount of nitrogen extracted increases linearly with increasing salt concentration. Extraction levels off at 65% of the meal nitrogen with either 0.7 N sodium chloride or 0.3 N calcium chloride. Acid without salt extracts only 2S and 7S components, but the amounts of these fractions extracted increase and reach maxima as the salt concentration is raised. At higher salt concentrations the 11S and 15S fractions also begin to dissolve but do not reach maximum extractabilities until after extraction of the 2S and 7S fractions is complete (Anderson and Wolf 1967).

Acid-precipitated globulins can be extracted at pH 4.8 with increasing concentrations of sodium chloride to yield 11S preparations approaching 90% purity (Wolf and Briggs 1959). Extraction of the acid curd with 0.2 N salt removes most of the 2S and 7S globulins, and subsequent extractions with 0.35 N salt solubilize the 11S component. Approximately 20% of the total protein in the acid curd failed to dissolve in phosphate buffer (pH 7.6, ionic strength 0.5, 0.01 M mercaptoethanol) as noted in other studies (Wolf et al 1964; Nash and Wolf 1967).

Chromatography

Hydroxylapatite.—Chromatography of water-extractable proteins, acid-precipitated globulins, cold-insoluble fraction, and several other soybean proteins on hydroxylapatite is described by Wolf and Sly (1965). This procedure yields a clean separation of a portion of the 2S fraction from the other ultracentrifuge fractions and reveals that the 7S fraction consists of at least two different proteins. Both 7S and 11S globulins of 80-85% purity are obtainable by chromatography on this adsorbent, but the 11S and 15S fractions do not separate from each other. Other soybean proteins that have been purified by hydroxylapatite chromatography include β-amylose (Gertler and Birk 1965), cytochrome c (Fridman et al. 1968), hemagglutinin (Lis et al. 1966B), and 2S globulins (Vaintraub 1965; Vaintraub and Shutov 1969).

Elution from hydroxylapatite can be effected by a gradient (Wolf and Sly 1965) or a stepwise (Wolf and Sly 1967) procedure. Chromatographic resolution is influenced by buffer cations (Wolf and Sly 1964) and slow flow rates are recommended (Vaintraub 1965; Koshiyama 1968A).
Modified Polysaccharides.—Ion-exchange chromatography of soybean proteins on modified polysaccharides is described in numerous papers, but most applications deal with separations of only minor proteins like those of soybean whey. Selected examples are cited to illustrate applications of the methods.

Dietzylaminoethyl (DEAE)-cellulose and DEAE-Sephadex.—Chromatography of whey proteins on DEAE-cellulose revealed more than 13 components in the mixture. Proteins identified include hemagglutinin, β-amylase, phosphatase, and trypsin inhibitors (Rackis et al. 1959). Subsequently 4 trypsin inhibitor fractions (Rackis and Anderson 1964) and 4 hemagglutinins were isolated (Lis et al. 1966A) by DEAE-cellulose chromatography. Separation of the four hemagglutinins illustrates the resolving power of DEAE-cellulose when proper conditions are known since these proteins were not separated by carboxymethylcellulose or hydroxylapatite chromatography or by polyacrylamide gel electrophoresis. Four hemagglutinins with different isoelectric points have also been isolated by isoelectric focusing, but they were immunochemically identical (Catsimpoolas and Meyer 1969).

A 2.3S and a 2.8S protein were purified by chromatography on DEAE-cellulose after preliminary fractionation of the globulins by ammonium sulfate precipitation and hydroxylapatite chromatography (Vaintraub and Shuto 1969).

Chromatography of cold-insoluble fraction on DEAE-cellulose at pH 7.6 yielded 11S preparations of 85–90% purity with 7S and 15S fractions still present (Wolf et al. 1962). Differences in charge between the various proteins apparently are too small to effect separations under the conditions used.

Chromatography of urea-treated 11S protein on DEAF cellulose in 4 M urea was used by Vaintraub (1967), Okubo and Shibasaki (1967), and Okubo et al. (1969) to separate protein subunits. Despite differences in pH, buffer concentration, and column size employed in the two laboratories, the results appear in good agreement with each other. Okubo and Shibasaki also used this technique to separate subunits of a 7S protein into two major fractions.

Catsimpoolas et al. (1967) utilized DEAE-Sephadex chromatography to purify 11S protein obtained by ammonium sulfate fractionation of cold-insoluble fraction (Wolf et al. 1962). Since the resulting 11S protein was homogeneous by disc electrophoresis, DEAE-Sephadex was presumed to remove the 15S contaminant normally present in the ammonium sulfate-fractionated preparation. Chromatography on DEAE-cellulose, however, failed to remove the 15S contaminant (Wolf et al. 1962).

Catsimpoolas and Ekenstam (1969) chose DEAE-Sephadex to isolate two proteins, designated β-conglycinin and γ-conglycinin on the basis of immunoelectrophoresis. Beta-conglycinin is proposed to be the major component of a 7S protein isolated by Roberts and Briggs (1965), whereas γ-conglycinin makes up the 7S protein prepared by Koshiyama (1965).
**Carboxymethyl (CM)-cellulose and CM-Sephadex.**—Birk *et al.* (1963) isolated the acetone-insoluble trypsin inhibitor (Bowman 1946) by CM-cellulose chromatography slightly below the isoelectric point of the inhibitor. Gertler and Birk (1965) found CM-cellulose useful for removing trypsin inhibitor and lipoxigenase from β-amylase, whereas Lis *et al.* (1966A) separated inactive proteins from hemagglutinins with this adsorbent. However, the multiple forms of hemagglutinin were not resolved from each other on CM-cellulose.

CM-Sephadex chromatography is the last purification step in the preparation of crystalline lipoxigenase (Mitsuda *et al.* 1967).

**Ion-exchange Resins.**—Ion-exchange resins have been used to only a limited extent for chromatography of soybean proteins. Cytochrome c from soybeans was purified on Amberlite-50 by the classical procedure for isolating this enzyme from other sources (Fridman *et al.* 1968). A glycopeptide with a molecular weight of about 4600 was obtained by Pronase digestion of hemagglutinin, gel filtration, and ion-exchange chromatography on Dowex-50 (Lis *et al.* 1966B). A glycopeptide of about 9870 in molecular weight was isolated in a similar manner from a 7S globulin (Koshiyama 1969A).

**Gel Filtration.**—The broad range of molecular sizes for soybean proteins is best demonstrated by gel filtration of the water-extractable proteins on Sephadex G-200. Hasegawa *et al.* (1963) observed 7 protein peaks in the effluent when water-extractable proteins were placed on a 200-cm column (Fig. 4.5A) as compared to only 4 fractions that are resolved by the ultracentrifuge (Fig. 4.5B). Peak 1 was attributed primarily to turbidity, but this fraction has an absorption maximum at 260 μm and gives a positive test for ribose with orcinol (Okubo and Shibasaki 1967; Obara and Kimura 1967). These results indicate that peak 1 contains ribonucleic acid. Ultracentrifugal compositions for other peaks are shown in Fig. 4.5B. The 1S protein eluted almost completely in peak 3 but was accompanied by a 7S fraction. Peaks 4, 5, and 6 also contained proteins of the 7S group while peaks 7 and 8 consisted of 2S proteins. Peaks 9-11 did not appear to contain protein.

Gel filtration of the high-molecular-weight portion of soybean globulins (corresponding to peaks 1 to 4 of Fig. 4.5A) showed that 7S protein occurred in peaks 2 to 4 (Koshiyama 1969B). The 7S protein was of 2 types: 1 dimerized when the ionic strength was changed from 0.5 to 0.1 while the other 7S protein did not. The dimerizing variety of 7S occurred in peaks 2 to 4 but was concentrated in peak 3; the leading half of peak 3 contained only this type of 7S protein. The nondimerizing 7S protein occurred in peak 2, the trailing half of peak 3 and peak 4. The dimerizing 7S protein represented about 75% of the total 7S protein and about 25% of the total protein in the globulin subfraction. These estimates are comparable with values for the dimerizing 7S in the water-extractable proteins: 60% of the total 7S and 20% of the total protein (Wolf and Sly 1967; Wolf 1969B). Heterogeneity of the 7S fraction is also demonstrated
by hydroxylapatite chromatography (Wolf and Sly 1965). From these results it is apparent that the 11S component is the major protein of soybeans.

Elution of 7S proteins with the 15S and 11S fractions (peaks 2 and 3 of Fig. 4.5A) is surprising because of the large differences in sedimentation rates for the 3 proteins. Further work is needed to explain this behavior of the 7S proteins.

Koshiyama (1965, 1968A) isolated the dimerizing 7S globulin and demonstrated that it corresponded to the protein that elutes with the 11S protein (peak 2, Fig. 4.5A) from Sephadex G-200. A synthetic mixture of 7S and 11S proteins tended to separate only slightly. Recycling gel filtration will probably separate the two proteins, but the process is slow, and yields of 11S are low (Eldridge and Wolf 1967). Column length is an important factor for resolution of the high-molecular-weight fractions.
Other uses of Sephadex G-200 include characterization of protein bodies (Saio and Watanabe 1966), heat denaturation of soybean proteins (Saio et al. 1968B), and purification of chalcone-flavanone isomerase (Moustafa and Wong 1967). Gel filtration of soybean whey proteins on Sephadex G-100 yielded six major fractions, which were heterogeneous by disc electrophoresis (Catsimpoolas and Leuthner 1969A); this technique should be a useful adjunct to other methods for fractionating these proteins.

Sephadex G-25 and G-50 easily separate glycopeptides from pronase digests of soybean hemagglutinin (Lis et al. 1966B) and of 7S globulin (Koshiyama 1969A).

CHMICAL PROPERTIES OF SOYBEAN PROTEINS

Nitrogen Content

Table 4.3 lists nitrogen contents for unfractionated proteins, several fractions, and various purified proteins. Since the purified proteins differ in nitrogen

TABLE 4.3
NITROGEN CONTENT OF SOYBEAN PROTEINS

<table>
<thead>
<tr>
<th>Protein Preparation</th>
<th>Nitrogen Content (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride-extractable proteins</td>
<td>16.42</td>
<td>Wolf et al. (1966)</td>
</tr>
<tr>
<td>Water-extractable proteins</td>
<td>16.51&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Wolf et al. (1966)</td>
</tr>
<tr>
<td>Acid-precipitated globulins</td>
<td>16.20&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Wolf et al. (1966)</td>
</tr>
<tr>
<td>Acid-precipitated globulins</td>
<td>16.24</td>
<td>Nash et al. (1967)</td>
</tr>
<tr>
<td>Alcohol-extracted, acid-precipitated globulins</td>
<td>17.04</td>
<td>Nash et al. (1967)</td>
</tr>
<tr>
<td>Glycinin</td>
<td>17.45&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Osborne and Campbell (1968)</td>
</tr>
<tr>
<td>Whey proteins</td>
<td>15.22&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Wolf et al. (1966)</td>
</tr>
<tr>
<td>Cold-insoluble fraction</td>
<td>17.46</td>
<td>Wolf et al. (1966)</td>
</tr>
<tr>
<td>Cold-soluble fraction</td>
<td>16.12&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Wolf et al. (1966)</td>
</tr>
<tr>
<td>11S</td>
<td>17.62&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Wolf et al. (1966)</td>
</tr>
<tr>
<td>7S</td>
<td>15.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Roberts and Briggs (1965)</td>
</tr>
<tr>
<td>7S</td>
<td>15.91</td>
<td>Koshiyama (1968B)</td>
</tr>
<tr>
<td>Hemagglutinin</td>
<td>13.2</td>
<td>Lis et al. (1966B)</td>
</tr>
<tr>
<td>β-Amylase</td>
<td>16.3</td>
<td>Gertler and Birk (1965)</td>
</tr>
<tr>
<td>Trypsin inhibitor (Kunitz)</td>
<td>16.74</td>
<td>Kunitz (1947)</td>
</tr>
<tr>
<td>Trypsin inhibitor (Bowman-Birk)</td>
<td>15.36</td>
<td>Frattali (1969)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean of three or more preparations.
content, mixtures of the proteins will vary in nitrogen contents depending on the relative amounts of each protein present. Nitrogen contents of the proteins are also influenced by nonprotein impurities, such as phytate and alcohol-extractable materials. For example, extraction of acid-precipitated globulins with aqueous ethanol increased nitrogen content from 16.24 to 17.04% (Table 4.3).

Comparison of the nitrogen content of glycinin reported by Osborne and Campbell (1898) with other values in Table 4.3 shows that glycinin must contain mainly of 11S protein. Indeed, the nitrogen content of cold-insoluble fraction (crude 11S) most closely agrees with the value for glycinin, and the two protein preparations are similar in composition by ultracentrifugation (Naismith 1955). Inability of Smiley and Smith (1946) to obtain preparations with nitrogen values similar to those of glycinin probably resulted from their use of acid to adjust the proteins to pH 4.1-4.5 after dialysis. Under these conditions the 2S and 7S globulins are precipitated with the 11S protein; whereas during dialysis of a neutral solution, pH drops slowly toward the isoelectric region, and the 11S component is precipitated preferentially (Wolf and Briggs 1959).

Nonprotein Constituents in Soybean Proteins

Carbohydrates are present in varying amounts in many preparations of soybean proteins (Wolf et al. 1966), but only hemagglutinin (Lis et al. 1966b) and 7S globulin (Koshiyama 1969A) have been shown to be glycoproteins. Hemagglutinin contains 3-5 glucosamine and 25 mannose residues per mole as compared to 12 glucosamine and 39 mannose residues per mole in the 7S globulin. These two proteins are among those with the lowest nitrogen contents in Table 4.3.

Noncovalently bound carbohydrates have also been isolated from soybean proteins (Smiley and Smith 1946). Nash et al. (1967) removed 3.6% of the weight of soybean globulins by alcohol extraction and identified saponins and sitosterol glycoside in the extract. Other components in the alcoholic extract were phosphatidyl choline, phosphatidyl ethanolamine, genistein, triglycerides and several unidentified compounds. The significance of these nonprotein materials in soybean globulins is still unknown. The phosphatides may originate from the membranes surrounding the protein bodies (Tombs 1967). Alcohol extraction does not remove all the nonprotein compounds from globulins since additional saponins were isolated from alcohol-extracted protein (Eldridge and Wolf 1969A).

In addition to phosphatides, soybean proteins may contain other phosphorus compounds. Phytate is a major contaminant of globulins prepared by isoelectric precipitation (McKinney et al. 1949; Smith and Rackis 1957).

Interaction of phytate with soybean proteins is influenced by pH and cations as calcium. Calcium phytate, which is insoluble above pH 6, remains soluble above pH 10 when protein is present. When a mixture of calcium ion,
phytate, and protein are filtered through Sephadex G-75, calcium and phytate elute with the protein (Saio et al. 1967). Equilibrium dialysis studies with crude 11S protein confirm an interrelationship between calcium ion and phytate in the binding of these ions by the protein (Saio et al. 1968A).

Puski and Melnychyn (1968) used 3 different methods to remove phytate from acid-precipitated globulins and found 70% of the phosphorus removed by each method. The treated globulins contained about 0.2% phosphorus. This residual phosphorus is not removed by alcohol extraction although phosphatides are removed by this process (Nash et al. 1967). The residual phosphorus may be mainly ribonucleic acid. Koshiyama and Iguchi (1965) reported 3.1% ribonucleic acid for acid-precipitated globulins, a percentage which corresponds to a phosphorus content of 0.26% for the protein assuming 8.5% phosphorus in the ribonucleic acid (DiCarlo et al. 1955). Shutov and Vaintraub (1967), however, report only 0.27% ribonucleic acid in acid-precipitated soybean globulins. Further studies are needed to resolve this discrepancy.

Bai and Pin (1964) isolated from soybeans proteins which yielded serine-O-phosphate on hydrolysis. Based on isolated yields these phosphoproteins occur as minor constituents.

Amino Acid Composition

Amino Acid Analyses.—Data for purified soybean proteins are assembled in Tables 4.4 and 4.5. Included are results for defatted soybean meal, the source material for the proteins. Analyses for meal are expressed as grams of amino acid residues per 16 gm N since the nitrogen-to-protein conversion factor is unknown and since no correction was made for nonprotein nitrogen in the meal. Cytochrome c, trypsin inhibitors, β-amylase, hemagglutinin, and lipoxygenase are minor proteins but are of interest because of their biological activities.

The Bowman-Birk trypsin inhibitor is unusually high in cystine but devoid of glycine and tryptophan. In contrast to the Bowman-Birk inhibitor, hemagglutinin is free of cystine. The 7S globulins isolated by Koshiyama (1968B) and Roberts and Briggs (1965) differ significantly in overall amino acid contents and molecular weights. The two 7S proteins were, however, remarkably low in methionine content, and both underwent a characteristic monomer-dimer reaction with change in ionic strength. Gel filtration of 7S globulins on Sephadex G-200 suggests that the protein capable of monomer-dimer formation is eluted over a broader range than is expected for a single protein (Koshiyama 1969B). Possibly there are two or more 7S proteins with the ability to dimerize at low ionic strength. Catsimpoolas and Ekenstam (1969) report that the 7S globulins prepared by the methods of Koshiyama and of Roberts and Briggs are different.

See Frattali (1959) and Steiner and Frattali (1969) for a description of this protein; it appears to be the same as the 1.9S trypsin inhibitor isolated by Yamamoto and Ikenaka (1967).
### Table 4.4

**Amino Acid Composition of Purified Soybean Proteins**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Defatted Meal, (^{1}) Gm (Bowman-Birk)</th>
<th>Trypsin Inhibitor (^{2})</th>
<th>Cytochrome c (^{3})</th>
<th>Trypsin Inhibitor (^{4}) (Kunitz)</th>
<th>2.85 Globulin (^{5})</th>
<th>(\beta)-Amylase (^{6})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gm Amino Acid Residue/ 100 Gm Protein</td>
<td>Residue/ 7,975 Gm</td>
<td>Residue/ 12,000 Gm</td>
<td>Residue/ 21,500 Gm</td>
<td>Residue/ 32,600 Gm</td>
<td>Residue/ 61,700 Gm</td>
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<tr>
<td>Arginine</td>
<td>2.34</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>13</td>
<td>20</td>
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<td>Histidine</td>
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<td>2</td>
<td>2</td>
<td>12</td>
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<tr>
<td>Lysine</td>
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<td>12</td>
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<td>2</td>
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**Notes:**
- Defatted Meal: \(^{1}\) Gm
- Trypsin Inhibitor: \(^{2}\) Gm
- Cytochrome c: \(^{3}\) Gm
- Trypsin Inhibitor: \(^{4}\) Gm
- 2.85 Globulin: \(^{5}\) Gm
- \(\beta\)-Amylase: \(^{6}\) Gm

**Protein Amino Acid Residues**

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<tr>
<th>Amino Acid</th>
<th>Protein Residue/ 16 Gm N</th>
<th>100 Gm Protein</th>
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<tr>
<td>Arginine</td>
<td>7.55</td>
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</tr>
<tr>
<td>Histidine</td>
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<td>1.56</td>
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<td>Tyrosine</td>
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<td>1.17</td>
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<tr>
<td>Phenylalanine</td>
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<td>3.58</td>
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</tr>
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</tr>
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<td>Isoleucine</td>
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<tr>
<td>Valine</td>
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**Ammonia**

<table>
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**Glutamic acid**

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<td>Methionine</td>
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1 Rackis et al. (1961).
3 Fridman et al. (1968).
4 Yamamoto and Ikenaka (1967).
5 Vaintraub and Shutov (1969). Residues/mole of protein are uncorrected for hydrolysis and chromatographic losses.
6 Gertler and Birk (1965).
7 Based on presence of only one 1/2-cystine/mole.
8 Wu and Scheraga (1962).
9 Does not include cysteine values.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Defatted Meal&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Gm Amino Acid Residue/16 Gm N</th>
<th>Gm Amino Acid Residue/100 Gm Protein</th>
<th>Residues/100,000 Gm</th>
<th>Gm Amino Acid Residue/100 Gm Protein</th>
<th>Residues/108,000 Gm</th>
<th>Gm Amino Acid Residue/100 Gm Protein</th>
<th>Residues/180,000 Gm</th>
<th>Gm Amino Acid Residue/330,000 Gm Protein</th>
<th>Residues/360,000 Gm</th>
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1 Rackis et al. (1961).
2 Lis et al. (1966B).
3 Stevens et al. (1970).
4 Koshiyama (1968B).
5 Roberts and Briggs (1965).
6 Shvarts and Vaintraub (1967).
7 Does not include cysteine values.
by disc electrophoresis and disc immunoelectrophoresis. However, Catsimpoolas and Ekenstam did not examine their 7S samples for the ability to dimerize at low strength in order to demonstrate identity with the preparations of the other workers.

The most significant differences between the 7S globulins and the 11S globulins are the 5- to 6-fold higher contents of tryptophan, methionine, and 1/2-cystine in the latter.

Other amino acid analyses on soybean proteins include: acid-precipitated globulins and whey proteins (Rackis et al. 1961), 11S protein and acid-precipitated globulins (Catsimpoolas et al. 1967), partially purified 7S and 11S globulins (Fukushima 1968), 11S protein and its basic subunits (Okubo et al. 1969), and a partially purified 2.3S protein (Vaintraub and Shutov 1969).

**Sulfhydryl Content.**—Available data for cysteine contents of purified proteins are included in Tables 4.4 and 4.5. Lipoxygenase contains four sulfhydryl groups per mole, but their reaction with 5,5'-dithiobis-(2-nitro-benzoic acid) requires that the enzyme be denatured (Stevens et al. 1970). Sulfhydryl contents of the major globulins have received little attention. The 7S globulin isolated by Koshiyama (1965; 1968B) appears to contain two disulfide bonds but no sulfhydryl groups although there is a 7S protein in the globulin fraction that forms polymers presumably linked by disulfide bonds (Nash and Wolf 1967). This protein in the depolymerized state contains at least two sulfhydryl groups per mole if polymers larger than the dimer are formed.

No data are available concerning the sulfhydryl content of the 11S protein. Kelley and Pressey (1966) report about 0.6 sulfhydryl equivalents per $10^5$ gm of acid-precipitated globulins as compared to 1.0-1.2 sulfhydryl equivalents per $10^5$ gm of glycinin (mixture of 7S and 11S globulins) obtained by Smirnova et al. (1959).

**Primary Structures**

Little is known about the primary structures of soybean proteins with one exception. Ikenaka et al. (1963) reported the sequence of the first five amino acids in the N-terminal position of Kunitz' trypsin inhibitor. Subsequently, Brown et al. (1966) described the sequence of amino acids around the two disulfide bonds of the inhibitor, while Ozawa and Laskowski (1966) reported an arginyl-isoleucine sequence at the active site. Thirty-nine amino acid residues out of a total of about 200 are accounted for by the sequences known at present. Ikenaka and coworkers in Japan are doing further work on the primary sequence of this protein (Sealock and Laskowski 1969). Hopefully, the entire sequence will be determined in the near future.

For other soybean proteins only terminal amino acids are known (Table 4.6). On the basis of N-terminal amino acids the 7S and 11S molecules contain a minimum of 9 and 12 polypeptide chains, respectively. Determination of the primary structure of these multichained proteins must, therefore, be preceded by isolation and purification of the subunits.
Primary structures of hemagglutinin and 7S globulin are more complex than those of other soybean proteins because they are glycoproteins. In hemagglutinin the carbohydrate units are attached to a single aspartic acid residue, probably through amido linkage with the \(\beta\)-carboxyl carbon of aspartic acid (Lis et al. 1969). Although the mode of attachment of the sugar residues to 7S globulin is not known, the carbohydrates appear to be linked as a single unit (Koshiyama 1969A).

### Disulfide Polymerization

**Polymers in Defatted Meal.**—When water-extractable proteins are ultracentrifuged with and without 0.01 \(M\) mercaptoethanol in the analysis buffer, the mercaptan causes the fast-sedimenting materials to disappear and increases the amounts of 7S and 11S proteins (Wolf and Sly 1967). The fast-sedimenting
proteins apparently are disulfide polymers of the 7S and 11S proteins. Approximately 30-35% of the total 7S and 10-12% of the total 11S protein are polymerized in this manner. Since the polymers are observed in the extracts which have received a minimum of manipulation (dialysis against buffer), they probably pre-exist in defatted meal. It is not known whether disulfide polymerization occurs during deposition of the proteins in the seed or during subsequent aging of the seed or meal.

**Polymerization During Protein Precipitation.**—Crude 11S protein isolated by cryoprecipitation and dialysis contains soluble and insoluble polymers that are depolymerized by 0.01 M mercaptoethanol and other disulfide-cleaving reagents (Briggs and Wolf 1957). Although disulfide polymers occur in the water-extractable proteins before cryoprecipitation, formation of insoluble polymers is favored by precipitation. Cryoprecipitation causes up to 40% of the 11S protein to polymerize as compared to only about 10% polymerization with N-ethyl maleimide present during precipitation. More extensive polymerization of 11S occurs during precipitation by dialysis against distilled water; some of the polymers are insoluble in buffer after this treatment.

Insolubilization of 7S and 11S proteins as a result of disulfide polymerization occurs during isoelectric precipitation of the globulins (Nash and Wolf 1967). Insoluble disulfide polymers of the 7S and 11S globulins are also noted in glycinin (Kretovich et al. 1956; Kretovich and Smirnova 1957).

**PHYSICAL PROPERTIES OF SOYBEAN PROTEINS**

**Solubility**

Solubility of soybean proteins is sensitive to pH and salts as discussed earlier (Protein Extraction; Fig. 4.4). The globulins are insoluble in the isoelectric region at low salt concentrations, but they are appreciably soluble if salts are added (Smith and Circle 1938). For example, the 11S protein is soluble at pH 4.0 in 1 M sodium chloride but precipitates at a lower concentration of ammonium sulfate than when precipitation is carried out at pH 7.6 (Wolf et al. 1962).

Solubility of the globulins is also influenced by phytate (Smith and Rackis 1957); phytate should be removed before attempting solubility studies. Another factor that affects solubility of some soybean proteins is disulfide polymerization, previously discussed. Solubilities of acid-precipitated globulins in buffer with and without 0.01 M mercaptoethanol have been described, but results were expressed as percentages of the total globulins rather than as absolute solubilities (Wolf et al. 1963; Kelley and Pressey 1966; Nash and Wolf 1967). Phosphate buffer of pH 7.6 and 0.5 ionic strength has been used extensively in solubility studies. This buffer containing 0.01 M mercaptoethanol is often referred to as standard buffer and tends to separate native (soluble) from denatured (insoluble) forms of the proteins as noted in alcohol denaturation studies (Roberts and Briggs 1963; Wolf et al. 1964). However, solubilization of denatured proteins by
standard buffer has been noted (Nash and Wolf 1967), and about 1/2 of
heat-denatured 11S protein remains soluble in the buffer (Wolf and Tamura
1969).
Kunitz (1947) applied the phase-rule solubility test to his crystalline trypsin
inhibitor; this test indicated a high degree of purity. Nonetheless, commercial
preparations of crystalline inhibitor may be impure (Eldridge et al. 1966).
Briggs and Mann (1950) applied the phase-rule solubility test to crude 11S protein
(cold-insoluble fraction) and obtained evidence of heterogeneity. Heterogeneity
is now known to arise from non-11S contaminants and disulfide polymers of
11S component (Briggs and Wolf 1957). Solubilities of 11S protein at 0°–2° C
as a function of ionic strength at pH 4.6 (in 0.01 M mercaptoethanol) are
reported by Eldridge and Wolf (1967).

Molecular Size

Molecular weights and other physical properties of purified soybean proteins
are listed in Table 4.7. Undoubtedly there are still many unidentified proteins in
soybean meal, but it is already evident that the proteins cover a wide range of
molecular sizes. At least five proteins have been isolated and shown to be
constituents of the 2S fraction of water-extractable proteins: Bowman-Birk
trypsin inhibitor, cytochrome c, Kunitz trypsin inhibitor, and two 2S globulins.
A sixth protein, chalcone-flavanone isomerase, reportedly has an s_{20,w} value of
1.6–2S but has not been characterized further (Moustafa and Wong 1967).

Molecular weight of the Bowman-Birk trypsin inhibitor is concentration-
dependent and various values are recorded in Table 4.7. A monomer-dimer-
trimer equilibrium with a monomer molecular weight of 8000 is proposed to
explain this behavior (Millar et al. 1969). Allantoinase, β-amylase, hemagglutinin,
lipoxygenase, and 7S globulin(s) are present in the 7S fraction of water-extract-
able proteins (Table 4.7). The two 7S globulin preparations agree in their
sedimentation properties but differ in their molecular weights. Further work is
necessary to clearly establish whether the 2 isolation procedures yield different
proteins or whether the molecular weight reported by Roberts and Briggs is too
high by a factor of 2.

Only one protein corresponding to the 11S fraction of the water-extractable
proteins has been isolated; the 11S globulin can thus be considered the major
protein of soybeans. The 11S protein has a molecular weight of about 350,000,
which is typical of major globulins of other seeds.

On the basis of its sedimentation rate, the 15S fraction is estimated to have a
molecular weight of 1/2 million or more. Catsimpoolas et al. (1969A) suggest
that the 15S fraction is a polymer of glycinin (11S half-molecules⁹); a trimer
and tetramer would correspond to respective molecular weights of 525,000 and
700,000. Polymers of 11S resembling the 15S fraction have been observed (Wolf
et al. 1962; Shvarts and Vaintraub 1967), but the 15S fraction observed in the

⁹In this terminology the 11S protein would be a dimer of glycinin.
<table>
<thead>
<tr>
<th>Protein</th>
<th>$V_{20}^*$ MI/Gm</th>
<th>$s_{20,w}^0 \times 10^{13}$</th>
<th>$D_{20,w} \times 10^7$</th>
<th>Mol Wt Method</th>
<th>Isoelectric Point</th>
<th>$E_{1%}^1$ Cm 280 M$\mu$</th>
<th>$[\eta]$ Dil/Gm</th>
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<td>9.06</td>
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<td>356,000</td>
<td>LS</td>
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1 AA—Amino acid analysis; AE—approach to equilibrium; GF—gel filtration; LS—light scattering; SD—sedimentation-diffusion; SE—sedimentation-equilibrium; SV—sedimentation-viscosity.
2 Value for 1% solution.
3 Value for solution with optical density of 0.2 at 267 μm.
4 Value for 1.5% solution.
5 Values for four hemagglutinins.
6 Based on solubility measurements.
water-extractable proteins has not been isolated and characterized to establish its chemical identity with 11S protein.

The water-extractable proteins often contain a small amount of material sedimenting ahead of the 15S fraction. Designated as the >15S fraction, this diffuse boundary probably represents a mixture of proteins with molecular weights approaching 1 million. Since soybean urease has an $s_{20\,w}$ value of 18S at pH 7.0 in a sucrose density gradient (Tanis and Naylor 1968), it is one of the proteins in the >15S fraction. The >15S fraction may also contain the nucleic acid or nucleoprotein that elutes near the exclusion volume of Sephadex G-200 (Okubo and Shibasaki 1967).

**Molecular Structure and Conformation**

Information about the secondary, tertiary, and quaternary structures of most soybean proteins is limited. Kunitz trypsin inhibitor was crystallized over 20 yr ago and has received more attention than any other soybean protein, but its detailed structure is still unknown. From the standpoint of size, trypsin inhibitor is a relatively simple molecule; consequently, the task of unraveling the structures of more complex molecules, such as the 7S and 11S globulins, is even greater.

**Bowman-Birk (1.9S) Inhibitor.**—Ultraviolet difference spectral studies indicate that the two tyrosine residues are accessible to solvent in the native state; high concentrations of urea or guanidine hydrochloride cause no changes attributable to exposure of buried phenolic groups (Steiner and Frattali 1969). Circular dichroism measurements revealed little, if any, $\alpha$-helical structure (Ikeda et al. 1968; Steiner and Frattali 1969). Its high cystine content (Table 4.4), however, suggests that the molecule is extensively crosslinked and, therefore, has a definite three-dimensional structure. At concentrations of 0.1% and higher the Bowman-Birk inhibitor also possesses quaternary structure since it associates into dimer and possibly trimer. A 1% solution contains approximately equal mole fractions of monomer and dimer and 7% trimer as estimated by sedimentation equilibrium studies (Millar et al. 1969). Further study of this system by osmometry confirmed a monomer-dimer equilibrium although no trimer was indicated at concentrations up to 0.3% (Harry and Steiner 1969).

**Kunitz Trypsin Inhibitor.**—This protein possesses a single polypeptide chain crosslinked by two disulfide bonds. Intrinsic viscosity (Table 4.7), fluorescence polarization, and other physical properties indicate that the inhibitor is compact, low in asymmetry, and rigid in structure (Steiner and Frattali 1969). Circular dichroism measurements failed to detect either $\alpha$-helical or $\beta$-structures in the native protein (Jirgensons et al. 1969). Nonetheless, large changes in the circular dichroism spectra occurred after cleavage of the disulfide bonds. Reduction of both disulfide crosslinks abolishes trypsin inhibitor activity, and activity is restored by reoxidation (Steiner 1965). However, one disulfide can be reduced selectively without loss of activity (DiBella and Liener 1969). Reducing both
disulfides also increases the intrinsic viscosity of the inhibitor in buffer as a result of increased unfolding of the molecule. Further unfolding occurs when reduced or reduced-alkylated inhibitor is treated with 8 M urea; the unfolded protein has an intrinsic viscosity of 0.2 - 0.27 dl per gm and an electrophoretic mobility (in polyacrylamide gel) 0.4 - 0.5 that of the native inhibitor (Elciidge and Wolf 1969B).

When Kunitz inhibitor interacts with trypsin, the arginyl-isoleucine peptide bond is cleaved at the reactive site of the inhibitor without loss of activity. However, removal of the resulting C-terminal arginine by treatment with carboxypeptidase B destroys activity of the inhibitor (Ozawa and Laskowski 1966). The arginyl residue at the reactive center was replaced enzymatically by a lysyl group with nearly complete recovery in activity (Sealock and Laskowski 1969).

Hemagglutinin.- Practically nothing is known about the structure of this glycoprotein. The absence of cystine crosslinks (Table 4.5) suggests that the molecule may be fairly flexible and subject to conformational changes under milder conditions than are required to alter conformation of such molecules as the trypsin inhibitors. The presence of 2 N-terminal residues is indicative of 2 polypeptide chains and, therefore, 2 subunits. Disruption of the quaternary structure with phenol-acetic acid-mercaptoethanol-urea yielded two subunits as detected by disc electrophoresis (Catsimpoolas and Meyer 1969).

Lipoxygenase.—This enzyme appears to consist of two subunits of 58,000 mol wt. Dissociation into subunits occurs with guanidine hydrochloride or sodium dodecyl sulfate (Stevens et al. 1970).

7S Globulin.—Fukushima (1968) investigated the secondary and tertiary structures of a partially purified 7S globulin that appears similar to the 7S proteins isolated by Koshiyama (1968B) and Roberts and Briggs (1965) on the basis of carbohydrate and amino acid analysis. Optical rotatory dispersion and infrared measurements failed to detect appreciable α-helical structure but suggested that antiparallel β-structure and disordered regions predominated in the molecule. Only about 40% of the peptide hydrogens exchanged with deuterium. Fukushima concluded that the molecules are folded compactly even though large regions of disordered (nonhelical) structure occur. Ultraviolet difference spectra in urea solutions showed that the tyrosine residues are buried in the interior of the molecule while the tryptophan residues are accessible to solvent. Fukushima proposed that hydrophobic bonding is important in maintaining the tertiary structure.

Koshiyama (1968B) reported an intrinsic viscosity of 0.0638 dl per gm for a purified 7S globulin. This value is consistent with a relatively compact structure. Isoelectrically precipitated globulins have an intrinsic viscosity of 0.052 dl per gm (Wolf et al. 1963).

—See Table III in review by Tanford (1968) for comparison of intrinsic viscosities of native and denatured proteins.
End group analysis (Table 4.6) indicates that there are at least nine polypeptide chains in the 7S globulin isolated by Koshiyama. Based on a molecular weight of 180,000, the 7S globulin has a quaternary structure of 9 subunits with an average molecular weight of 20,000. The extent of disulfide crosslinking between polypeptide chains is unknown but must be small since only two cystines occur per mole of 7S globulin (Table 4.5).

Changes in the quaternary structure of 7S globulin(s) as a function of pH and ionic strength are reported by Roberts and Briggs (1965) and Koshiyama (1968C). Figure 4.6 summarizes changes in sedimentation properties of 7S globulin under various conditions as described by Koshiyama. At neutrality the 7S protein undergoes a distinctive reversible reaction with changes in ionic strength. At 0.5 ionic strength the protein has a molecular weight of 180,000-210,000, but at 0.1 ionic strength the protein sediments at a rate of about 9S (at 1% concentration) and has a molecular weight of 370,000. The 9S form is thus a dimer of the 7S protein. Based on formation of the 9S form at 0.1 ionic strength, about 60% of the total 7S fraction in water-extractable proteins can undergo dimerization (Fig. 4.1) (Wolf and Sly 1967). At pH 2 and low ionic strength the 7S globulin is converted into 2S and 5S species presumably as a result of dissociation into subunits. Conversion into the 2S and 5S forms is inhibited by ionic strengths of 0.1 and higher and is reversed by dialysis of the protein against pH 7.6, 0.5 ionic strength buffer. In 0.01 N sodium hydroxide the protein is irreversibly converted to a slowly sedimenting form (0.4S).

The 7S globulin isolated by Roberts and Briggs (1965) exhibited some of the same reactions shown in Fig. 4.6 but they reported respective molecular weights of 330,000 and 660,000 for the monomer and dimer forms. They also observed conversion of their 7S preparation to slow sedimenting species by the detergent, sodium octyl benzene sulfate, and by high concentrations of urea. Dissociation

\[ 2S + 5S \xrightarrow{\mu = 0.01} 7S \xrightarrow{\mu = 0.1} 9S \]

\[ \begin{align*}
\text{pH 2} & \quad \mu = 0.1, \\
\text{pH 7.6} & \quad \mu = 0.5
\end{align*} \]

\[ 0.01 \text{N NaOH} \]

\[ 0.4S \]

\[ \text{From Koshiyama (1968C)} \]

FIG. 4.6. SCHEMATIC DIAGRAM OF REACTIONS OF 7S GLOBULIN ACCORDING TO KOSHIYAMA
of 7S globulin into subunits by treatment with urea or sodium dodecyl sulfate has been confirmed by molecular weight determinations. In 8 M urea the protein has a molecular weight of only 22,500 (Koshiyama 1970).

Disc electrophoresis of 7S globulin (Koshiyama preparation) in phenol-acetic acid-mercaptoethanol-urea yielded 9 major and 5 minor bands. In the absence of mercaptoethanol 6 major and 13 minor bands occurred; some of the minor bands were attributed to aggregates formed by thiol-disulfide interchange (Catsimpoolas et al. 1968B).

11S Globulin.—Optical rotatory dispersion and infrared measurements suggest that the secondary and tertiary structures of 11S protein are similar to the structures of the 7S globulin (Fukushima 1968). The 11S protein contains little, if any, \( \alpha \)-helical structure but may consist of antiparallel \( \beta \)-structure and disordered regions. As noted in the 7S globulin, only about 40% of the peptide bond hydrogens in the 11S globulin exchanged with deuterium. Ultraviolet difference spectral studies indicated that the tyrosine and tryptophan residues of the 11S protein are buried in hydrophobic regions of the molecule (Fukushima 1968; Catsimpoolas et al. 1969A). Fukushima further demonstrated that denaturation of the protein by alcohols depends on hydrophobicity of the alcohols; \( n \)-butanol was a much more effective denaturant than ethanol or methanol. Native 11S protein is attacked very slowly by the proteinase of \textit{Aspergillus sojae} but is readily hydrolyzed if the protein is denatured by alkaline treatment (pH 12.6). The 11S molecule, therefore, appears to be a compact structure stabilized by hydrophobic bonds.

The 12 N-terminal groups of 11S protein (Table 4.6) indicate that the molecule has a complex quaternary structure. Changes in the quaternary structure as a function of experimental conditions are shown schematically in Fig. 4.7. At 0.1 ionic strength the 11S protein is partially associated into faster sedimenting forms (reaction A); this reaction is reversed by increasing the ionic strength (Naismith 1955). Breakdown into subunits (reactions B and C) through the formation of a 7S intermediate (half molecules) occurs under a variety of conditions (Wolf and Briggs 1958). Dissociation under the mildest conditions (pH 7.6, 0.01 ionic strength) is reversible, but irreversibility was noted when the protein was dissociated at pH 8.6 (Eldridge and Wolf 1967). Urea in high concentrations, anionic detergents, and extremes of pH are effective dissociating agents. Because dissociation processes generally are irreversible, unfolding of the subunits also must occur. A more detailed discussion of conditions causing irreversible conformation changes is given elsewhere (Denaturation of Soybean Proteins). Although only a single step is shown for conversion from half molecules to unfolded subunits (Fig. 4.7), a multistep process may be involved. For example, in dissociation of 11S at pH 2.2 the slowest sedimenting fraction is 5S at 0.2 ionic strength, 4S at 0.1 ionic strength, and 2S at 0.01 ionic strength (Wolf et al. 1958). The 4S and 5S forms may be aggregates (dimers, trimers, etc.)
of the 2S form. The extent of disulfide crosslinking between polypeptide chains is also unknown.

An interesting result of starch gel electrophoresis of 11S protein in 5-7 M urea is the observation of basic protein bands (Shibasaki and Okubo 1966; Puski and Melnychyn 1968). Vaintraub (1967) reported 18 bands on polyacrylamide gel electrophoresis of 11S protein after treatment with 6 M urea at pH 6.76. Several of the bands migrated slightly at pH 8.6. Chromatography of urea-dissociated 11S protein on diethylaminoethyl-cellulose yielded 4 fractions, each consisting of 2 subfractions. The first fraction was not adsorbed on the column at pH 8.0 and contained one band that migrated toward the cathode plus several others that moved only slightly toward the anode. These results also suggest that some of the subunits of the 11S molecule are basic.

On analysis of the chromatographic fractions for N-terminal amino acids, Vaintraub found that the first (basic) fraction contained predominantly N-terminal glycine. The other (acidic) fractions contained N-terminal glycine, isoleucine (leucine), and phenylalanine with clear evidence of redistribution of N-terminal residues as compared to the parent 11S molecule. Apparently then, there are four kinds of subunits in the 11S protein based on differences in the
N-terminal analyses. Vaintraub attributed the 18 gel electrophoretic bands to isomerization of the 4 groups of subunits, but heterogeneity within each kind of subunit is a distinct possibility. Because the subunit mixture obtained after urea treatment of the 11S protein had an $s_{20,w}$ value of 2.56S in pH 6.9, ionic strength 0.1 buffer, urea dissociation of the protein is irreversible.

Independently, Okubo and Shibasaki (1967) treated 11S protein with 8 M urea followed by chromatography on diethylaminoethyl-cellulose and also separated 4 major fractions in good agreement with Vaintraub's results. Approximately 20% of the 11S protein was recovered as the basic subunits with isoelectric points of pH 7-9, a sedimentation coefficient of 1.21S, molecular weight of about 36,000, and with glycine in the N-terminal position (Okubo et al. 1969). The acidic subunits contained leucine (isoleucine) and phenylalanine N-terminal groups.

Catsimpoolas (1969C) confirmed the existence of basic subunits in the 11S molecule by isoelectric focusing in 6 M urea and 0.2 M mercaptoethanol. Among the 6 fractions were: 3 acidic that had isoelectric points of pH 4.75, 5.15, and 5.40; while 3 were basic with isoelectric points at pH 8.00, 8.25, and 8.50. The isolated fractions agreed with the six bands after disc electrophoresis of the 11S protein in phenol-acetic acid-mercaptoethanol-urea.

Discovery of basic subunits in the 11S protein is an unexpected finding and its significance in terms of the quaternary structure of the protein is still unknown. Electron microscopy of the 11S molecule suggests that it is made up of 2 annular-hexagonal structures, each containing 6 subunits (Catsimpoolas 1969C). Alternation of acidic and basic subunits is proposed as an aid to stabilizing the molecule through ionic bonding. Saio et al. (1970) recently proposed an 11S structure consisting of two oval split-rings facing each other.

Heat will also disrupt the quaternary structure of 11S protein. Changes in 11S protein induced by heat are discussed later (Denaturation of Soybean Proteins).

15S Globulin.—Although this protein has not been isolated and characterized, there is evidence that it also has a quaternary structure. In studies on 11S protein where the 15S fraction occurred as a contaminant, dissociation into subunits apparently took place under conditions similar to those outlined in Fig. 4.7 (Wolf and Briggs 1958; Wolf et al. 1958).

Urease.—Unpurified soybean urease sediments as an 18S form in a pH 7 sucrose density gradient, but at pH 4.8 it is converted into a 13.3S form. Similar changes in jackbean urease are attributed to a splitting of the 18S unit into halves (Tanis and Naylor 1968).

Electrochemical Properties

Electrophoresis.—Use of this technique to determine the electrochemical properties of soybean proteins has been very limited. Reasons for this paucity include low solubility of the globulins in the isoelectric region (pH 4-5), lack of
purified proteins until recently, and a greater interest in the use of gel electrophoresis as a tool for assessing homogeneity and for detecting subunits.

Moving Boundary Electrophoresis.—Briggs and Mann (1950) demonstrated heterogeneity of glycinin and other globulin preparations by moving boundary electrophoresis, but their resolution of components was poor. Likewise, Kondo et al. (1953) and Smith et al. (1955) observed only limited separations of proteins now known to be mixtures. The latter workers, for example, obtained 4–5 peaks on electrophoresis of acid-precipitated globulins; 1 peak accounted for nearly 90% of the total protein. However, this protein mixture separates into 4 peaks in the ultracentrifuge and the major component (11S protein) is less than 50% of the total protein (Wolf and Sly 1965). A synthetic mixture of 7S and 11S globulins did not separate electrophoretically at pH 7.6, thereby showing limitations of this technique (Fukushima 1968).

Moving boundary electrophoresis is, however, useful for determining isoelectric points if solubility problems can be overcome. Isoelectric points for 2.8S globulin, hemagglutinin, lipoxygenase, and 7S globulin (Table 4.7) were determined by this method. Electrophoresis has been used to study interaction of phytate with soybean globulins, but results are difficult to interpret because the protein components and protein-phytate complexes did not resolve well (Smith and Rackis 1957). This system needs further study since the presumed protein-phytate complexes migrated slower than the uncomplexed protein, whereas one would predict the opposite behavior on the basis of the high negative charge of phytate at pH 7.6.

Gel Electrophoresis.—Shibasaki and Okubo (1966) observed 13–14 bands on starch gel electrophoresis of soybean proteins in tris-citrate buffer, pH 8.6, containing 7 M urea and 0.02 M mercaptoethanol. Crude 11S component (cold-insoluble fraction) formed 3 major bands plus 3 minor bands that migrated toward the cathode. These three minor fractions were subsequently isolated and shown to be basic polypeptide chains (Okubo et al. 1969). In the absence of urea, the crude 11S migrated as a major band plus 4–5 minor bands.

Puski and Melnychyn (1968) analyzed soybean globulins by starch gel electrophoresis using 5 M urea in tris-hydrochloride buffer, pH 8.7, or in 1 N acetic acid, pH 3.5. The globulins separated into 14 bands in alkaline gels and into 15 bands in acid gels. Purified 7S globulin (Roberts and Briggs preparation) migrated as 7 bands in alkaline and as 12 bands in acid gels, whereas purified 11S protein formed 18 bands in alkaline and 10 bands in acid gels. Puski and Melnychyn also observed the basic protein bands detected by Shibasaki and Okubo.

Vaintraub (1967) obtained 18 bands by polyacrylamide gel electrophoresis of 11S protein after treatment with 6 M urea and he isolated basic subunits as described earlier (Molecular Structure and Conformation—11S Globulin). Polyacrylamide gel electrophoresis with 8 M urea has also been used to study soybean whey proteins and trypsin inhibitors (Eldridge et al. 1966; Eldridge and Wolf 1969B).
Catsimpoolas et al. (1967) introduced polyacrylamide gel disc electrophoresis for characterizing soybean proteins. They purified the 11S protein until a single major band resulted, but on treatment with guanidine hydrochloride, the protein separated into more than 12 bands. The method was later applied to soybean proteins during germination (Catsimpoolas et al. 1968B), soybean whey proteins (Catsimpoolas et al. 1969B), Kunitz trypsin inhibitor (Catsimpoolas et al. 1969C), hemagglutinin (Catsimpoolas and Meyer 1969), purification of 2S and 7S globulins (Catsimpoolas and Ekenstam 1969), and association-dissociation of 11S protein (Catsimpoolas et al. 1969A).

The disc electrophoretic technique was modified by using phenol-acetic acid-mercaptoethanol-urea as a dissociating solvent to detect subunits in proteins, quaternary structures (Catsimpoolas et al. 1968B). In this solvent, 11S component formed 12 bands while 7S globulin (Koshiyama preparation) separated into 14 bands. Changes in the proteins during germination were followed with this method.

Genetic variants of an unidentified protein (Larsen and Caldwell 1968) and of trypsin inhibitor (Singh et al. 1969) have been detected by polyacrylamide gel electrophoresis.

**Immunoelectrophoresis.**—Combinations of immunochemical techniques with gel electrophoresis are discussed later (Immunochemical Properties of Soybean Proteins).

**Isoelectric Focusing.**—Catsimpoolas and coworkers have extensively studied usefulness of this technique as a tool for isolating and characterizing soybean proteins. Isoelectric focusing of whey proteins yields a spectrum of proteins with isoelectric points ranging from pH 3.38 to 10. Isolation of the isoelectrically focused proteins and analysis by disc electrophoresis shows that many of the fractions are heterogeneous. Multiple forms of trypsin inhibitor and hemagglutinin reported by others were confirmed by isoelectric focusing (Catsimpoolas et al. 1969B). Kunitz trypsin inhibitor isolated from whey and commercial inhibitor samples by isoelectric focusing is pure by disc electrophoresis and immunoelectrophoresis (Catsimpoolas et al. 1969C). Four hemagglutinins isolated by isoelectric focusing of whey proteins had isoelectric points in the range of 5.85-6.20 (Table 4.7) (Catsimpoolas and Meyer 1969).

Isoelectric focusing of whey proteins also yielded lipoxygenase homogeneous by disc electrophoresis, and immunoelectrophoresis (Catsimpoolas 1969B). Multiple forms of lipoxygenase as reported by Guss et al. (1967) and Christopher et al. (1970) were not observed.

The most significant finding by isoelectric focusing is the separation of 11S protein (referred to as glycinin) into acidic and basic subunits by Catsimpoolas (1969C) as described earlier (Molecular Structure and Conformation—11S Globulin).

Advantages of isoelectric focusing are the ability to test for homogeneity and to obtain isoelectric points with small amounts of protein. Isoelectric focusing in polyacrylamide gels requires only 0.2-0.4 mg of protein (Catsimpoolas 1968D).
The method is limited, however, by the small samples that can be purified and by the insolvability of some proteins at their isoelectric points. The latter limitation prevents fractionation of the major soybean globulins with their quaternary structures intact since solvents, such as 6 M urea, are required to dissolve the proteins.

Proteins with small differences in isoelectric points can be separated. For example, Kunitz and Bowman-Birk trypsin inhibitors whose isoelectric points differ by only 0.2 pH unit were separated by using narrow pH gradients (Catsimpoolas 1969D).

**Titration Studies.**—Few studies are available on hydrogen ion equilibria of soybean proteins. Wu and Scheraga (1962) measured the titration curve for Kunitz trypsin inhibitor and found the curve reversible over the entire pH range. Total number of ionizable groups including guanidyl groups was 62 per mole of inhibitor. Harry and Steiner (1969) published a titration curve for Bowman-Birk trypsin inhibitor but did not analyze their data in terms of numbers of various ionizable groups involved. The titration curve was reversible below neutrality, but the curves were no longer superimposable after titration to high pH.

Malik and Jindal (1968) titrated a preparation referred to as glycinin and believed to be a single protein. The method of isolation, however, indicates that their material consisted of acid-precipitated globulins, which are a mixture of at least four proteins (Wolf and Sly 1965) and which were probably denatured by the high pH used during extraction of the proteins from defatted meal. Their sample likely was also contaminated by phytate since isoelectric precipitation causes phytate to interact and precipitate with the proteins (Smith and Rackis 1957). Notwithstanding these limitations, the data should be a useful approximation of the ionic groups in soybean globulins.

**DENATURATION OF SOYBEAN PROTEINS**

Current information indicates that soybean proteins have compact structures as opposed to random coil-like conformations typical of milk caseins (McKenzie 1967). Denaturation studies of soybean proteins, therefore, provide several types of information: (a) the limits of the conditions under which the native structures are stable, (b) the nature of the conformation changes occurring during denaturation, and (c) information about the structures of the native protein molecules. For example, a study of proteins in urea or guanidine hydrochloride may reveal the presence of subunits. If subunits are found, the native protein has a quaternary structure and therefore a higher degree of complexity than a protein that contains only a single polypeptide chain.

For purposes of definition, denaturation is interpreted here as a major change from the native structure without alteration of the amino acid sequence (Tanford 1968). Limitations of this definition are discussed by Tanford.
Heat Denaturation

Denaturation of soybean proteins by moist heat is well known and has long been used to eliminate antinutritional factors (believed to be proteins) in soybean meals and flours used in feeds and foods (Chap. 9). Even though heat is a common physical treatment given to most foods either during processing or cooking, surprisingly little is known about the reactions that soybean proteins undergo when they are heated. Early studies dealt with the effects of heat and moisture on defatted meal and were primarily concerned with measurements of the amounts of soluble protein remaining after heating (Beckel et al. 1942; Belter and Smith 1952). Changes in extractability of the different protein components as a function of heating time are reported by Shibasaki et al. (1969). Water, buffer, buffer-mercaptoethanol, and buffer-mercaptoethanol-urea were used as extraction solvents. The various extracts were analyzed by starch gel electrophoresis.

When soybean meal with an equal weight of water is autoclaved above 100°C, the water-soluble protein decreases to a minimum and then increases again as heating is continued (Fukushima 1959A). Likewise, heating at 100°C with greater amounts of water solubilizes large amounts of protein even after heating for 30 min. Fukushima (1959B), therefore, developed a proteolytic digestion method for measuring extent of denaturation to overcome difficulties inherent in solubility methods.

Mann and Briggs (1950) observed that heating water extracts of defatted meal precipitated protein which appeared to be primarily nonglobulin (whey proteins) as determined by electrophoresis. Heating the whey proteins in buffer (pH 7.6, 0.1 ionic strength) prevented precipitation, and, instead, a single, nearly symmetrical peak formed. This electrophoretic peak apparently is an aggregate formed by interaction between the different components. When cold-precipitable protein (now known to be primarily 11S protein) was added to the whey proteins, it also was incorporated into the aggregate by heating.

Watanabe and Nakayama (1962) heated water-extractable soybean proteins at pH 7.0 and confirmed formation of aggregates. After the proteins were heated for 10 min at 100°C, only a 5S and a 2S fraction were detectable by ultracentrifugation; after 30 min of heating the 5S fraction disappeared leaving only the 2S fraction. Saio et al. (1968B) presented additional evidence for heat aggregation of soybean proteins. Gel filtration of water-extractable, acid-precipitated, and calcium-precipitated proteins, before and after heating, showed that the slowly eluting (low molecular weight) fractions were converted to the fraction that eluted first; i.e., the high-molecular-weight materials.

Circle et al. (1964) studied the effect of heat on aqueous dispersions of a commercial preparation of soybean globulins (sodium soy proteinate form).
Gels formed when protein dispersions of 8% or higher were heated for 10-30 min at 70° C. In the concentration range of 8-12% the gels broke down when heated at 125° C. This behavior may be related to resolubilization of protein observed by Fukushima (1959A) when meal is heated at 100° C for prolonged times with excess water or at temperatures about 100° C.

Circle and co-workers also examined the influence of additives on gelation; included were salts, lipids, starch, gums, and reducing agents. Sodium sulfite and cysteine markedly reduced viscosity of unheated 10% dispersions and inhibited gelation. Evidently, cystine crosslinks contribute to the gel structure, but other interactions undoubtedly also play a part.

Catsimpoolas and Meyer (1970) propose that heating soybean globulins at concentrations greater than 8% converts them to a progel state that gels on cooling. Gel-to-progel conversion is reversible on heating. Excessive heat or addition of chemicals, such as disulfide-cleaving agents, forms a "metasol" state that does not gel. Effects of pH and ionic strength on viscosities of the progests and gels were examined.

Heat gelation of soybean globulins, precipitated by hydrochloric acid or calcium chloride, is reported by Aoki (1965A,B,C). Methods are described for evaluation of the gels and effects of time, temperature, protein concentration, and salt concentration were examined. Most studies were conducted with 20% protein solutions. Sodium bisulfite markedly decreased gel strength in the range of 3-11mM. Effects of low concentrations of urea and guanidine hydrochloride on gelation were complex, but at high concentrations they tended to weaken the gels (Aoki and Sakurai 1968).

Proteins isolated from a defatted meal with a nitrogen solubility index (NSI) of 63 gave stronger gels than proteins from meals with NSI values of 27, 42, or 83. If the extracts from the meals of different NSI values were heated to 95° C for 5 min before precipitating the proteins with acid, gels subsequently prepared (at 90° C for 50 min) were similar in strength and other properties. Although solubilities of the thermal gels in phosphate buffer, 6 M urea, or 0.01 M mercaptoethanol were low, combination of the three solvents dissolved 90% or more of the gels (Aoki and Sakurai 1969). This solubility behavior indicates that gel structure depends upon disulfide, plus hydrogen, and/or hydrophobic bonds.

Only limited data are available on heat denaturation of purified soybean proteins. Kunitz (1948) made a classical study of the kinetics and thermodynamics of heat denaturation of crystalline trypsin inhibitor. This protein, consisting of a single polypeptide chain crosslinked by two disulfide bonds (Wu and Scheraga 1962), is inactivated when heated in solution but regains its activity reversibly when the solution is cooled. Side reactions must occur, however, when soybean meal is heated since there are no reports of reversibility of inhibitor activity in heated meal.

Liener (1958) studied kinetics of heat inactivation of soybean hemagglutinin
from pH 4 to 9.5. Maximum stability occurred at pH 6–7. No evidence for reversibility of denaturation was reported.

Catsimpoolas et al. (1969A) heated 11S protein solutions and followed the reaction by turbidity measurements. Turbidity increased rapidly above 70°C and protein precipitated at 90°C. Disc electrophoresis showed that dissociation into subunits occurred at 90°C but that undissociated 11S also remained; immunodiffusion likewise indicated incomplete denaturation of the protein.

Wolf and Tamura (1969) heated 11S protein at 100°C and followed changes in ultracentrifugal composition as a function of heating time (Fig. 4.8). In 5 min of heating the 11S component disappeared and was converted into a soluble aggregate and a 3-4S fraction. However, on continued heating the aggregate precipitated and only the 3-4S fraction was left in solution. Heating in 0.01 M mercaptoethanol hastened formation of the precipitate and no soluble aggregate was detectable as an intermediate form. Since precipitation still occurred in 0.5 M mercaptoethanol, sulfhydryl-disulfide interchange does not appear to contribute to the precipitation reaction. When the protein was heated in 0.01 M

![Graph showing changes in ultracentrifugal composition of 11S protein as a function of time at 100°C.](image)
N-ethylmaleimide, the 11S protein again disappeared, but no precipitation of protein took place. Instead, a soluble aggregate of 58-67S and a 3-4S fraction formed. It was concluded that heating disrupts the quaternary structure of the 11S protein and separates the subunits into a soluble and an insoluble fraction by the following three steps:

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

In reaction a, A-subunits represent the 3-4S fraction, which remains soluble, while B-subunits represent that portion of the 11S molecule which is converted into aggregates through reactions b and c. Reaction b apparently is very rapid since B-subunits were not detected in an unaggregated state. Reaction c is catalyzed by sulfhydryl groups but blocked by N-ethylmaleimide. It was proposed that reaction c involves hydrophobic interactions which are promoted by cleaving the disulfide bonds in the molecule.

Additional reactions probably occur when the 11S protein is heated in the presence of other soybean proteins since Watanabe and Nakayama (1962) did not observe that the 3-4S fraction was formed when water-extractable proteins were heated.

Catsimpoolas et al. (1970) followed changes in turbidity of 11S protein solutions as a function of heating time under various conditions. Rate and extent of aggregation were increased by low ionic strength and by mercaptoethanol. Maximum aggregation occurred between pH 4.0 and 6.0.

Denaturation by Extremes of pH

The overall effect of high or low pH on the major proteins (7S and 11S globulins) appears to be dissociation of the molecules into subunits possibly by electrostatic repulsions between the high positive or negative charges on the proteins at extreme pH values. The subunits are often irreversibly altered by conditions necessary for complete dissociation.

Acid pH.—Soybean globulins prepared by acid precipitation were progressively converted from a mixture of 2S, 7S, 11S, and 15S fractions observed at alkaline pH values to 2-3S and 7S fractions as the pH was lowered from 3.8 to 2.0 at ionic strength, 0.06 (Rackis et al. 1957). The 11S fraction was observed at pH 3.0 when the ionic strength was 0.15, but at higher ionic strengths the 11S fraction was not stable and appeared to aggregate. A complex series of association-dissociation reactions influenced by pH and ionic strength is suggested by this behavior in acid solution.
Similar conclusions were reached by Kretovich et al. (1958) in studies on
glycinin (prepared according to Osborne and Campbell 1898) and a glycinin
subfraction in acid solutions. At pH 2.2 the glycinin subfraction, which con­
sisted of 7S and 11S components in 10% sodium chloride, sedimented at a rate
of 2.36S that corresponded to a molecular weight of 19,800. Dissociation of the
7S and 11S globulins into subunits in acid is thus established. However, the
concept of a system of identical subunits in reversible states of aggregation as
proposed by Kretovich and co-workers is now known to be incorrect on the
basis of differences in physical, chemical, and immunochemical properties be­
tween the various globulins and their subunits (Wolf et al. 1962; Okubo and
Shibasaki 1967; Puski and Melnychyn 1968; Fukushima 1968; Catsimpoolas and
Ekenstam 1969).

The 11S protein undergoes changes in sedimentation rate and optical rotation
as pH and ionic strength are lowered below pH 4. Dissociation into 2-3S
subunits occurs through a 7S intermediate. Only a 2S species is detectable at pH
2.2, 0.01 ionic strength, and levorotation (sodium D line) is 81-82° as compared
to 43-47° in the undissociated state. The tendency of the 11S to dissociate into
subunits is counteracted by increasing ionic strength, but conformation changes
still occur if the pH is low enough. For example, at pH 2.2, ionic strength 1.0,
no 11S component was detectable and the protein precipitated on standing
(Wolf et al. 1958). Low pH treatment of 11S protein is irreversible as measured
by ultracentrifugation (Wolf and Briggs 1958). Catsimpoolas et al. (1969A)
observed exposure of tyrosine and tryptophan residues in the 11S molecule at
pH 2.0 and found the protein to be irreversibly modified as detected by disc
electrophoresis.

A 7S globulin isolated by Koshiyama (1968C) sedimented as 2 peaks of 1.92S
and 5.47S in 0.01 N hydrochloric acid (pH 2). On dialysis of the acid solution to
pH 7.6 the protein exhibited the 7S ⇔ 9S monomer-dimer reaction with change
in ionic strength that is typical of the native protein (Fig. 4.6). On prolonged
acid treatment part of the protein precipitated when it was brought back to pH
7.6, but the soluble portion still underwent the monomer-dimer reaction. In 0.1
N hydrochloric acid, conversion to the slowly sedimenting form was less com­
plete and the protein was irreversibly modified to a greater extent than by 0.01
N acid. Addition of salts to acid solutions of 7S globulin inhibited the confor­
mation changes observed at low ionic strength.

Alkaline pH.—Solutions of soybean globulins in sodium hydroxide (pH 12)
increase in viscosity and form gels if protein concentration is 14.5% or higher
(Kelley and Pressey 1966). The globulin mixture dissolved in alkali sediments
with an $s_{20,w}$ value of about 3S. Conversion of the globulins to the 3S form is
nearly complete in 15 min at pH 12. If the alkaline solution is dialyzed against
phosphate buffer (pH 7.6, ionic strength 0.5, 0.01 M mercaptoethanol), only 3S
and 7S peaks are observed. When the alkali treatment is stopped by dilution and
pH is adjusted to 4.5 to precipitate the protein, large decreases in protein solubility occur. Alkali thus causes irreversible changes in the molecules.

Partial dissociation of 11S protein into subunits occurs even near neutrality if ionic strength is low and divalent cations, such as calcium, are absent. Thus dialyzing the 11S protein at pH 7.6, ionic strength 0.01, dissociates it into 7S and 2-3S units (Wolf and Briggs 1958; Wolf and Tamura 1969). Dissociation is increased by raising the pH from 7.6 to 9.0, but reassociation into 11S is low when the sample is brought back to pH 7.6, ionic strength 0.5. Disc electrophoresis also indicates that alkali-induced (pH 11) changes in the 11S molecule are irreversible (Catsimpoolas et al. 1969A).

Optical rotatory dispersion measurements suggest that alkali disrupts the internal structure of the 11S molecule. Such disorganization of the structure makes the 11S molecule more susceptible to attack by a protease from Aspergillus sojae (Fukushima 1968).

When 7S globulin is dissolved in 0.01 N sodium hydroxide (pH 12) it is converted into a very slow sedimenting form (Fig. 4.6). Adjusting the alkaline solution to pH 7.6, ionic strength 0.5 buffer did not restore the 7S form (Koshiyama 1968C). Evidently dissociation of the protein into subunits under these conditions is also accompanied by irreversible conformational changes as noted in studies on the globulins (Kelley and Pressey 1966).

Denaturation by Organic Solvents

Smith et al. (1951) studied the effects of time, temperature, and concentration of methanol, ethanol, isopropanol, and acetone on extractability of proteins in defatted meal. Aqueous solutions of the organic solvents were more effective than water or the pure solvents in denaturing the proteins. Denaturation of soybean proteins by organic solvents is complete in about 5 min. Fukushima (1969A) measured denaturation of the proteins in defatted meal by a variety of organic solvents. Water-immiscible solvents were weak denaturants but water-miscible solvents in combination with water were stronger denaturants than the pure solvents. Solvents completely miscible with water, such as the lower alcohols, were most effective as denaturants at characteristic water:solvent ratios. The ability of the lower alcohols to denature the proteins increased as the hydrocarbon chain length increased; the order of effectiveness was: methanol < ethanol < propanol < butanol.

Electrophoretic studies by Mann and Briggs (1950) indicated that the globulin fraction is the group of proteins that is most markedly affected by alcohols. Treatment of the globulins with ethanol showed that maximum denaturation occurred at 60% alcohol and that the 7S fraction was the most sensitive while the 2S fraction was most stable to ethanol (Roberts and Briggs 1963). With isopropanol, maximum denaturation of the globulins occurs with 40% alcohol (Wolf et al. 1964). Again, the 7S fraction was most readily denatured (insolubilized) while the 2S fraction was comparatively stable as demonstrated by ultracentrifugation and hydroxylapatite chromatography.
Fukushima (1968) treated 7S and 11S globulins with eight different alcohols and found that denaturation increased as the hydrocarbon chain length of the alcohols increased as noted in studies on meal (Fukushima 1969A).

Fukushima (1969A) proposed that the major proteins have structures with hydrophobic groups, buried in the interior, which help stabilize the molecules to heat in water. Alcohols presumably are able to penetrate to the interior and can disrupt the hydrophobic bonding while water breaks hydrogen bonds in more polar regions near the surface. The combination of water and alcohol is, therefore, more effective than either solvent alone. This conclusion is supported by the fact that soybean meal proteins denatured with aqueous ethanol are more completely hydrolyzed by proteolytic enzymes than are proteins denatured by moist heat (Fukushima 1969B).

Denaturation by Detergents

Data on denaturation of soybean proteins by detergents are scarce. Wolf and Briggs (1958) measured the binding of sodium octyl benzene sulfonate by 11S protein by equilibrium dialysis and analyzed the detergent-protein complexes in the ultracentrifuge. Binding of detergent caused dissociation of the 11S species into a 3S form through a 7S intermediate. Dissociation was complete when approximately 375 moles of detergent were bound per mole of protein. Dissociation of 15S contaminant in the 11S sample also occurred. Removal of the detergent by dialysis did not reform the 11S component; irreversible conformational changes in the subunits caused aggregates to form instead.

Detergents have also been used to precipitate proteins from soybean whey (Smith et al. 1962). Enzymes normally associated with whey proteins were inactive in the detergent-protein precipitates. Soybean hemagglutinin, a whey protein, is inactivated by sodium decylbenzene sulfonate (Liener 1958).

Effects of Urea and Guanidine Hydrochloride

A major effect of urea on soybean globulins is dissociation of the high-molecular-weight proteins into subunits. Treatment of 11S protein with 1.5 and 3.0 M urea caused partial dissociation into 7S and 3S forms (Wolf and Briggs 1958). Kelley and Pressey (1966) confirmed these results in studies on the globulin fraction and found that the reactions were reversible on removing the urea. In 6 M urea the globulin fraction was largely converted into 1-2S material plus a small fraction of 4S, but only partial reversal of the protein to its original sedimentation distribution occurred when the urea was removed by dialysis.

Aoki and Sakurai (1968) studied the effects of urea and guanidine hydrochloride denaturation on thermal gelation of soybean globulins prepared by acid or calcium chloride precipitation. High concentrations of the two denaturants weakened the gels.

Starch-gel electrophoresis with 5-7 M urea separates soybean globulins and globulin fractions into a large number of bands presumably as a result of dissociating the 7S and 11S molecules into subunits (Shibasaki and Okubo 1966;
Puski and Melnychyn (1968). Vaintraub (1967) likewise observed a large number of protein bands on polyacrylamide gel electrophoresis of 11S protein after treatment with urea.

Optical rotatory dispersion and ultraviolet difference spectral studies on the 7S and 11S indicate that high concentrations of urea disrupt native structures. In the 7S globulin, tyrosine groups appear buried in the interior of the molecule, whereas in the 11S globulin both tyrosine and tryptophan residues are exposed to solvent until they are denatured (Fukushima 1968). Catsimpoolas et al. (1969A) confirmed these effects of urea on the 11S protein and found that treatment of the protein with 6 M urea is irreversible as measured by disc electrophoresis and immunochemical techniques.

In contrast to the effects of urea on the 7S and 11S globulins, soybean trypsin inhibitor changes little in conformation in 9 M urea unless high temperatures or alkaline pH are included in the treatment (Edelhoch and Steiner 1963). If the two disulfide bonds are reduced, the inhibitor unfolds in 8 M urea as measured by viscosity and gel electrophoresis (Eldridge and Wolf 1969B).

High concentrations of urea are required to inactivate soybean hemagglutinin while guanidine hydrochloride is a much more effective denaturant for this protein (Liener 1958).

**IMMUNOCHEMICAL PROPERTIES OF SOYBEAN PROTEINS**

Introducing immunodiffusion and immunelectrophoresis to characterize soybean proteins, Catsimpoolas and Meyer (1968) have published extensively on applications of these techniques. Their initial study demonstrated at least 5 components in water-extractable proteins and 3 components in the isoelectrically precipitated globulins. Purified 11S protein formed only one precipitin band when diffused against antibodies for the water-extractable proteins. The 11S protein retained its immunochemical properties on heating up to 80° C; at higher temperatures the protein precipitated. Single diffusion measurements indicated that quantitative estimates of 11S protein are possible by this method.

Single immunodiffusion techniques for estimation of Kunitz trypsin inhibitor are also described (Catsimpoolas et al. 1969D; Catsimpoolas and Leuthner 1969B). This approach offers the possibility that the amounts of various proteins present in soybean meal can be determined quantitatively.

Immunochemical techniques were used to demonstrate homogeneity of soybean lipoxygenase (Catsimpoolas 1969B) and hemagglutinins (Catsimpoolas and Meyer 1969). Although four different hemagglutinins were isolated by isoelectric focusing, they were immunochemically identical.

Protein bodies contained six antigenic components by disc immunelectrophoresis. One of the components was 11S protein while another was identical to the 7S globulin isolated by Koshiyama (1965). On germination, the 11S component was detectable up to the 16th day, whereas the 7S component disappear-
ed after the 9th day (Catsimpoolas et al. 1968A). Immunochemical techniques also proved useful in following fractionation of soybean globulins (Catsimpoolas and Ekenstam 1969) and to study dissociation of 11S protein into subunits (Catsimpoolas et al. 1969A).

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