

Miscibility in Lipid-Protein Monolayers

Lipid-protein monolayers of various lipids mixed with either β -lactoglobulin, bovine serum albumin, or β -casein were transferred from the air-water interface to mica substrates and examined by electron microscopy. Phospholipids and triglycerides which gave condensed films at the air-water interface did not mix with proteins in monolayers as shown by electron micrographs of the transferred films. Heterogeneous films were observed with as little as 5 mole (residue) percent phosphatidic acid or tristearin in the mixed films, suggesting little, if any, solubility of condensed lipids in protein before the onset of phase separation. Homogeneous lipid-protein films were observed when the mixed monolayers were prepared from phospholipids or triglycerides that exhibit expanded behavior at the air-water interface. Fatty acids did not always follow this behavior pattern. Stearic acid, which forms a condensed film at the air-water interface, did not separate from the protein in a mixed film until the lipid content exceeded 10-15 mole (residue) percent. Nervonic acid, which exhibits condensed behavior at high pressure and room temperature, also did not separate from protein in mixed films under these conditions. Film balance experiments suggest that lipid-protein interactions may be hindering segregation of nervonic acid from proteins in monolayers. © 1985 Academic Press, Inc.

INTRODUCTION

Monolayers of lipids and proteins have been studied as models for biological surfaces by numerous investigators. In studies of pure lipid films, consideration has been given to structural factors and dynamic properties in which the approaches have been both experimental and theoretical (1-3). Likewise, protein films have received much attention over a span of many years (4-6). In addition to investigations on pure components, studies of lipid-protein mixtures have assumed increasing importance in the efforts to understand the structure and function of biomembranes (7, 8).

Previously, we reported on phospholipid-protein interaction in monolayer films (9) and electron microscopic examination of a few of the films to answer questions concerning phase separation vs. homogeneous mixing (10). It was observed that phospholipids which exhibit condensed behavior at the air-water interface

did not mix homogeneously with β -lactoglobulin in monolayers, whereas homogeneous lipid-protein films resulted under conditions where the lipid exhibited expanded behavior (10). These observations are consistent with the predictions from a lattice model of a dipalmitoylphosphatidylcholine bilayer containing integral protein (11). The theoretical and experimental studies dealt exclusively with phosphatidic acid and phosphatidylcholine mixed with protein, and in view of the importance attached to phase behavior in films of biochemical interest, the question of whether or not the observations apply to other classes of lipids mixed with proteins naturally arises. In this work we extend the investigations to include phosphatidylethanolamines, triglycerides, and fatty acids plus two additional proteins, β -casein and bovine serum albumin. The current work also includes the examination of mixed films with low lipid content and a more thorough investigation of lipid-protein monolayers in which the lipid exhibits a liquid expanded-liquid condensed

¹ Agricultural Research Service, U. S. Department of Agriculture.

phase change over the temperature range accessible to film balance measurements. The latter work involves the examination of both pressure-area curves and electron micrographs of transferred films.

MATERIALS AND METHODS

Materials

The fully automated recording film balance has been described (9). Deionized, double-distilled water was used for the subphase with the pH adjusted by sulfuric acid, 10^{-4} N, pH 4. β -Lactoglobulin A (β -lg) was prepared by the method of Aschaffenburg and Drewry (12) and β -casein by the method of Groves and Gordon (13). Fatty acid-free bovine serum albumin (BSA) was purchased from Miles Laboratories,² Elkhart, Indiana. The saturated fatty acids were from Applied Science Laboratories (State College, Pa.), the distearoylphosphatidylcholine (DSPC) was from Avanti Polar Lipids (Birmingham, Ala.), and all other lipids were from Sigma (St. Louis, Mo.).

Methods

Lipid and protein were mixed together in acid chloroform-methanol (2:1 v:v with 1 ml conc. HCl/250 ml solvent) immediately prior to spreading. Monolayer films were transferred to freshly cleaved mica for all systems for examination under the electron microscope. Monolayer films of a few systems were also transferred to collodion substrate for examination. Films containing either BSA or β -lg were transferred at 15 mN/m. Films containing β -casein were transferred at 8–10 mN/m because of the lower collapse pressure of this protein (M. C. Phillips, private communication). Additional details about solvents, monolayer preparation, and film transfer have been given (9). The transferred films were air dried and placed in a vacuum evaporator. Platinum from Pt-carbon pellets was laid

down at an angle of $\arctan \frac{1}{5}$ at a distance of 7.5 cm. Carbon was deposited vertically from a distance of 10 cm. The Pt-carbon replicas were lightly scored and floated onto a clean water surface, picked up on a 200 mesh copper grid, and examined in a Zeiss 10-B electron microscope operating at 60 kV.

RESULTS

In the course of these investigations 500–600 individual electron micrographs were examined in an effort to eliminate ambiguous observations insofar as possible. For certain systems, such as stearic acid-protein this meant multiple replications of the entire process from film balance experiments to the final micrographs. Thus, despite the small area depicted in each micrograph, we feel that an adequate sample of each system has been examined.

Films containing phosphatidylethanolamine or fatty acids did not always detach cleanly from the mica substrate during EM workup. Although sufficient fragments were obtained for EM examination, these systems were also transferred to collodion substrate for examination to guard against possible bias in the observations on incompletely separated films. No evidence for bias was found; observations with the two substrates were in agreement. Freshly cleaved mica is the substrate for all figures shown in this report.

Electron micrographs of replicas of lipid-protein monolayers containing PE and tristearin mixed with β -lg are shown in Figs. 1a and b. Segregation of the lipid and protein components is clearly evident with the circular patches of PE (Fig. 1a) surrounded by a continuous phase of protein. From the shadowing direction (arrows), it can be seen that the patches are raised above the continuous phase thus identifying the discontinuous phase as the lipid component. This is because the lipid component forms a thicker film than does the protein as discussed fully in Ref. (10). The tristearin- β -lg films is also heterogeneous as shown in Fig. 1b. A replica of a mixed film containing only a small amount of DPPA (Fig.

² Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

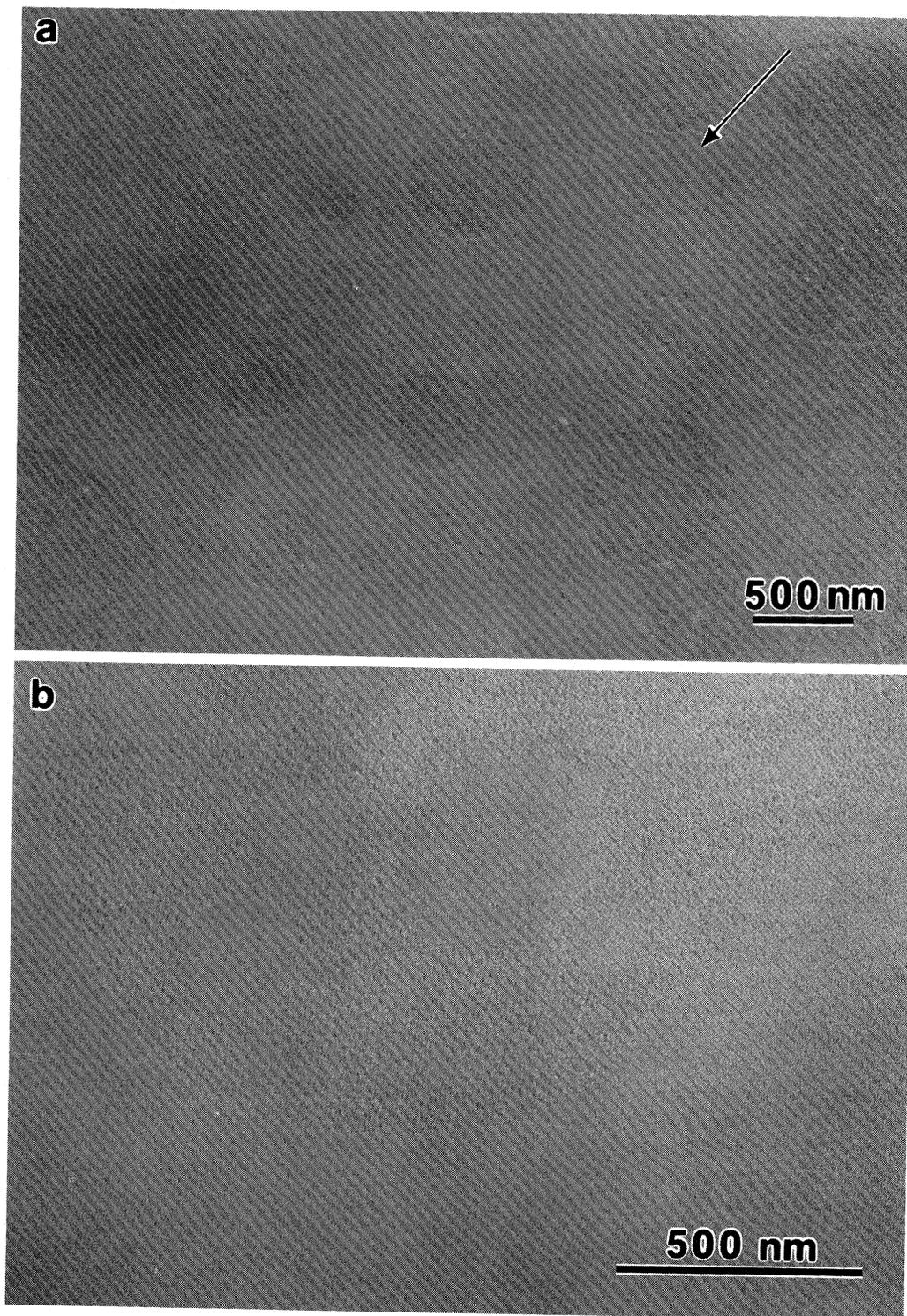


FIG. 1. Mixed film of (a) DSPE and β -lactoglobulin, 15 mole (residue) percent, and (b) tristearin- β -lactoglobulin, 10 mole (residue) percent lipid. Both films transferred at 15 mN/m and room temperature. The shadowing direction is indicated by the arrow in (a) and is the same for all micrographs in Figs. 1-3. Mica substrate.

2) also shows a heterogeneous structure with BSA as the continuous phase.

In Fig. 3, results are presented for mixed films containing β -lg which were prepared at (a) low temperature (9°C) where the lipid, 15-*cis*-tetracosenoic (nervonic) acid, exhibits liquid condensed behavior and (b) 29°C where the pure lipid is expanded at the air-water interface (14). For these runs, the film balance was controlled ($\pm 0.5^{\circ}\text{C}$) at the required temperature by water flowing through the aluminum block on which the Teflon trough was mounted. The transferred films were stored at 4°C until preparation for electron microscopy. The results show that segregation of the components occurs when the lipid is condensed (Fig. 3a) but homogeneous films result when the temperature is high enough for the lipid to exhibit expanded behavior (Fig. 3b).

A summary of the behavior observed in mixed films as a function of preparation con-

ditions is given in Tables I and II. In column 3, N_1 is the mole (residue) fraction of lipid in the mixed film, calculated on the basis of molecules of lipid and amino acid units (residues) of protein. Table I summarizes the results obtained when phospholipids or triglycerides were mixed with protein. For these two classes of lipids heterogeneous films were observed when the transfer was made under conditions where the lipid exhibited condensed behavior. When conditions were such that the lipid exhibited expanded behavior, homogeneous films were obtained.

Generally, unequivocal results were also obtained with the fatty acids listed in Table II. Stearic acid, however, gave variable results, but only over a narrow range of concentrations which probably represents the miscibility limit of the lipid with protein in mixed films. For example, 5 mole (residue) percent stearic acid in lipid-protein monolayers always gave clear

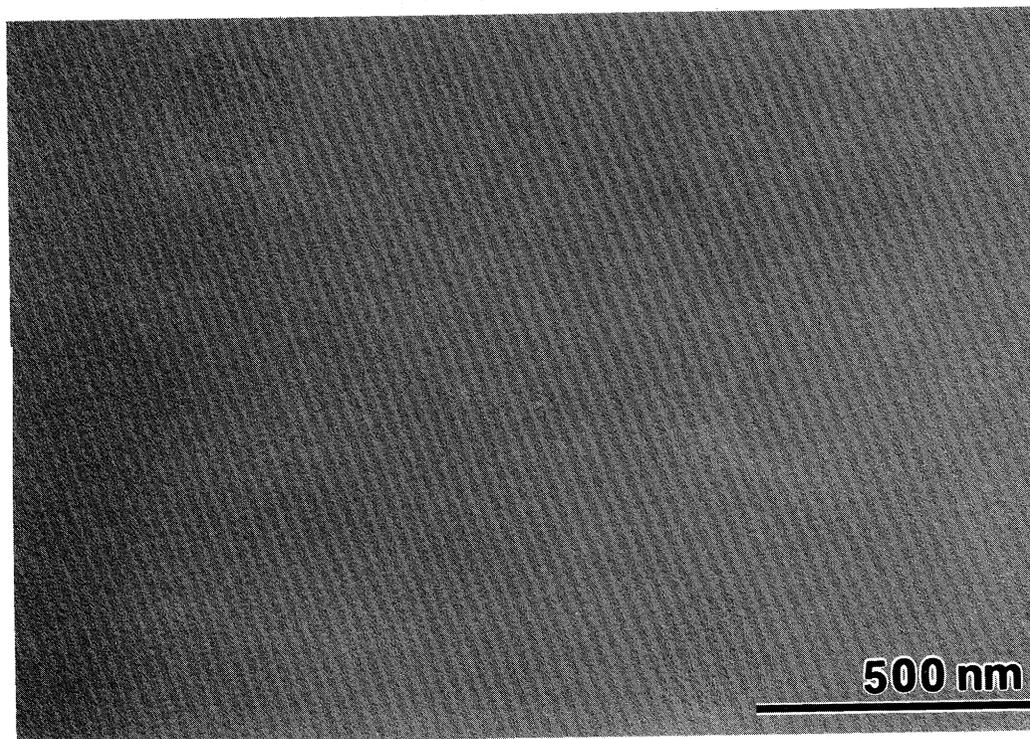


FIG. 2. Mixed film of DPPA-BSA containing 5 mole (residue) percent lipid and transferred at 15 mN/m and room temperature. Mica substrate.

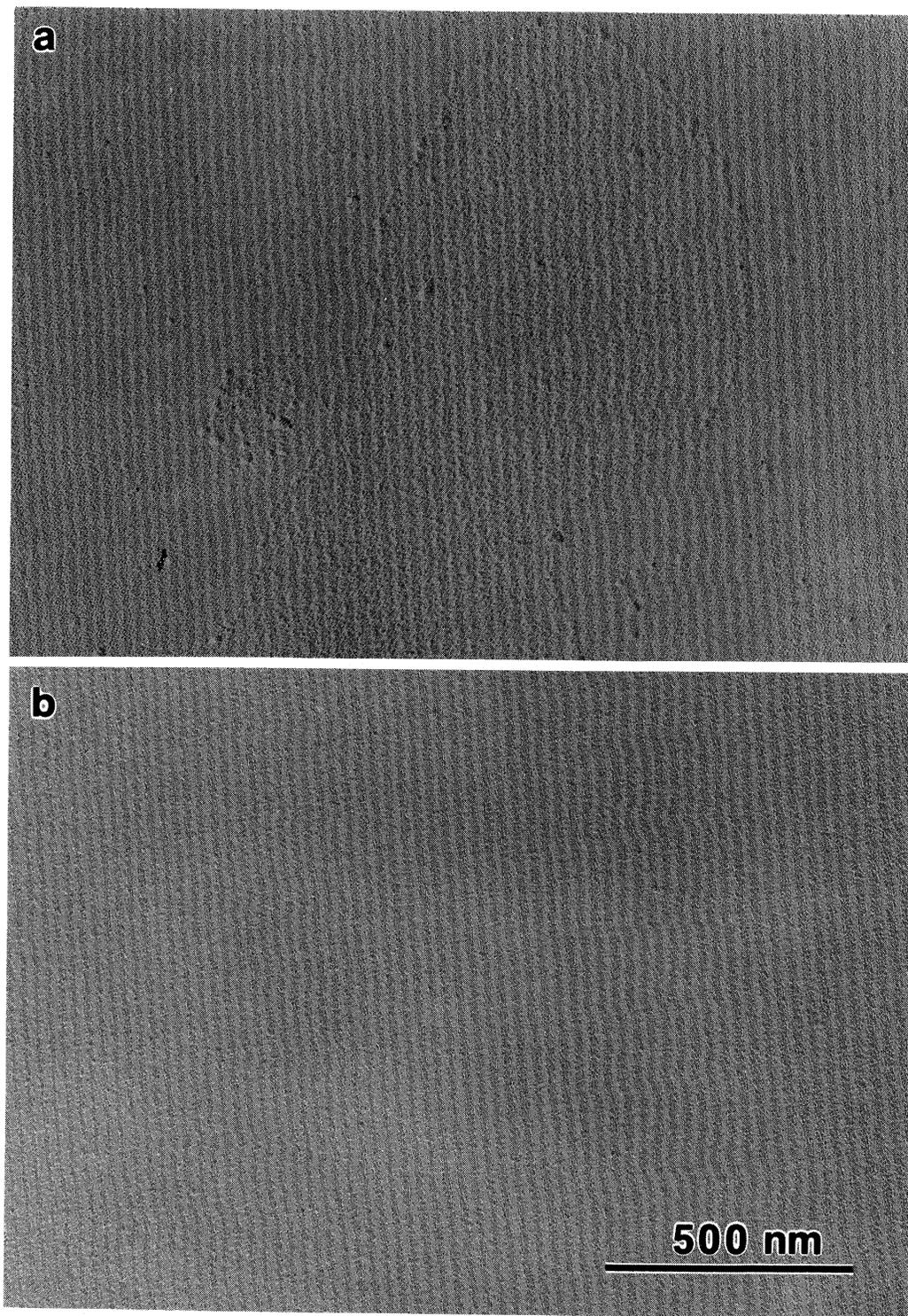


FIG. 3. Mixed films of nervonic acid and β -lactoglobulin containing 20 mole (residue) percent lipid and transferred at 15 mN/m and (a) 9°C or (b) 29°C. Mica substrate.

LIPID-PROTEIN MONOLAYERS

TABLE I

Summary of Electron Microscope Observations and Preparation Conditions for Lipid-Protein Monolayers at $22 \pm 1^\circ\text{C}$

Lipid	Chain length: double bonds	N_1	Lipid monolayer type	Phase separation detected?		
				β -lg	BSA	β -Casein
DPPA ^a	2 × 16:0	0.05–0.2	C ^b	Yes ^c	Yes	Yes
EYPA	Mixture	0.2	E	No ^c	No	No
DSPC	2 × 18:0	0.1–0.2	C	Yes	Yes	Yes
DOPC	2 × 18:1	0.2	E	No	No	No
DSPE	2 × 18:0	0.15	C	Yes	—	—
DOPE	2 × 18:1	0.2	E	No	—	—
Tristearin	3 × 18:0	0.05–0.2	C	Yes	Yes	Yes
Trierucin	3 × 22:1	0.1	E	No	No	No

^a Abbreviations: DPPA, dipalmitoylphosphatidic acid; EYPA, egg yolk phosphatidic acid; DSPC, distearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine.

^b C = condensed, E = expanded.

^c Reference (10).

micrographs, but 10% stearic in BSA gave an occasional micrograph with a single patch probably representing lipid while 15% stearic in BSA gave micrographs with a few heterogeneous structures with an occasional micrograph showing a completely clear film. Thus, for stearic acid-BSA, the onset of phase separation occurs when the lipid content exceeds 10–15 mole (residue) percent.

DISCUSSION

Most of this work was performed at room temperature ($22 \pm 1^\circ\text{C}$) where the majority of lipids exhibit either pure condensed or pure expanded behavior with little effect of temperature on their characteristic areas. One lipid which is sensitive to temperature changes around ambient is nervonic acid. Different temperatures were used in this case merely to

TABLE II

Summary of Electron Microscope Observations and Preparation Conditions for Fatty Acid-Protein Monolayers

Fatty acid	Chain length: double bonds	N_1	T ($^\circ\text{C}$)	Lipid monolayer type	Phase separation detected?		
					β -lg	BSA	β -casein
Lignoceric	24:0	0.05–0.2	RT ^a	C ^b	Yes	Yes	—
Nervonic	24:1	0.2	29	E	No	—	—
		0.2	21	E-C	—	No	—
		0.2	9	C	Yes	—	—
Erucic	22:1	0.1–0.15	RT	E	No	No	No
Stearic	18:0	0.05	RT	C	No	No	No
		0.10			No	No	—
		0.15			—	Yes	Yes
		0.20			Yes	—	—
		0.30			—	Yes	—

^a RT = room temperature ($22 \pm 1^\circ\text{C}$).

^b C = condensed, E = expanded.

establish conditions under which the pure lipid exhibits condensed (9°C) or expanded (29°C) behavior. No attempt was made to determine phase diagrams or thermodynamic quantities.

The previous observation that phospholipids which exhibited expanded behavior at the air-water interface mix with proteins in monolayers while phospholipids exhibiting condensed behavior did not (10) also holds for the lipids examined in this work with the exception of two fatty acids. The earlier work was restricted to phosphatidylcholines (PC) and phosphatidic acids (PA) mixed with β -lactoglobulin (β -lg). The current investigation expands the list of compounds to include phosphatidylethanolamines (PE), triglycerides, fatty acids, and two additional proteins, β -casein and bovine serum albumin (BSA). The list of compounds studied thus includes representatives of the major classes of lipids plus proteins which are high in the three principle conformations, namely: α -helix (BSA), β -sheet (β -lg), and unordered (β -casein and β -lg). Judged from the results obtained here, it appears that for phospholipids and triglycerides, the surface behavior of the lipid in single component monolayers serves to predict its miscibility behavior when mixed with proteins in films. As previously suggested, this indicates that factors such as Van der Waals forces which are responsible for the close packing in condensed films of pure lipid also contribute to the separation of the components when lipids and proteins are spread together in monolayers (10). Phase separation was noted with as little as 5 residue percent phospholipid or triglyceride in the mixed films, suggesting little if any "solubility" of the lipid in protein before the onset of phase separation.

It is interesting to compare the lipid-protein miscibility in films containing phospholipids and triglycerides with the predictions of a lattice model of a lipid-protein bilayer recently described (11). The model identifies two characteristic temperatures, T_K and T_c , where $T_K < T_c$. For temperatures above T_c , a bilayer of dipalmitoylphosphatidylcholine (DPPC) containing integral protein is predicted to exist as

a single homogeneous phase. For $T_K < T < T_c$ the system separates into an essentially pure lipid phase and a protein-rich phase unless the lipid-protein interaction parameter is "large." At $T < T_K$ the system is stabilized with respect to changes in size of the lipid and protein areas. Although Lookman *et al.* dealt with a phospholipid-protein bilayer, there are striking qualitative similarities between features of their model and results reported in this work on lipid-protein monolayers. For example, above a characteristic temperature (gel-liquid crystal transition for phospholipids) lipids exhibit expanded behavior at the air-water interface and, as reported here, appear to mix homogeneously with proteins, while at sufficiently low temperature lipids exhibit condensed behavior and segregate from proteins in mixed monolayers. Furthermore, in certain cases discussed below, lipid-protein interactions appear to be hindering segregation of components just as is discussed in the theoretical model.

The miscibility of the fatty acids with proteins in monolayers did not always follow the pattern of behavior observed with phospholipids and triglycerides. Although tetracosanoic (lignoceric) acid forms condensed films when pure and segregates from proteins in monolayers in accordance with the previous observations, octadecanoic (stearic) acid, which also exhibits condensed behavior, gives no sign of segregation from protein until the lipid content exceeds 10-15 residue percent. Pressure-area curves of stearic acid mixed with BSA obeyed the additivity rule, implying either ideal mixing or phase separation of the lipid and protein components. This means either that a small amount of stearic acid is "soluble" in BSA before the onset of phase separation or that any segregation resulted in patches of stearic acid that were too small to be seen with the electron microscope. Likewise, the systems stearic acid- β -casein and stearic acid- β -lg exhibited phase separation at 15 mole (residue) percent lipid but homogeneous micrographs were obtained at 5% lipid in the mixed monolayer.

Mixed systems containing a lipid which exhibits a pressure-induced expanded-condensed transition when pure are interesting since one might speculate that at low pressure, where the pure lipid is expanded, homogeneous films might be obtained which then could demix at high pressure where the pure lipid is condensed. Indeed, phase separation was observed at 15 mN/m in the case of dipalmitoylphosphatidylcholine (DPPC)- β -lg mixtures (10). In the present work, however, nervonic acid-protein gave homogeneous films both below and above the "transition pressure" of 2-4 mN/m (at 21°C) for the pure lipid. In preliminary work with nervonic acid-protein films of various compositions, pressure-area curves deviated from additivity at all pressures indicating that some mixing must be occurring. One might speculate that the lipid-protein interactions observed at low pressure, possibly combined with mechanical entanglement of the fatty acid in the protein, could prevent or hinder the diffusion required for coalescence of the lipid into a separate phase as the pressure increased.

Lipid-protein interactions in monolayer films containing β -lg or BSA are interesting in light of the known ability of these proteins to bind lipids in solution. However, binding sites of a protein in solution are a function of its tertiary structure (15), which is undoubtedly affected by the unfolding process at the air-water interface. Hence, detailed discussion of matters such as binding constants, number of sites, etc., in protein monolayers should be deferred until more complete knowledge of the structure of these films is available.

The results presented here suggest that lipids tend to segregate from proteins in films whenever conditions are such that the lipids exhibit condensed behavior, but that in certain cases lipid-protein interactions may hinder separation of the components.

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