VEGETABLE PROTEIN: LIPID INTERACTIONS

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ABSTRACT

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Soybean protein, phospholipids, and neutral lipids interacted under cavitational force fields in essential accordance with the published methodology for reassembly of lipids and apoproteins of naturally occurring lipoproteins. Native soybean globulins do not form lipoproteins. Prior dissociation with denaturing agents, *e.g.*, urea, guanidine hydrochloride, acid pH, or alkaline pH, yielding predominantly a population of subunits providing increased surface area and greater proportion of hydrophobic residues is necessary to activate the globulins to form lipoproteins of characteristic buoyant densities. This principle seems to hold true for other vegetable proteins. Denatured globulins are shown to provide greater emulsion stability than do native globulins. The pH dependence of emulsion stability has been established and results are discussed in terms of current knowledge of multisubunit vegetable proteins.

Many natural and processed foods contain lipid protein complexes in which apolar and polar lipids are dispersed and often held by proteinaceous networks. Important among these may be mentioned the lipoproteins of egg yolk (1) and milk fat globule membrane (2). The nature of these disperse systems and the mutual interactions between lipids and proteins are fundamental to the organoleptic quality of various food items. For instance, the structural integrity of egg yolk lipoproteins has been demonstrated to be vital to baking performance of egg and to the texture of baked goods (3,4). In view of the increasing costs and growing shortages of animal structural proteins in recent years, considerable emphasis has been placed on use of vegetable proteins, particularly soybean, for fabrication of analogues that simulate conventional food products. Most of the effort, however, has been devoted to replacing protein or extending animal protein with analogues based on soybean; little information on the nature of interaction of lipids with vegetable proteins is on record. We believe that an understanding of such interactions is important in achieving acceptable food characteristics.

Lipoproteins of egg yolk, milk, and animal blood have been studied extensively by biochemical and physical methods (5,6). From these studies, certain rigorous as well as complementary criteria of lipoprotein structure can be deduced. The bonds between lipids and proteins in a naturally occurring lipoprotein are such that they cannot be readily dissociated by simple manipulations of pH (except extremes), ionic strength, and ultracentrifugal force fields. A rigorous criterion of lipoprotein structure is its equilibrium or buoyant density, which is intermediate between that of free lipid and free protein. Lipoproteins can be associated with free (externally adsorbed) lipids, which cannot be distinguished in the case of low density lipoproteins ($d < 1$) by ultracentrifugation techniques. Such lipids can, however, be removed by apolar solvents, *e.g.*, *n*-hexane. Integral (core) lipids of lipoproteins, on the other hand, resist extraction by apolar solvent under mild conditions. Resistance to extraction by apolar solvent therefore can be used as a complementary criterion of lipoprotein stability and structure. These criteria are of general applications

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for lipid-protein disperse systems; biochemists have used them for purification and characterization of lipoproteins (7–10).

In this investigation, we have used these criteria as our guidelines to develop experimental approaches for generating lipoproteins based on soybean and other vegetable proteins, and to study the stability of emulsions of oils made with soybean as the emulsifier. Some lipoproteins have been characterized morphologically. Here we report and discuss results of our studies.

MATERIALS AND METHODS

Soybean Proteins

Soybean proteins were prepared routinely from hexane-defatted soybean meals by aqueous extraction in the presence and, in typical cases, in the absence of 1% sodium sulfite; clarification of the extract by preparative centrifugation; and acidification to pH 4.8. The precipitated proteins were washed thoroughly with distilled and deionized water and stored frozen at −20° C until use. They were labeled soybean isolate (SI).

Our colleague Dr. A. Badley prepared and supplied partially purified glycinin and γ-conglycinin.

Other Vegetable Proteins

Defatted meals of cottonseed, rapeseed, and groundnut were extracted by aqueous 10% NaCl solutions and clarified by centrifugation; the proteins were precipitated by acidification of salt extracts to pH 4.8. Precipitated proteins were redissolved in $10^{-2}M$ tris buffer at pH 7.2 and reprecipitated. After redissolving the protein precipitates in tris, they were clarified by centrifuging at 8,000 rpm for 30 min to remove residual lipid, filtered through asbestos filter, and freeze-dried.

Effects of Protein Denaturants on Soybean Proteins

Thawed portions of SI prepared in the presence of sulfite were dissolved in $10^{-2}M$ tris pH 8 ($0.05M$ NaCl) to obtain a near native 1% solution of soybean globulins. Denaturing treatments applied were dissolution of the isolate in 2, 4, 6, or $8M$ urea or exposure to pH 2, 10, or 12 for varying lengths of time. In typical cases, urea was removed by dialysis, and urea-treated isolate (UI) was freeze-dried. Similarly, after exposure to pH extremes, the protein samples were readjusted to pH 8. Effects of various treatments were examined by following changes in the sedimentation distribution profiles. Analytic ultracentrifugation was done in the Beckman L 2.75B equipped with a preparative optics accessory. All centrifugations were done at 20° C and 60,000 rpm. Observed sedimentation coefficients were converted to $S_{20,w}$ by the method of Schachman (11) using approximate values of partial specific volumes that Badley *et al.* (12) determined.

Lipids

Glycerophospholipids, namely, 1,2 diacyl-Sn-glycero 3 phosphorylcholine (PC) and 1,3 diacyl 3-Sn-phosphatidylserine (PS), were purchased from Lipid Products, Epsom, Surrey. 1,2 Diacyl 3-Sn-phosphatidylethanolamine (PE) was obtained from Koch Light (13). Tripalmitin, trielaidin, and palmitolein were purchased from Serva Feinbiochimica, Heidelberg. Our colleagues in the oils and fats division supplied purified triolein, crude soybean lecithin, and sunflower oil.

Complexing of Lipids With Proteins

Aqueous dispersions of PC yielding predominantly unilamellar vesicles were prepared by known methods (14,15), employing ultrasonic force fields. They were added to clarified water extract of soyfluff (Oppenheimer Casing, London), salt solutions of soybean globulin isolates, and soybean proteins in the presence of 6*M* guanidine hydrochloride or 8*M* urea, the denaturing agent being removed subsequently by dialysis. Alternatively, the soybean proteins were dissolved in increasing concentrations of urea and the denaturing agents were removed by dialysis to yield partially or totally scrambled proteins. These were codispersed ultrasonically with aqueous suspensions of PC—and in typical experiments, with equimolar mixtures of PC plus PE plus PS—and neutral lipids (*e.g.*, triolein, tripalmitin, oils) and compared with corresponding products obtained by codispersions with native proteins. Samples of protein exposed to pH 2 or pH 12 were brought to pH 8 prior to dispersion with phospholipids.

Assessment of Lipoprotein Formation

The complexes of lipids and protein were layered on top of buffered sodium bromide gradients ranging in density between 1 and 1.4 prepared by layering

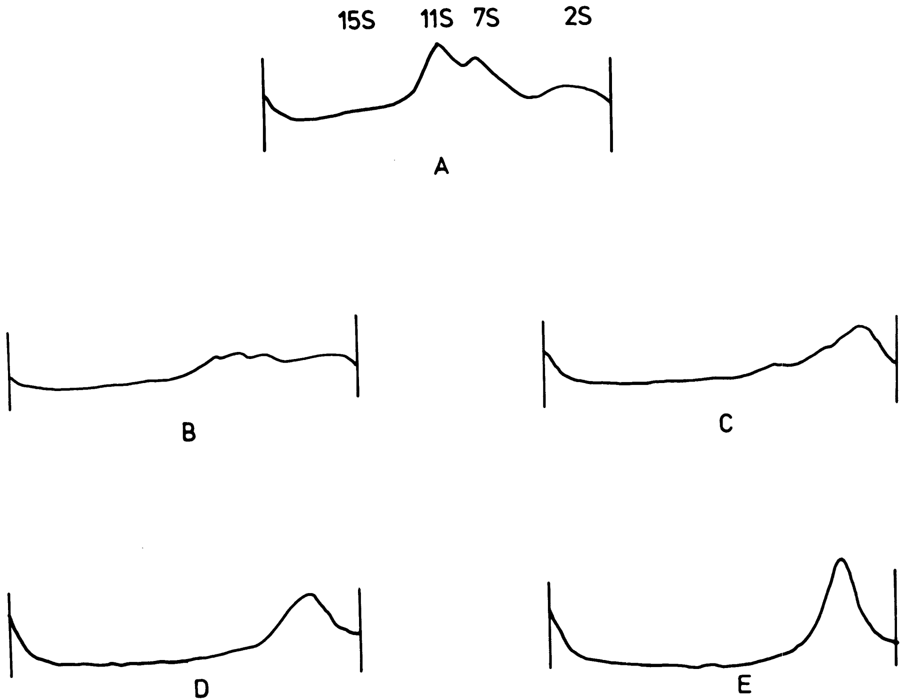

Fig. 1. Ultracentrifuge patterns obtained from soybean isolate (SI) in urea solutions. Runs were done as described in text. A, 1% SI in $10^{-2}M$ tris pH 8 containing 0.01*M* mercaptoethanol; B, 1% SI in 2*M* urea; C, 1% SI in 4*M* urea; D, 1% SI in 6*M* urea; E, 1% SI in 8*M* urea.

solutions of decreasing sodium bromide concentrations in a polycarbonate tube and centrifuged at 58,000 rpm for 24 hr in a titanium rotor (3 × 6.5-ml swing out) in the MSE preparative ultracentrifuge Superspeed 75. The tubes were cut into 500-μl fractions using a Lang-Levy micropipette. The fractions were analyzed by known methods for density (using refractive index measurements), protein, and in typical cases, lipid phosphorous and triglycerides (16–18). The lipoproteins formed were examined for morphology by negative staining with phosphotungstic acid, adjusted to pH 7.

Stability of Oil-Water Emulsions Made With Soybean Globulins as Emulsifiers

Emulsions of sunflower oil and soybean lecithin (2:1) were made by homogenizing in an Atomix with water and SI previously adjusted to different pH values between pH 2 and 10.5 with either 0.1*N* HCl or 1*N* NaOH. The final ionic strength ($\Gamma/2$) was 0.1. The disperse phase concentration was 25%. Known weights of these emulsions were extracted with $CHCl_3$:CH_3OH (1:1), to determine total fat, and *n*-hexane, to determine the degree of instability. The amounts extracted were determined by drying in a vacuum rotary evaporator to constant weight; hexane-accessible lipids were expressed as percent of the total fat in the sample.

RESULTS AND DISCUSSION

The sedimentation distribution of native soybean globulins and the effects of urea or extremes of pH on the sedimentation distribution profile are essentially

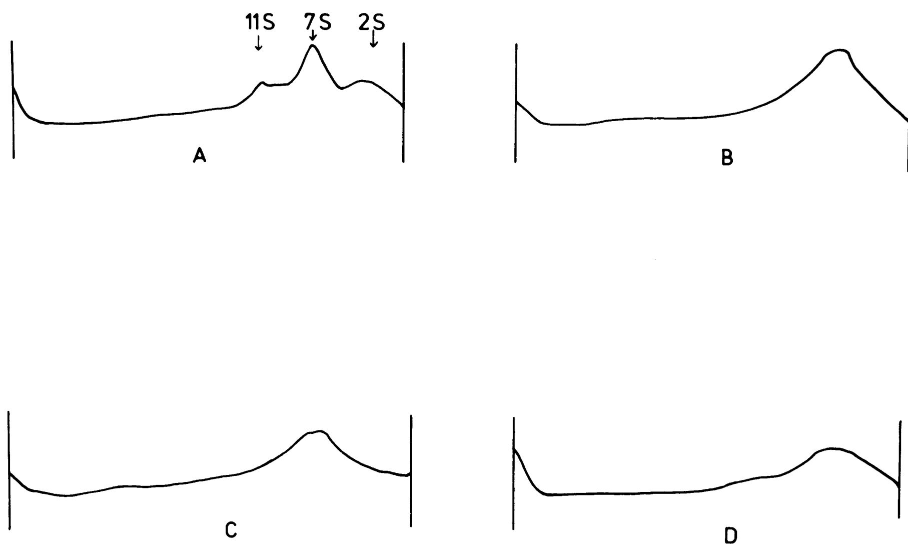

Fig. 2. Ultracentrifuge patterns obtained when samples B-E of Fig. 1 were dialyzed against $10^{-2}M$ tris pH 7.2. A, 2*M* urea sample dialyzed; B, 4*M* urea sample dialyzed; C, 6*M* urea sample dialyzed; D, 8*M* urea sample dialyzed.

similar to those that other investigators (19–22) reported. The profile of the native globulins shows the typical 15S, 11S, 7S, and 2S components (Fig. 1A). With increasing urea concentrations, a progressive disruption of the 15S, 11S, and 7S proteins is observed. Treatment with 8*M* urea disaggregates soybean to 2S species (Fig. 1E). On removal of urea by dialysis, changes observed in the sedimentation distribution profile reflect a dependence on urea concentration used for denaturation (Fig. 2, A–D). After 8*M* urea treatment and subsequent dialysis, no marked aggregation is observed, and the urea-free protein appears to be predominantly 2-3S. Spray drying of UI causes massive aggregation that can be reduced partially by ultrasonic irradiation. The effects of pH and drying on $S_{20,k}$ of SI and UI are listed in Table I. These results are important for understanding the lipid binding behavior of soybean and other multisubunit vegetable proteins.

Biological membranes (23) and natural lipoproteins of serum, egg, and milk are lipoprotein complexes made up of neutral lipids, phospholipids, and proteins in varying proportions and forming multiphase systems. In membranes, the neutral lipid is predominantly free cholesterol, whereas in lipoproteins it is cholesteryl esters or triglycerides or both. Choline-containing lipids, PC, and

TABLE I
Effects of pH and Drying on Sedimentation Pattern

Treatment	$S_{20,w}$	Comments
pH 10.0	...	All original components, some 7S dimerized to 9S
pH 12.0	2.0	Homogenous except for some insoluble material
pH 10.0 $\xrightarrow{\text{titrated}}$ pH 8.0	...	2, 7, 11, and 15S present
pH 12.0 $\xrightarrow{\text{titrated}}$ pH 8.0	2.1	Fairly homogenous, some insoluble material
pH 2.0/1 hr	1.8S	Fairly homogenous, some insoluble material
pH 2.0/20 hr	1.9S	Fairly homogenous, some insoluble material
pH 2.0/1 hr $\xrightarrow{\text{titrated}}$ pH 8.0		Polydisperse, major component > 2S
pH 2.0/20 hr $\xrightarrow{\text{titrated}}$ pH 8.0	2.0	Polydisperse, major component > 2S
Urea isolate (UI)		
Freeze-dried UI	...	Polydisperse, no boundaries formed
Spray-dried UI	...	Polydisperse, no boundaries formed
Freeze-dried UI (sonicated, 5 min)	1.8S	Soluble material homogenous, much insoluble material
Spray-dried UI (sonicated, 5 min)	2.0S	Soluble material homogenous, much insoluble material
Nondried UI	1.9S	Soluble material homogenous, little insoluble material
Soy isolate (SI)		
Freeze-predried SI treated pH 12.0 for 10 min	3.54	Homogenous peak, little insoluble material
Freeze-predried SI treated with 0.5 NaOH for 10 min	3.83	Homogenous peak, little insoluble material

sphingomyelin represent a sizeable proportion of phospholipids. In various studies on separation and reassembly of molecular moieties of lipoproteins and membranes, use is made of cavitational force fields; invariably an investigator chooses to start with complexes of PC plus apoproteins to study underlying lipid protein interactions involved in the formation of organized structures (24).

When aqueous dispersions of PC are merely added to water extracts of defatted soyfluff (soybean globulins) or salt solutions of SI and the resulting mixture is analyzed by equilibrium ultracentrifugation, the bulk of the protein bands at a buoyant density of 1.3. A typical result is shown in Fig. 3. This situation is unaltered even if PC and native soybean globulins are codispersed, or if instead of PC, a mixture of zwitterionic PC and charged phospholipids (PE plus PS) is used. However, SI, which was previously denatured by either $\geqslant 6M$ urea or $6M$ guanidine hydrochloride followed by subsequent removal of the denaturant or disaggregated by exposure to pH 10.5 or pH 2-3, can be codispersed with phospholipids to yield lipoproteins of d~ 1.17. As one could

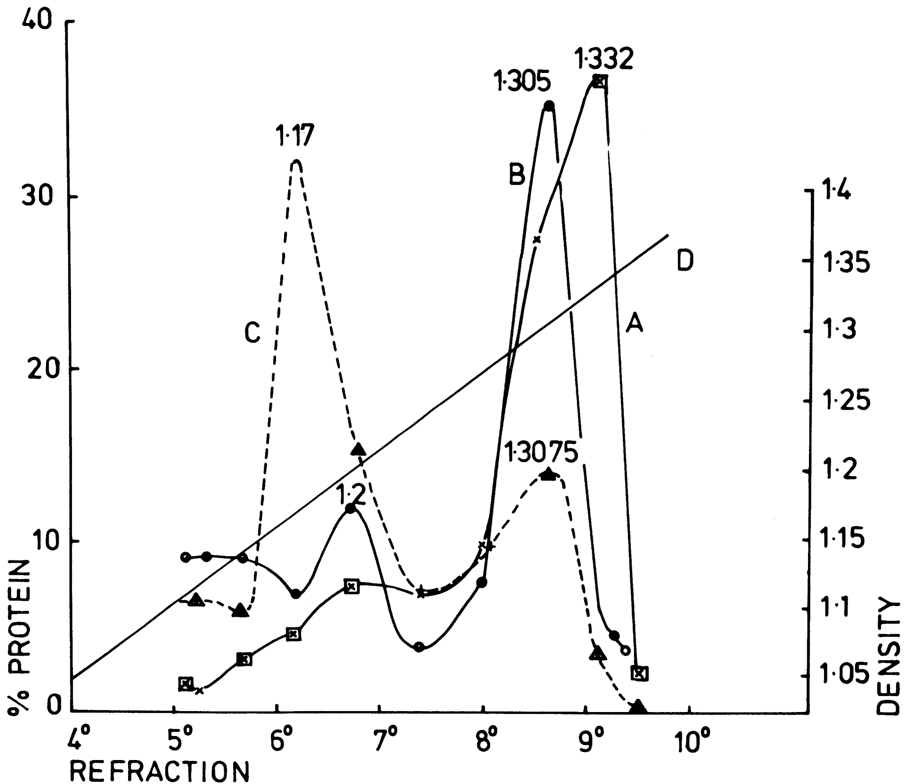

Fig. 3. Typical protein distribution profile at or near equilibrium of A, soybean protein, native or dissociated; B, native soybean codispersed with phospholipids; C, dissociated soybean codispersed with phospholipids. D is plot of refractive index versus density of NaBr solutions.

expect, lipid phosphorous distribution (not shown) follows the protein distribution of lipoproteins. Dissociation of the native soybean multisubunits into a population consisting predominantly of single subunits, folded or unfolded, is apparently necessary for lipoprotein formation.

Prior dissociation of soybean protein is also necessary for incorporating triglycerides into lipoproteins. Triolein, trielaidin, tripalmitolein, or sunflower oil can be incorporated readily into soybean-based lipoproteins made with PC or PC plus PE plus PS. Figure 4 illustrates that triglyceride distribution follows the protein distribution in the buoyant density distribution profile. PC alone does not favor the incorporation of tripalmitin into lipoprotein. PC plus PE plus PS, however, allows incorporation of significant amounts of tripalmitin. Furthermore, presence or absence of sulfite during preparation of SI makes little difference in activating it for lipoprotein formation via amide denaturation. This suggests a minor role for disulfide bridges in lipid-soybean protein interactions. Ionic and hydrophobic bonds may dominate such interactions.

Figure 5 is an electron micrograph of lipoprotein complexes based on phospholipids and UI. It reveals closed vesicles with one or more layers. The

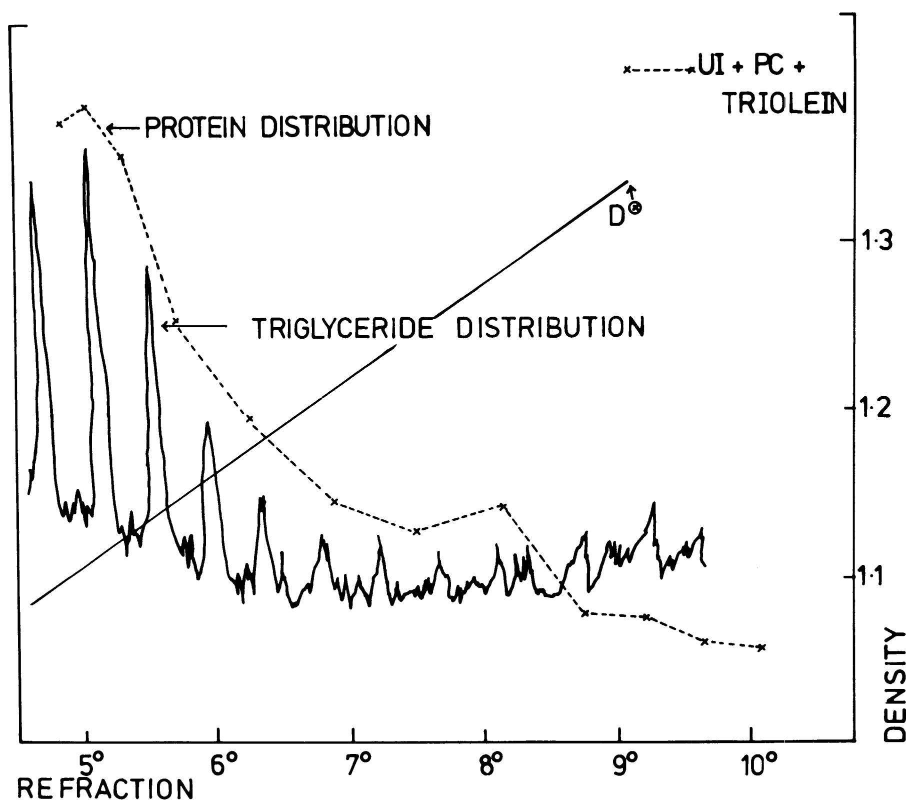

Fig. 4. Protein and triglyceride distribution profile after complexing UI with PC plus triolene. D is plot of refractive index versus density of NaBr solutions.

vesicles distribute between 200 and 1,000 Å, and the vesicle wall thicknesses vary between 30 and 70 Å. These structures are distinctively different from those obtained with UI solution or free phospholipid aqueous dispersion (unpublished observations) and are membranous in their morphologic appearance.

Incorporation of neutral lipid such as triolein or sunflower oil into a

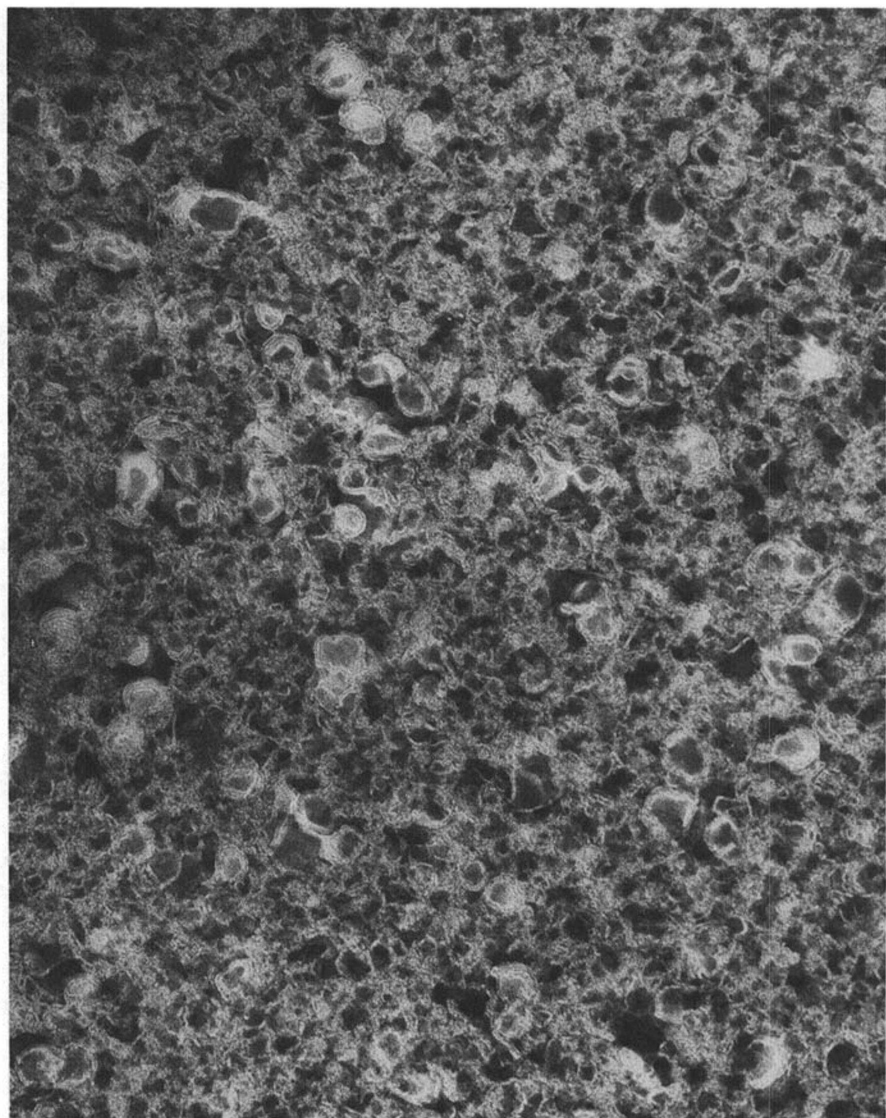

Fig. 5. Electron micrograph of codispersion of disaggregated soybean protein (UI) with mixed phospholipid-water systems (PC plus PE plus PS). Magnification, ×62,000.

lipoprotein complex of UI plus PC reveals oil bodies stabilized by irregular structures of proteins or phospholipids and proteins (Fig. 6). These bodies range in diameter between 500 and 2,000 Å. The lipoproteins are asymmetric, polydisperse, and irregular when compared with the published electron micrographs of many natural lipoproteins (3,5).

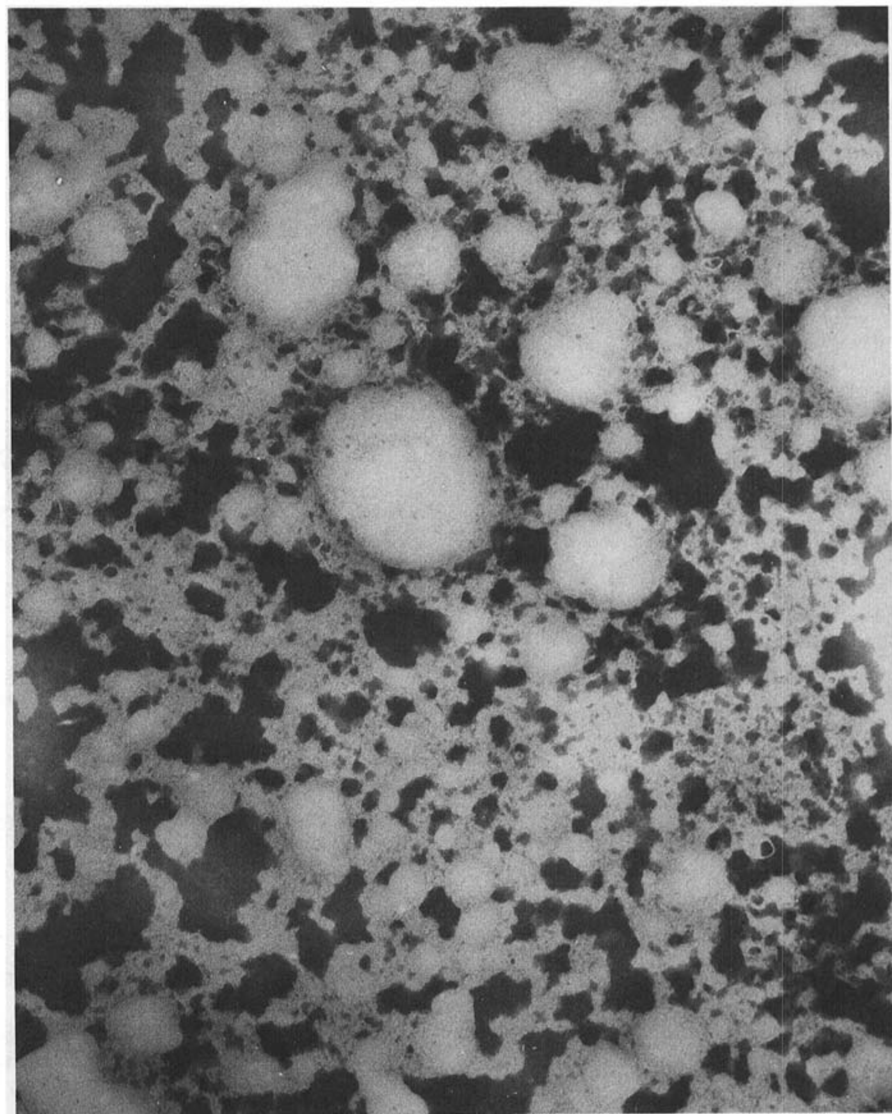

Fig. 6. Electron micrograph of lipoprotein formed by codispersion of UI, PC, and sunflower oil. Magnification, ×62,000.

Exposure of soybean to pH 2 or 12, followed by reversal to pH 8 and codispersion with phospholipid water systems, also yielded lipoproteins of characteristic buoyant densities.

Native soybean glycinin 11S, conglycinin 7S, and native proteins of groundnut, rapeseed, and cottonseed do not form lipoproteins unless they are disaggregated or scrambled into subunits prior to interaction with lipids. Other seed proteins need to be activated with 8*M* urea or extremes of pH in the same way as soybean before they will form lipoproteins.

Current models (12,25) of the major soybean protein 11S glycinin suggest that it consists of two similar, but not identical, monomers—each monomer consists of three different kinds of acidic and basic subunits. There are also distinguishable differences in the tertiary structures of 7S and 11S. In addition to disulfide, hydrogen, and ionic bonds, hydrophobic bonds play a dominant role in subunit interactions and stabilization of the native dimer. Furthermore, Lillford's (26) nuclear magnetic resonance data show that amino acid side chains of leucine, isoleucine, and valine are specifically involved in some element of structural order in native glycinins and γ-conglycinin. Urea disrupts this structure. Moreover, removal of urea causes reaggregation of subunits, but the original proteins are not reformed. These results are consistent with our observations and support our thesis that prior dissociation of soybean in the presence of suitable denaturants and scrambling of the native proteins after

TABLE II
Stability of Sunflower Oil-in-Water Emulsions Made With Native Soy Globulins/SI and Denatured Soya Globulins/UI

Composition of Emulsion	Disperse Phase (%)	Initial pH of Protein Solution	Treatment Prior to *n*-Hexane Extraction	Theoretical Lipid Extracted (%)
Aqueous extract of soy meal plus soy lecithin plus sunflower oil	30	6.9	Room temperature	0.89
			Room temperature plus 1*M* NaCl	21.89
			100°C in 1*M* NaCl for 0.5 hr	26.32
UI plus soy lecithin plus sunflower oil	30	6.0–6.5	Room temperature	0.30
			Room temperature plus 1*M* NaCl	1.30
			100°C for 0.5 hr in 1*M* NaCl	3.05
	25	4.76	Room temperature	100.00
SI plus soy lecithin plus sunflower oil	25	7.0	Room temperature	3.68
			Room temperature plus 1*M* NaCl	4.86
			100°C for 0.5 hr in 1*M* NaCl	6.06
	25	5.0	Room temperature	92.50
UI plus sunflower oil	25	6.0–6.5	Room temperature	0.11
			Room temperature	0.35
			100°C for 0.5 hr in 1*M* NaCl	0.67
Soy lecithin plus sunflower oil	25	4.8	Room temperature	4.56
		(water)	Room temperature plus 1*M* NaCl	100.00

denaturant treatment, followed by its subsequent removal, are necessary to activate the protein for lipoprotein formation. Unfolding not only allows greater surface area but also exposes numerically larger numbers of ionic and hydrophobic residues for interaction with polar and apolar regions of lipids.

A recent report (27) claims that purified 11S globulins can be renatured in high yield from nonreductive denaturation in 8*M* urea. Our results on the effects of urea were obtained with whole SI prepared in the presence of sulfite. Hence, they are not directly comparable with the claim, especially since interactions between various proteins of the isolate in the denatured states are not understood.

The advantages of unfolding soybean globulins for lipid binding are manifested by the ease with which they stabilize emulsions of oil and water. Results in Table II show that aqueous extracts of soybean globulin and UI stabilize emulsions at room temperature and near-neutral pH. On raising the $\Gamma/2$ and heating at 100° C for 10 min, however, the emulsion made with native globulins, unlike those made with UI, become demulsified as judged by the criterion of accessibility of fat to hexane. A salt solution of SI (pH 4.8) gives unstable emulsions even at room temperature. The effect of pH on SI and UI at a constant $\Gamma/2$ of 0.1 is illustrated in Fig. 7. Clearly, both the native globulins and scrambled globulins, represented by UI, are influenced by pH with respect to a constant $\Gamma/2$ in their ability to stabilize sunflower oil phospholipid

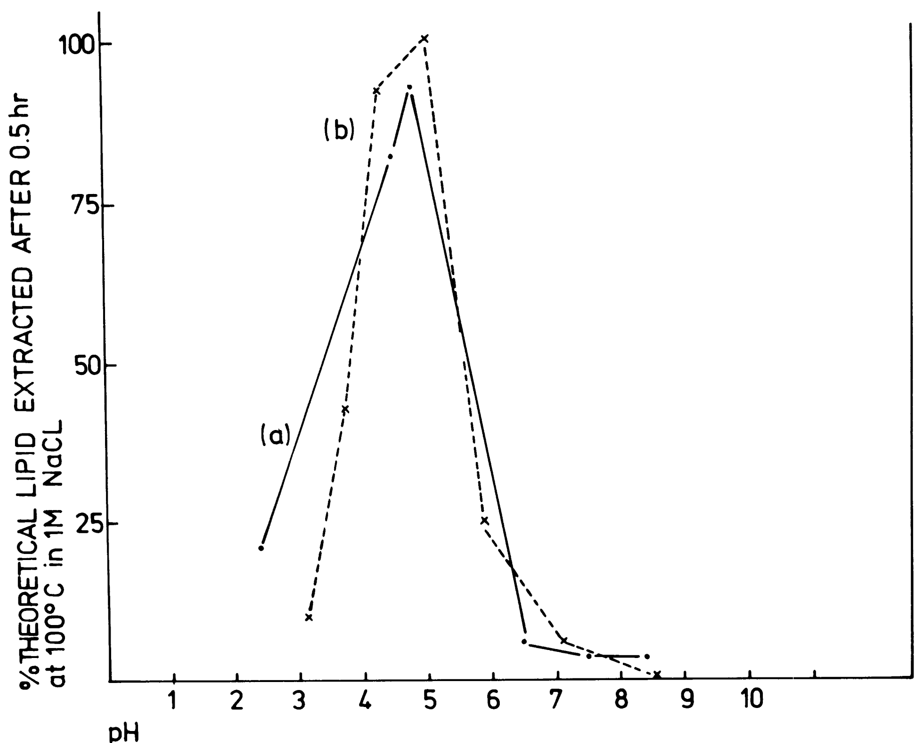

Fig. 7. Effect of pH on emulsifying ability of SI (a) and UI (b).

and water emulsions. It is also clear from the results that the initial pH of soybean, native or scrambled, is critical for emulsion stability. pH ranges between 3 and 6 are close to the isoelectric range and are unsatisfactory as regards emulsion stability. These results are in agreement with the observations of Franzen and Kinsella (28), who demonstrated that the pH-emulsifying capacity (volume of oil emulsified by 1 g of protein) profile followed the pH-solubility profile of soybean protein.

Since protein adsorption and viscoelasticity at the oil-water interface is maximum at the isoelectric pH of the protein, one would expect maximum resistance to coalescence and also to lipid extractability by *n*-hexane. The observation that the emulsions are least resistant to *n*-hexane could be explained in terms of a distance effect between protein molecules. In the isoelectric pH range, the distance between molecules is minimum with minimum repulsion due to zero net charge. This reduces the thickness of water films between protein molecules, with the result that clumping occurs, thereby rendering the fat accessible to *n*-hexane.

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