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Interaction of Phosphatidylcholine Vesicles with Soybean 7S and 11S Globulin Proteins

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Soy phosphatidylcholine (PC) vesicles prepared by a reverse-phase evaporation method in the presence of ^{14}C-labeled sucrose were mixed with 7S or 11S globulin solutions of varying concentrations. Since both protein preparations contained iron, controls in 2.5–5 mM $FeCl_3$ and controls without protein or iron saturated with air or nitrogen were used to estimated oxidative degradation of lipid species. Both 7S and 11S reduced the level of sucrose entrapment, but results varied widely among preparations. The level of reduction had no significant dependence up globulin type or protein concentration. Ferric ion reduced the level of sucrose encapsulation by approximately 25% after 4–5 days at 4 °C. With *N*-[^{14}C]-methyl-labeled soy PC, liposomes preformed by sonication were added to protein solution at known mole ratios of PC to protein. After about 12-h storage at 4 °C, preparations were subjected to an ultracentrifugal separation procedure. Gradient fractions were compared to those of a liposome control for label content. These results confirm the existence of protein–lipid interactions, with the inference that the extent of association is different for both globulin preparations.

The 7S and 11S soy protein globulins, so designated on the basis of sedimentation behavior, comprise about 70% of the total seed protein content, with a major protion of the proteins located in protein bodies within a matrix of lipid spherosomes. Although lacking specific biological activity, each globulin is composed of smaller subunits, and the 7S globulins are glycoproteins containing mannose and *N*-acetylglucosamine residues. The physical and chemical properties and amino acid composition for these globulins were reviewed by Wolf (1972), Hill and Breidenbach (1974), and Koshiyama (1983).

Soy globulin–phosphatidylcholine (PC) complexes are formed when systems containing protein and PC are sonicated together (Kanamoto et al., 1977). Reports of similar associations of liposomes with either 7S or 11S proteins (Ohtsuru et al., 1979; Ohtsuru and Kito, 1983) implied that the lipid interactions occur at hydrophobic regions within the protein and lead to alteration in protein conformation and that the association does not take place at specific amino acid residues.

The general effects of protein interaction with lipid vesicles have been studied (Guo et al., 1980: Ohki and Leonards, 1982; Schenkman et al., 1981; and many citations given therein). Large Liposomes have been a means of delivering nucleic acid polymers to plant protoplasts (Lurquin et al., 1981; Matthews and Cress, 1981; Lurquin and Sheehy, 1982) and have served as an immobilizing substrate for trypsin (Goldmacher, 1983). By using the encapsulating property of lipid vesicles, this investigation reevaluated the extent of soy globulin–soy PC interactions and considered the possible detrimental effects produced by protein–lipid interaction and oxidation of lipid residues upon the soy phospholipid vesicle dynamics.

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MATERIALS AND METHODS

Soybean Materials. Soy PC type 111-S in chloroform solutions (100 mg/mL) was purchased from Sigma Chemical Co., St Louis, MO. *N*-[^{14}C]-Methyllabeled PC, prepared by the procedure of Patel et al. (1979) from soy phosphatidylethanolamine, was supplied by C. G. Crawford of this laboratory as a chloroform solution (ca. 12 mg of PC/mL). The 7S and 11S protein fractions, isolated by the method of Thanh and Shibasaki (1976) and Thanh et al. (1975), were provided by D. H. Honig of this laboratory.

Reagents and Solvents. Ficoll, a nonionic polymer of sucrose, types 70 and 400 DL were obtained from Sigma Chemical Co., St Louis, MO. Aqueous solutions of [^{14}C]sucrose (250 μCi) were purchased from the Amersham Corp., Arlington Height, IL. Maxifluor, a liquid scintillation cocktail for use with aqueous media, was from the J. T. Baker Chemical Co., Phillipsburg, NJ. All other chemicals used were commercially available and of reagent-grade quality. Organic solvents were used directly or redistilled.

High-Pressure Liquid Chromatography (HPLC) of Lipids. A C-18 μBondapak stainless steel semipreparative

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column (Waters Associates, Milford, MA) with a C-18 Corasil precolumn filter was used to separate oxidized from unoxidized molecular species in soy PC preparations (Crawford et al., 1980). Column effluents were monitored in the ultraviolet (UV) at 206 and 234 nm with a Beckman Model 155 variable-wavelength detector (Backman Instruments Inc., Fullerton, CA) and with a Model ALC/GPC-201 differential refractometer (Waters Associates). To isolate unoxidized PC, 40–50 mg of PC in metanol–water (95:5 v/v) was injected onto the column. The column was eluted at 2 mL/min with this solvent for 20 min to separate the rapidly eluting oxidized material. A solvent change to straight methanol stripped the column of unoxidized PC in a single fraction (ca. 40 mL). This fraction, taken to near dryness on a rotary evaporator, was redissolved in chloroform–methanol (2:1 v/v) and taken to dryness on the evaporator to remove small amounts of water present. Evaporator vacuum was broken with a nitrogen supply. When isolating molecular species of PC or when just comparing chromatograms of different preparations, the column was eluted with methanol–water (95:5 v/v) only.

Preparation of Vesicles. The reverse-phase evaporation procedure and phosphate-buffered saline (PBS) system of Szoka and Papahadjopoulos (1978) was employed to prepare PC vesicle dispersions (30 μmol of PC/mL) encapsulating ^{14}C-labeled sucrose. The aqueous phase also contained 3 mM unlabeled sucrose. The average formula weight for soy PC was taken as 780 daltons. Liposomes containing ^{14}C-labeled and unlabeled PC were prepared in glass vessels employing a bath-type sonifier (Laboratory Supplies Co., Inc., Hicksville, NY, Model G 112 SPI-T). After removal of chloroform under a nitrogen stream, a preselected volume of nitrogen-saturated cold PBS buffer containing 3 mM sucrose was added, and the glass vessels were sealed to maintain a nitrogen atomosphere above the buffer. Samples were then sonicated for 5-min intervals at room temperature with cooling on ice between intervals until clear or slightly translucent dispersions were obtained. The total lipid content of the preparation varied from 10 to 30 μmol/mL.

Preparation of Mixtures. Reaction vessels consisted of 5-mL vials equipped with aluminum-lined screw caps that had been prewashed with chloroform–methanol (2:1 v/v). Protein stock solutions of 7S and 11S (2–3% w/v) in cold, nitrogen-saturated PBS–sucrose buffer were mixed with the vesicle preparations to give 1–2 mL of a mixture containing 0.5, 1.0, or 1.5% w/v protein and 7–10 μmol of PC. after being swept out with nitrogen, the vials wre capped tightly, sealed with Parafilm (American Can Co., Greenwich, CT) and placed in a refrigerator at 4 °C for 4–11 days. Controls containing no protein and controls containing 2.5–5 mM $FeCl_3$ were both maintained under nitrogen for the same period and also were saturated with air before storage to study the effect of lipid oxidation upon release of encapsulated sucrose.

Radiolabeled liposomes were mixed with protein solution to yield mole ratios of PC to protein of approximately 90:1, 45:1, 25:1 and 1:1. Protein concentrations were maintained at 1% w/v. Molecular weights for 7S and 11S are considered to be 186 000 and 360 000 daltons, respectively. Protein controls without lipid and liposome controls (2.5 μmol of PC/mL) were used. All samples under nitrogen atmosphere were stored overnight at 4 °C.

Isolation of Liposomes and Analysis of Mixtures. Discontinuous Ficoll step gradients were prepared in 5-mL cellulose nitrate centrifuge tubes (Dellaporta and Frayley, 1981) by first thoroughly mixing 0.5 or 1 mL of reaction mixture with 0.5 mL of 30% or 50% w/v Ficoll. Then 2.5 or 3 mL of 10% w/v Ficoll was carefully layered on top, followed by a 1-mL layer of buffer. All Ficoll solutions were made from 50% w/v solutions containing buffer and kept at 4 °C under a nitrogen atomsphere. Samples were centrifuged in a Beckman L8-80 ultracentrifuge employing an SW55Ti six-place rotor at 40 000 rpm (RCF = 151 kg) for 2 h at 5 °C. Lipid, which had encapsulated sucrose, collected as a heavy film at the 10% Ficoll buffer interface and was easily removed along with a major portion of the buffer layer; it then was made up to a 2-mL dispersion with fresh buffer. The remainder of the tube contents, including protein pellet, was transferred to a 5 mL volumetric flask and made to volume with fresh buffer.

For sonicated PC preparations, when the ratio of PC to protein was 45:1 or less, a distinct film at the interface was barely visible or nonexistent. Consequently, the upper 2 mL of the step gradient was used as a single fraction; the remaining Ficoll layer was made to a 5-mL volume as a second fraction; the protein pellet, dispersed in 0.5 mL of fresh buffer, constituted the third fraction.

The radioactivity distribution among the fractions was estimated by scintillation counting (CPM) in Beckman Model L8-250 or LS-9800 scintillation counters using 100 μL of fraction and 3 mL of Maxifluor in Beckman Snap-Cap Bio-Vials.

Lipid isolates were extracted from aqueous contaminants by the method of Folch et al. (1957) and then subjected to HPLC for comparative purposes.

Levels of protein content in pellets found during the isolation of lipids were estimated by the method of Lowry et al. (1951).

Other Procedures. Unoxidized molecular species of soy PC, fractionated by HPLC, were analyzed for fatty acid content by gas chromatography (GC) (Crawford et al., 1980). To determine the extent of tightly bound contaminanting lipid, chloroform–methanol (2:1 v/v) extracts of the 7S globulin were also subjected to GC analysis.

Soybean meals contain iron (Osborn, 1977), so dilute (0.3% w/v) solutions of 7S and 11S in PBS buffer were analyzed by atomic adsorption spectroscopy (AAS) (Varian, Sunnyvale, CA, Model AA-126) for iron content in excess of blanks of buffer alone.

Electron microscopy (TEM) of undialyzed lipid vesicles was performed on an Hitachi H-500 electron microscope (Hitachi, Ltd., Tokyo) operated at 75 kV. Lipid dispersions were applied to Formvar carbon-coated Cu grids for 1 min and then drained of excess liquid by edge on contact to tissue paper. Grids were negatively stained with sodium phosphotungstate (2% w/v, pH 6.5) for 1 min, with excess staining agent removed as before, and then examined as soon as possible.

RESULTS AND DISCUSSION

Lipid and Protein Purity. As evaluated by HPLC and GC, the molecular species content and fatty acid composition of the soybean PC used here differed very little from that reported by Crawford et al. (1980). The retention times indicated in Figure 1 of Crawford et al. (1980) are about half those found when using a semipreparative column and methanol–water (95:5 v/v) at the same flow rate. As purchased, the PC was 98% pure (based on RI profile areas) but contained small amounts of 234-nm absorbance, indicating a low-level fatty acid oxidation. The influence of lipid oxidation upon liposome membrane integrity has been examined from the standpoint of autoxidation and oxidation induced by specific agents, e.g., iron salts (Barclay and Ingold, 1981; Bucher et al., 1983; Edwards and Quinn, 1982; Kunimoto et al., 1981; Weiss

et al., 1983; Wu et al., 1982). As determined by AAS, the 7S globulin used contained 130–140 ppm of Fe and the 11S fraction and 30–40 ppm of Fe, as compared to 129–280 ppm for defatted soybean meal (Osborn, 1977). This protein-associated Fe did not have a significant effect upon the extent of lipid oxidation. Furthermore, residues from chloroform–methanol extracts of the globulin proteins contained 234 nm absorbing species, eluting on HPLC at similar retention times as the oxidized species observed in PC samples. The level of contamination was about the same for 7S and 11S fractions on a weight basis, and was 1.5–2% w/w for the 7S fraction by GC analysis. The protein precipitates remaining after the extraction were insoluble in typical globulin solvents.

Preparation of Vesicles. The reverse-phase evaporation procedure of Szoka and PPahadjopoulis gave levels of sucrose encapsulation of 19 ± 5% on a batch-to-batch basis in preparation of soy PC vesicles. The wide range for the encapsulation of sucrose solution noted here could not be directly related to low levels of oxidized lipids present in different commericial isolates of soy PC based upon comparisons to results obtained following HPLC cleanup of soy PC. Redistillation of reagent-grade organic solvents was also without effect. A problem frequently encountered in the procedure occurred during the removal of diethyl ether from the sonicated aqueous ether emulsions. The emulsions were partially transparent and stable for at least 30 min when the sonication time for the preparation of the emulsions was at least 5 min. Removal of the organic phase under mild vacuum conditions at 30–35 °C readily produced the expected gels. The gels liquified only slowly with increasing vacuum. Frothing was a very minor problem because ether removal generally caused little disturbance in the gel structure. The procedure led to opaque dispersions upon complete removal of ether. The clarity did not improve when dispersions were incubated at 40–45 °C for 30 min. After 1–2 h in the cold, amorphous precipitates formed. After cold storage, lipid vesicles produced translucent stable dispersions when treated with repeated 30–60-s bursts of sonications (5-min total). For soy PC, this finding would suggest that the reverse-phase method produces primarily random-sized multibilayer structures. The disruption of vesicle dispersions by sonication reduced the level of entrapped sucrose to a value of 3–4%. Repeated freezing in dry ice–acetone and thawing at room temperature of the sonicated samples restored the level of encapsulation to initial values (16–19%), but once again the dispersions were opaque and produced precipitates upon standing. The freeze–thaw method has been used to prepare liposomes having large trapping capacities (Pick, 1981). Although the range of sucrose entrapment was quite broad (19 ± 5%), multiple determinations with single preparations had precissions of 2% or better.

Chromatography of Vesicle Isolate. The differential refractive index profiles for several vesicle lipid isolates separated by HPLC appear in Figure 1. In this instance the column loading was low (ca. 2 mg of PC) so that the levels of oxidation as measured by absorbance at 234 nm among the various lipid isolates could be compared qualitatively. The oxidized species initially present were removed by HPLC from the PC used to prepare the vesicles, and all organic solvents used in the preparation, extraction, and elution steps were redistilled. The chromatograms in Figure 1 were obtained after 11 days of storage at 4 °C. The profiles for the controls stored under both air and N_2 (Figure 1A) have essentially the same relative areas for the three major fractions. Fractions I,

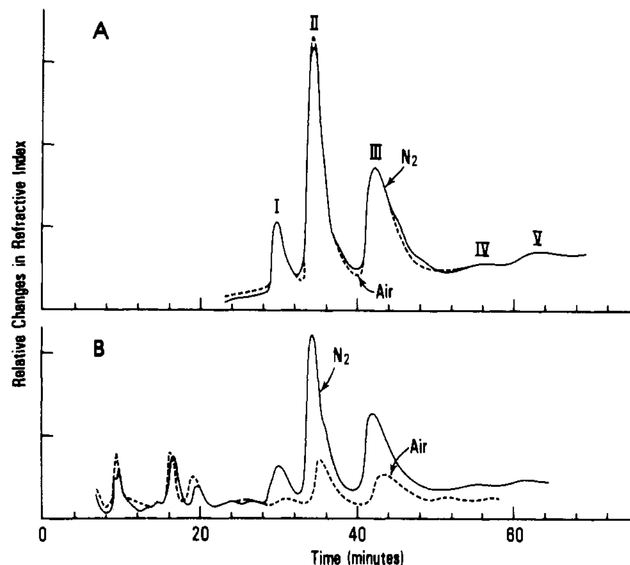

Figure 1. Relative refractive index changes on HPLC for extracts of lipid vesicle isolates: (A) nitrogen and air-saturated controls after 11 days at 4 °C; (B) from 5 mM $FeCl_3$ after 11 days at 4 °C in nitrogen or air.

II, and III shown in Figure 1A account for 90% of the total lipid in soy PC. A small amount of 234-nm absorbance eluting as three peaks at 15–21 min (0.06 AU maximum) was detected in both isolates. The level of sucrose entrapment for the controls was 19.5 ± 0.2%.

Storage of vesicle dispersions in the presence of 7S and 11S globulins under N_2 at 4 °C did not markedly alter the lipid refractive index chromatograms. The 234 nm absorbance profiles were also very similar to those for the controls, with small differences due to the extraction of absorbing species associated with protein contamination (Crawford et al., 1980). However, though no differences in HPLC profiles were observed, the level of entrapped sucrose could be reduced by as much as 20% by storing in the presence of 7S or 11S proteins. Furthermore, when chloroform–methanol extracts of protein–PC preparations were partitioned against 1% w/v NaCl solutions (Folch et al., 1957) to obtain samples for HPLC analysis, an insoluble film developed at the aqueous–organic interface. The films were insoluble in the original solvent and in neutral salt solutins. When dispersed by sonication in a small volume of saturated sodium bicarbonate (0.5–0.6 mL), the films gave a positive ninhydrin color upon being heated in a boiling water bath, which indicated that the films were proteinanceous. When standard solutions of 7S and 11S in saturated bicarbonate and the Lowry procedure were used, the films amounted to about 3% of the protein in the original preparations. Per gram of lipid isolate, this value amounts to 43–44 mg of protein for both 7S and 11S and possibly is due to the presence of similar chemical structures or subunits in both globulin proteins.

As indicated in Figure 1B, extensive lipid degradation occurred upon cold storage in the presence of 5 mM ferric chloride. The absorbance profile for preparations stored in both air and N_2 had a multiplicity of peaks, evidenced by refractive index differences eluting prior to the three main peaks. Two peaks for the N_2-saturated system (9.6 and 16.5 min) had absorbance values at 234 nm of 0.84 and 0.87, respectively. The corresponding 234-nm peaks for the sample stored in air was greater than 1 and 0.9 AU. After storage for 5 days in the presence of Fe, the isolated lipid vesicles stored under nitrogen contained 14.4% of the sucrose initially added to the sample, whereas the corresponding isolate stored in an air atmosphere contained

13.7% of the sucrose. After 11 days, the values were 14% and 6.9%, respectively.

Soy vesicles in the presence of 2.5 mM $FeCl_3$ degraded when maintained in a water vapor, N_2-saturated atmosphere in a bubble-trapped, all-glass system. The control was allowed to stand in a loosely sealed vessel exposed to atmospheric oxygen. After 24 h at room temperature, HPLC of the extracted lipid pointed to significant amounts of UV-absorbing species in both samples eluting from the column within 24 min. The total amount of UV absorbance detected in the N_2-saturated sample was about half that found in the control. The RI profile confirmed the existence of degradation products. The results obtained in the presence of Fe appear quite similar in general features to those obtained by Edwards and Quinn (1982) for the peroxidation of polyenoic multibilayer lipid structures, suggesting that a major portion of the sucrose is entrapped deep within multilamellar vesicles. In the absence of ferric ions, cold storage of soy PC reverse-phase vesicles reduced oxidation and the effect oxidation had upon the retention of entrapped sucrose, regardless of the gaseous atmosphere used for storage. After 3 and 7 days at room temperature, the sucrose content for vesicles under N_2 remained constant, whereas those stored under an air atmosphere contained 9.8% and 28% less sucrose, respectively. However, a temperature of 4 °C is probably well above the main liquid-crystal transition temperature for a highly unsaturated lipid (Jain and Wagner, 1980), and the lipid membranes still exist in the fluid state.

Electron Microscopy. A negatively stained TEM micrograph of an undialyzed soy PC vesicle isolate appears in Figure 2a. At low concentrations (0.2–0.5 mg/mL) only diffuse structures, appearing as background in Figure 2a, were observed. Detection of any well-defined structure in this preparation required a concentration of 2–2.5 mg of PC/mL in contact with the grid film for at least 1 min. Micrographs were taken after 5 days of cold storage, and this particular preparation contained 23.7% of the sucrose originally present in the aqueous phase used to prepare the vesicles. Parts b and c of Figure 2 are the corresponding micrographs obtained with the same vesicle preparation after cold storage in the presence of 1% w/v solution of 7S and 11S globulins, respectively. In the presence of 7S, the entrapped sucrose was reduced to 19.6% and to 21.2% with 11S. The micrographs in Figure 2 show a clear alteration of vesicle structures, indicating that an interaction has taken place between protein and lipid. The same concentration of lipid was used to prepare all grids shown in the figure; a lot of background structures (Figure 2b,c) present were observed only for preparations stored in the presence of protein. The level of sucrose encapsulation was always reduced after vesicles were stored in the presence of protein, but the amount by which the level was reduced varied from as little as 5% to as much as 18–19%. The extent of sucrose release was essentially independent of protein concentration (0.5–1.5% w/v). Also, the disruption of lipid structures when stored in protein solutions was not always as marked as indicated in Figure 2.

A single isolate of 7S globulin and another of 11S was used throughout this investigation, so the observed variability in the foregoing results is not due to differences in protein sample preparations. Consequently, a study with ^{14}C-labeled PC liposomes was used to obtain a more quantitative estimate of the extent of protein–lipid interactions.

Interaction of Liposomes with Soybean Protein. Soy PC dispersed in aqueous media by sonication yields poorly defined structures having diameters of 100–200 nm and larger, as determined from TEM micrographs (not shown). Systems of this type containing ^{14}C-labeled PC were preformed by extended periods (30–35 min) of intermittent sonications, then mixed with 2% w/v solutions of 7S or 11S globulins in amounts to give known mole ratios of PC to protein (1% w/v protein after mixing), and stored overnight at 4 °C. With the discontinuous step gradient ultracentrifugation used in the isolation procedure, the lipid–protein mixture was placed in the lower half of the centrifuge tube, where the average relative centrifugal force (RCF) exceeded 151 kg at 40000 rpm. A tendency of the liposomes to float to the buffer–10% Ficoll layer interface would be counteracted by any protein–lipid interaction, tending to carry the lipid along with protein toward the bottom of the tube, where the maximum RCF is 194 kg. In the near-neutral solutions used here (pH 7.2) at low ionic strength (ca. 0.015), the 7S proteins could dimerize, whereas the 11S globulins might undergo a reversible dissociation (Wolf, 1972). Because these latter phenomena may or may not occur in a highly viscous medium such as 15–16% w/v Ficoll, protein controls dissolved in PBS and in the absence of added lipids served as qualitative estimated for protein sedimentation behavior. A liposome control (2 mg of PC/mL) in the ab-

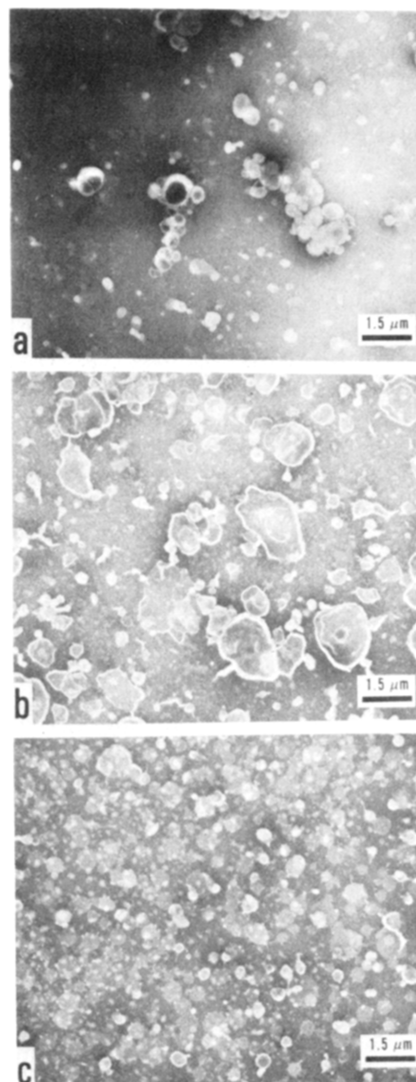

Figure 2. TEM micrographs of vesicle isolates: (a) soy PC control; (b) from 1% w/v 7S preparation; (c) from 1% w/v 11S preparation. See the text for preparation of grids for TEM examination.

Table I. Soy Phosphatidylcholine Distribution among Three Ficoll-Containing Fractions

protein preparation[a]	7S				11S				PC control
mol of PC/mol of protein	88/1	44/1	25/1	1/1	86/1	43/1	25/1	1/1	
% of ^{14}C in fraction 1	11	8	8	2	8	6	8	3	35
% of ^{14}C in fraction 2	81	87	81	65	83	84	79	66	65
% of ^{14}C in fraction 3	8	6	11	33	9	10	13	31	
mol of PC/mol of protein (fraction 3)	41/1	15/1	16/1	2/1	24/1	14/1	10/1	1/1	

[a] Fraction 1: buffer layer and 1 mL of 10% Ficoll layer. Fraction 2: 3 mL of Ficoll layer. Fraction 3: protein pellet dispersed in 0.5–0.6 mL of buffer.

sence of protein produced no sediment in the bottom of the tube after a 2-h ultracentrifugal separation at 40 000 rpm and 5 °C. The relative ^{14}C activity for the upper 2 mL of centrifugate (Fraction 1), 3 mL of Ficoll layer (fraction 2), and dispersed protein pellet (fraction 3) for various ratios of PC to protein appears in Table I. Also shown are the corresponding ratios of PC to protein determined for the protein pellets. The PC control indicates that the bouyant density for the major portion of the liposome is probably close to that of the 10% Ficol solution, since most of the radioactivity is in the second fraction.

Protein determinations undertaken by the Lowry procedure with fractions 1 and 2 showed that Ficoll blanks, diluted in the same manner as protions of the fractions, also produced a 750-nm absorbance that increased with time and masked the contribution made by small amounts of protein. The UV spectra (200–400 nm) for fraction 2 at a PC to protein ratio of 1/1 had absorbance bands at 206 nm (lipid and protein) and 276 nm (protein) for both 7S and 11S preparations. The Lowry procedure was used, however, to estimate the amount of protein in the pellet of protein controls by comparison to standard solutions of 7S and 11S. On this basis, it was determined that 16.4% of the 7S and 32.9% of the 11S sedimented from solution with a 20–25% uncertainty in these average percentage values. Therefore, if protein dimerization and dissociation phenomena are negligible, these average percentage values indicate that the same number of moles of 7S and 11S have sedimented from solution.

The values given in Table I for the mole ratio of PC to protein found in fraction 3 indicate that the extent of lipid–protein interaction is greater for the 7S globulins than for the 11S proteins. For the results with 11S protein, this mole ratio varies linearly with the initial mole ratio, and for a lne through the origin the slope has a value of 0.29 ± 0.02. With the 7S preparations, the mole ratio relationship is more scattered about a line; but for the limited data, a corresponding line through the origin has a slope of 0.45 ± 0.04, a value 1.6 times that found with the 11S isolate. As noted earlier, the 7S isolate has 3–5 times the iron content of the 11S preparation. The amount of Fe detected in the 7S isolate corresponds to approximately $^1/_2$ mol of Fe/mol of protein. If a portion of this Fe is associated with enzymes such as lipoxygenase, then the 3–5-fold difference in Fe content points to a significant difference in macromolecular composition of the two globulin isolates. Whereas the action of lipoxygenase upon phosphatidylcholine lipids is slight at neutral pH values (Sessa, 1979), this enzyme or other Fe-containing proteins could serve as specific subunits for the interaction of the proteins with liposomes, which would account for the results presented in Table I.

Even though protein subunits that sequester and render Fe incapable of catalyzing lipid oxidation might serve as sites of interaction, the extent of protein–lipid association would depend upon the physical character and dimensions of the lipid dispersions, as well as upon the molecular species content. Thus, sucrose release from multibilayer structures could be initiated by association with specific protein sites; however, conformational changes within the proteins, which result from the protein–lipid interaction or binding of lipid to protein, would inhibit a higher level of interactions or binding. Consequently, the extent of interaction appears to be independent of protein concentration when the mole ratio of PC to protein was high (>100/1), as was the case for the initial portion of this investigation. The release of entrapped sucrose from within lipid vesicles by the action of soy globulin proteins does not appear to involve a chemical degradation of lipid membrane moieties, as was suggested by the findings in the presence of ferric ions. Since the soy globulins possess other ionizable groups on amino acid residues, the protein–lipid association may be of a more general hydrophobic type similar to that proposed for local anesthetics with small phospholipid vesicles (Schlieper and Michaelis, 1983; Wang et al., 1983), which could also lead to protein conformational changes in the proteins limiting the extent of association at high mole ratios of phosphatidylcholine to protein.

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Effect of Temperature on the Conformation of Soybean Glycinin in 8 M Urea or 6 M Guanidine Hydrochloride Solution

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The effect of temperature on soybean glycinin in 8 M urea or 6 M Gdn·HCl solution has been studied by CD and ORD measurements. At room temperature the protein assumes an unordered conformation in the presence of the denaturant. With an increase in temperature, $[\Theta]_{MRW}$ in the range 250–210 nm assumes more negative values, suggesting formation of ordered conformations at higher temperatures.

The study of denaturation of proteins by various agents is aimed at understanding the molecular forces responsible for maintaining the native state of the protein (Kauzmann, 1959; Tanford, 1968). Urea, guanidine hydrochloride (Gdn·HCl), and heat have been extensively used as denaturants (Joly, 1965; Lapanje, 1978). Urea is less effective than Gdn·HCl in denaturing proteins (Tanford, 1968; Pace, 1975). Thermodynamics of denaturation of proteins as also the mechanism have been the subject of extensive studies (Tanford, 1968, 1970; Pace, 1975; Privalov, 1979, 1982).

There are only a few reports on the effect of temperature on proteins in concentrated urea or guanidine hydrochloride solution (Pace and Tanford, 1968; Ahmed and Salahuddin 1974; Cortijo et al., 1973). In 6 M Gdn·HCl solution, the proportion of α-helix in charged polypeptide chains appears to increase with temperature (Cortijo et al., 1973). Proteins of unordered conformation show conformational anomalies in denaturing solvents at higher temperatures that are independent of amino acid composition, chain length, and the nature of the denaturing solvent (Ahmed and Salahuddin, 1974). In the present investigation, the effect of temperature on soybean glycinin (also called the 11S protein of soybean) in 8 M urea and 6 M Gdn·HCl solution has been studied by CD and ORD measurements. The effect of temperature on the structure of glycinin has been reported (Fukushima, 1968; Koshiyama et al., 1980–81; German et al., 1982).

MATERIALS AND METHODS

Soybean (Bragg variety), grown in the State of Karnataka, India, was purchased from the local market. Bovine serum albumin (recrystallized), Gdn·HCl, and *N*-ethylmaleimide were from Sigma Chemical Co., and mercaptoethanol was from Fluka, Switzerland. Urea from British Drug House Co., India, was recrystallized from ethanol. Gdn·HCl was recrystallized by the method of Nozaki (1972). The buffer salts and NaCl were of analytical reagent quality.

Soybean glycinin was prepared by the method of Appu Rao and Narasinga Rao (1977). It was found to be homogeneous (>95%) by gel filtration on Sepharose 6-B, sedimentation velocity, and polyacrylamide gel electrophoresis on 7.5% gels at pH 7.8.

CD and ORD measurements were made with Jasco J20-C automatic recording spectropolarimeter. The instrument was calibrated with (+)-10-camphorsulfonic acid for CD and sucrose solution for ORD measurements. Quartz cells of different path lengths (1, 0.1, or 0.05 cm) were used for measurements in the region 330–200 nm. Slits were programmed to yield a 10-Å bandwidth at each wavelength. Protein concentration in the range 0.3–2.0 mg/mL was used. Mean residue rotation, $[m]_{MRW}$, and mean residue elipticity $[\Theta]_{MRW}$, were calculated by the standard procedure (Adler et al., 1973). On the basis of the amino acid composition of glycinin, determined with an LKB α-amino acid analyzer, a value of 115 was used for the mean residue weight (MRW). For measurements at different temperatures, water from a preset water thermostat was circulated through the double-walled cell and the temperature maintained for 15–20 min for thermal equilibrium before making measurements. The temperature was controlled to ±0.05 °C.

All the measurements were made in 0.05 M phosphate buffer of pH 7.8 containing 0.35 M NaCl and 0.01 M β-mercaptoethanol and in triplicate.

Protein concentration was estimated by absorbance measurements at 280 nm, using a value of $E^{1\%}_{1cm} = 7.9$

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