Surface Tension of Protein and Insoluble Lipids at the Air–Aqueous Phase Interface

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ABSTRACT: The surface activity of bovine serum albumin (BSA) in water and aqueous solutions of ethanol (0.5, 1.0, and 2.0 M) and sucrose (0.5 and 1.0 M) has been investigated over a range of protein concentrations (5–1.10^{-5}, % w/w). The surface tension data were determined by the Wilhelmy plate method. Surface data at low protein concentrations indicate a low surface activity, which rises to a plateau as the monolayer is saturated at higher protein concentrations. The protein concentration and surface tension at the plateau depend on the aqueous phase composition. The effect of aqueous phase composition on BSA–lipid interactions has been investigated by spreading an insoluble lipid (monostearin or monoolein) on a film of BSA previously adsorbed on the interface. The existence of protein–lipid interactions depends on the protein/lipid ratio. The surface activity of mixed BSA–lipid films is determined by the lipid because the surface pressure of the mixed film is the same as the lipid equilibrium spreading pressure, and the monolayer is not saturated by BSA. However, the surface activity of mixed BSA–lipid films is determined by BSA as the monolayer is saturated by the protein.
JAOCS 75, 1233–1239 (1998).

KEY WORDS: Adsorption, air–water interface, bovine serum albumin, food emulsifiers, lipid, monoolein, monostearin, protein–lipid interactions, surface tension, tensiometry.

Most foods are dispersed systems that contain particles of colloidal size. The stability and mechanical properties of these systems depend on the way in which the constituent particles and macromolecules interact (1–3). To stabilize food emulsions and foams, emulsifiers (lipids and proteins) must be placed at the interface, so they can form a film around droplets or bubbles, respectively (1). The optimal use of emulsifiers depends on the knowledge of their interfacial physicochemical characteristics—such as surface activity, structure, stability, and superficial viscosity—and the kinetics of film formation at fluid-fluid interfaces (4–7). The lipids and proteins at the interface reduce the interfacial tension between the phases and thus stabilize (1,3) and improve the formation of food emulsions and foams (8–10). Proteins, in addition to lowering the interfacial tension, can form a continuous film at the interface *via* complex intermolecular interactions and thus impart structural rigidity (1,7). Emulsifier-based foods, such as traditional foods or low-fat products (especially water–oil concentrated emulsions), instant foods, or alcohol-free and low-alcohol beverages, are some examples of food systems in which the data obtained in this research are of practical interest (11–13).

As a result of systematic studies of model systems, the colloidal and intermolecular interactions themselves are now becoming reasonably well understood (14). What seems to be needed now, if real progress is to be made, is the establishment of the generic link between these model systems and more representative food formulations by using an approach that takes into account key variables, such as processing conditions (temperature) and the concentrations of lipids and/or proteins, and the presence of typical food solutes (sugars, ethanol, salts, etc.) in the aqueous phase.

There have been many studies of protein–lipid interactions in relation to the formation and stability of food emulsions and foams. Several groups have studied the interactions between proteins and soluble lipids (14–17), but much less is known about the details of protein-insoluble lipid interactions (8,18,19). In previous papers, we studied the interfacial characteristics—surface rheological properties, drainage, and diffusion in thin films—of protein–lipid mixed films as a function of interfacial and aqueous phase compositions (20–23).

We report here surface tension data of a protein (bovine serum albumin, BSA) and its mixtures with two insoluble lipids (monostearin and monoolein) at equilibrium. The surface tension data were used to analyze the effect of aqueous phase composition on the adsorption isotherm of a protein and the existence of protein–lipid interactions at the interface. Temperature and the aqueous phase (ethanol and sucrose aqueous solutions) and interfacial compositions (BSA and BSA–lipid mixed films at different protein/lipid ratios) have been studied as variables. These experiments mimic the behavior of emulsifiers in food emulsions in which an oil-soluble lipid (monostearin or monoolein) diffuses to the interface where a protein film is adsorbed from the aqueous bulk phase, followed by protein–lipid interactions in the interfacial region.

MATERIALS AND METHODS

Materials. The water used in this study was purified by means of a Millipore (Milford, MA) filtration device (Mille-Q). BSA (>96% pure, Fluka, Buchs, Germany), monostearin (1-monooctadecanoyl-*rac*-glycerol, >99%; Sigma, St. Louis,

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MO), monoolein [1-mono(*cis*-9-octadecanoyl)glycerol, Sigma, >99%], analytical-grade ethanol [Merck (Darmstadt, Germany), >99.8%], hexane (Merck, 99%), sucrose (Fluka, >99.5%), potassium dihydrogen phosphate (99.5% pure, Merck), and dipotassium hydrogen phosphate (99% pure, Merck) were used as supplied.

Surface tension. Surface tension measurements were used to determine BSA adsorption and to explore the presence of BSA–lipid interactions at the interface. The surface activity was expressed by the surface pressure, $\pi = \sigma_o - \sigma$, where σ_o and σ are the aqueous subphase surface tensions (σ_o was taken as the surface tension of water or aqueous solution of ethanol and sucrose prior to addition of lipid or protein) and the surface tension of the aqueous solutions of BSA and BSA–lipid mixed films, respectively. Measurements were performed with a Krüss digital tensiometer K10 (Hamburg, Germany), based on the Wilhelmy method, with a roughened platinum plate. The measurements were carried out in a circular, thermostated dish with a surface area of 63.6 cm^2. A humid atmosphere was maintained by putting a glass of water in the enclosing box. The water was replaced by ethanol in experiments with aqueous ethanol solutions in the bulk phase. Because adsorption measurements are sensitive to the presence of impurities, extreme care was taken to ensure that all materials and instruments used in this study were clean. All glassware in contact with the sample was previously cleaned in ammonium persulfate–sulfuric acid and rinsed in deionized water. The platinum plate was washed with ethanol, then rinsed with deionized water, heated in a Bunsen burner flame, and left to cool to room temperature. As the experiments lasted a long time, the dish was maintained in the enclosed box after sample preparation and during measurements to minimize the presence of impurities from the atmosphere.

The experiments for BSA adsorption on water were carried out at 5, 20, and 30°C. The aqueous phase composition dependence on BSA adsorption and BSA–lipid interactions were monitored at 20°C. The temperature of the system was maintained constant within 0.5°C by a circulating Heto thermostat. All aqueous subphases were prepared in 50 mM phosphate buffer and adjusted to pH 7.0 (0.05 wt% sodium azide was added as antimicrobial agent). Water and aqueous ethanol (0.5, 1.0, and 2.0 M) and sucrose (0.5 and 1.0 M) solutions were studied as variables. Several stock solutions of BSA at different concentrations (5–1.10^{-5}%, w/w) were prepared and measured. In experiments with a solute in the subphase, solutions were prepared at room temperature by stirring for 30 min. The solutions were placed in the dish and then in an enclosing box and were allowed to stand for 24 h to reach the desired adsorption equilibrium.

Measurements were also performed to study BSA–monostearin and BSA–monoolein at the air–water interface. Fifty µL of monostearin at $5.2.10^{-4}$ M and monoolein at $3.8.10^{-4}$ M—dissolved in a mixture of hexane and ethanol, 9:1 (vol/vol)—was spread on a film of protein previously adsorbed from the subphase bulk. After the hexane–ethanol evaporated in 10–15 min, the solutions were placed in the dish and then in an enclosing box and were allowed to stand for 24 h to reach the desired protein–lipid interactions at steady state. Previous experiments (data not shown) showed that the surface tension of the protein–lipid mixed systems were practically the same (within experimental error) at 24, 48, and 72 h for protein–lipid interactions. Measurements were performed a minimum of five times. Surface tension measurements were reproducible within ±0.5 mN/m.

RESULTS AND DISCUSSION

Adsorption of BSA at the air–aqueous phase interface. Figure 1 shows the adsorption isotherm of BSA on water as a function of temperature. The BSA concentration dependence on surface pressure showed classical sigmoidal behavior. At low BSA concentrations, the initial solutions caused only a small increment in the surface pressure. The surface pressure increases with BSA concentration and tends to a plateau. This plateau commences at the point where surface pressure reaches its maximal value over the range of protein concentrations from 10^{-3} to 10^{-1} wt%. The surface pressure values determined in this work are practically the same as those previously reported for BSA at the same temperatures (24–28). The general characteristics of BSA adsorption are practically the same at the three temperatures studied. However, the surface pressure value at the plateau decreased as the temperature was increased. That is, the surface activity of the protein decreases with temperature (Table 1).

The behavior of adsorbed BSA films can be interpreted in terms of monolayer coverage by comparing the data of surface pressure determined here with that of surface concentration determined by ellipsometry (25) or surface radioactivity

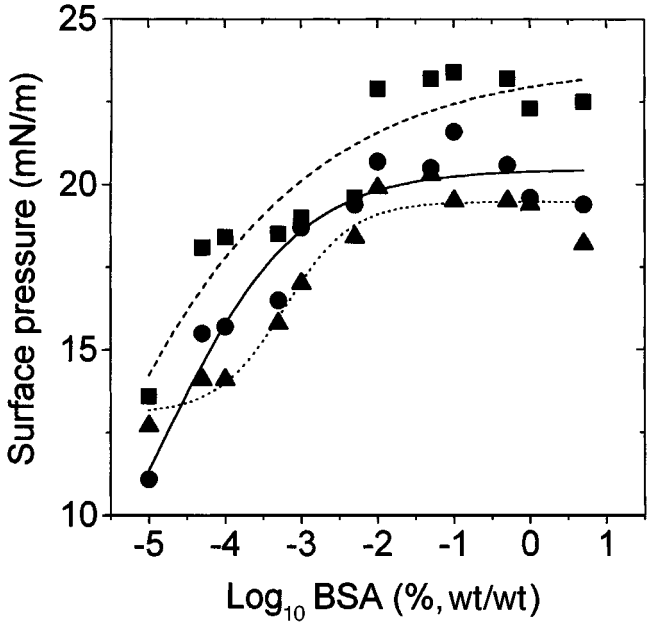

FIG. 1. Adsorption isotherm of bovine serum albumin (BSA) on water as a function of temperature: (▲) 5, (●) 20, and (■) 30°C.

TABLE 1
Steady-State Surface Pressure at the Plateau, π^{∞} (mN/m), for BSA Adsorbed Films from Aqueous Solutions at 5 wt%

Aqueous phase composition	Temperature (°C)	π^{∞} at BSA wt 5% (mN/m)
Water	5	23.0
Water	20	20.5
Water	30	19.5
Ethanol 0.5 M	20	21.0
Ethanol 1 M	20	14.5
Ethanol 2 M	20	10.4
Sucrose 0.5 M	20	30.1
Sucrose 1.0 M	20	37.0

(24,25). Adsorption of BSA at lower concentrations than that of the plateau forms a monolayer of irreversibly adsorbed molecules. As the plateau is attained, the monolayer is saturated by protein that is irreversibly adsorbed. At higher protein concentrations, the BSA molecules form multilayers beneath the primary monolayer, but these structures do not contribute significantly to surface pressure (25).

The effect of aqueous phase composition on the surface activity of BSA is shown in Figure 2. These results indicate that the adsorption of BSA depends greatly on the solutes in the aqueous phase. In ethanol aqueous solutions, the surface activity of BSA decreased, and a higher protein concentration was necessary to reach the plateau. In ethanol aqueous solutions at 2 M, the plateau was not attained at the highest BSA concentration studied here (1 wt%). However, the surface activity of BSA at the plateau was independent of the presence of ethanol in the aqueous phase at concentrations below 0.5 M. A similar trend in casein adsorption at the oil–water interface was observed by Dickinson and Woskett (15).

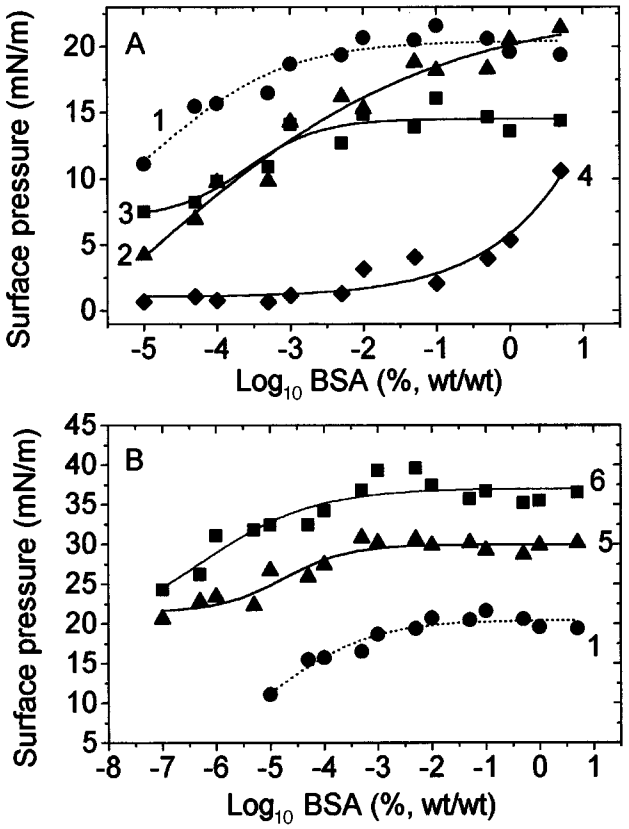

FIG. 2. Adsorption isotherm of BSA on (1) water and aqueous solutions of ethanol at (2) 0.5 M, (3) 1.0 M, and (4) 2.0 M, and sucrose at (5) 0.5 M and (6) 1.0 M, at 20°C. See Figure 1 for abbreviation.

The presence of sucrose in the bulk aqueous phase also strongly affects the adsorption behavior of BSA (Fig. 2). Under these conditions, the adsorption isotherms were displaced toward the surface pressure axis, especially at the higher sucrose concentration in the aqueous phase. That is, monolayer saturation by BSA is easier in the presence of sucrose. Moreover, the surface activity of BSA increased with sucrose concentration in the aqueous phase (Table 1).

The adsorption characteristics of BSA as a function of aqueous phase composition could be interpreted in terms of the competitive BSA–ethanol adsorption at the interface and by the effect of sucrose on the structure of BSA in the bulk phase and at the interface. Briefly, as a surfactant, ethanol molecules in the aqueous phase may be adsorbed at the interface. So, during BSA adsorption from aqueous ethanol solutions, BSA and ethanol molecules can be present at the interface. The surface activity may be determined by the protein, due to its higher hydrophobicity. However, at higher ethanol concentration, BSA can be partially displaced from the interface, as was observed by Dussaud *et al.* (29). As a consequence of this phenomenon, the development of a strong viscoelastic film of adsorbed protein could be inhibited (30), which agrees with data in Figure 2. On the other hand, sucrose limits protein unfolding and protein-protein interactions (31,32), which allows more protein to be involved in film formation. As a consequence of these effects, the BSA concentration drops at the point where the plateau commences (Fig. 2), and the surface pressure of the plateau increases (Table 1). The higher surface activity of BSA in aqueous sucrose solutions at 20°C could also be due to the higher surface activity of the aqueous sucrose solutions—the surface tensions of aqueous sucrose solutions at 0.5 and 1.0 M are 73.4 and 74.1 mN/m, respectively—in relation to water (72.7 mN/m) and aqueous solutions of ethanol at the same temperature—64.2, 57.2, and 51.0 mN/m for aqueous solutions of ethanol at 0.5, 1.0, and 2.0 M, respectively. The existence of aggregated, noninteracting BSA molecules at the interface in the presence of sucrose was observed recently by drainage in thin liquid films (23) and correlated with superficial diffusion data in thin liquid films (23) and with a decreased surface dilational viscosity (33).

BSA–lipid interactions at the air–water interface. The effect of the protein/lipid ratio on the surface activity of mixed BSA–lipid systems at 20°C is shown in Figure 3. In these experiments, the lipid spread on a previously adsorbed BSA film was maintained constant at 11.9 and 8.9 molecules · nm^{-2} for monostearin and monoolein, respectively. So, the variation of the protein/lipid ratio is due to the BSA added in the bulk phase over the range 5 to 1.10^{-5} wt%. The BSA concentra-

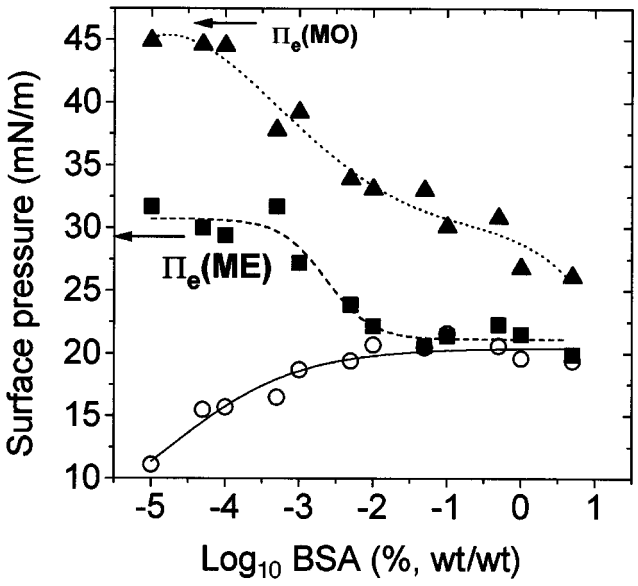

FIG. 3. The effect of spreading monostearin, ME (■), and monoolein, MO (○), on a film of BSA, (▲), previously adsorbed on the air–water interface. Temperature: 20°C. Lipid superficial density (molecule · nm^{-2}): monostearin (11.9), monoolein (8.9). The arrows indicate the equilibrium spreading pressure for monostearin, Πe (ME), and monoolein, Πe (MO). See Figure 1 for other abbreviation.

tion dependence on surface pressure for BSA–lipid mixed systems showed a sigmoidal behavior. The surface activity of the BSA–lipid mixed systems depends on the protein/lipid ratio and the lipid spread on the interface (Fig. 3 and Table 1). The surface pressure values approach that of pure BSA films at higher relative BSA concentrations in the mixed systems as the monolayer is saturated by the protein (see Fig. 1).

At lower relative BSA concentrations, the surface pressure is practically the same as the equilibrium surface pressure (Π) of the pure lipid (34), which is indicated in Figure 3 by arrows. That is, at BSA concentrations below 1.10^{-4} wt%, the surface activity of BSA–lipid mixed systems remains practically unchanged at the values of Πe of the pure lipids. So, we suggest that, at the lipid surface densities spread here, the protein is removed and the interface is saturated by a collapsed monostearin or monoolein film (35,36) with liquid-condensed or liquid-expanded structure (34), respectively. Confocal scanning laser microscopy (37) showed that the displacement of caseinate from the oil–water interface correlated with the interfacial concentration of monoglyceride (monostearin or monoolein).

Above this protein concentration (1.10^{-4} wt%) and up to monolayer saturation by BSA (over the range 10^{-2}–10^{-1} wt%), significant further reduction in the subphase pressure was observed. The effect resulted in an inflection in the surface pressure curve in the intermediate region. The general features described earlier support indirect evidence of the existence of BSA–lipid interactions at the interface.

The interfacial behavior analyzed previously is practically similar to that observed with monocaproin + ovalbumin (18) and with mono- and diglycerides and casein (19). As a consequence of these results, we can speculate that, at higher BSA relative concentrations in BSA–lipid mixed systems, the surface activity is determined by the protein. In contrast, the surface activity of BSA–lipid mixed systems is determined by the lipid (monostearin or monoolein) at the lower BSA relative concentrations. In the intermediate region, the surface activity is determined by the existence of a BSA–lipid interacting complex. Further confirmation of this explanation comes from measurements of surface dilational experiments with the same systems (20,21), as will be discussed later.

BSA–lipid interactions at the air–aqueous solution interface. To investigate the effect of aqueous phase composition on the surface activity of BSA–lipid mixed systems, we considered the influence of ethanol and sucrose. Figures 4 to 8 show the effect of the protein/lipid ratio on the surface activity of BSA–lipid mixed systems in aqueous solutions of ethanol and sucrose, respectively, at 20°C.

The general features described earlier for BSA–lipid mixed systems in water are evident here for aqueous ethanol (Figs. 4, 5, and 6) and sucrose (Figs. 7 and 8) solutions. That is, (i) the surface activity of protein–lipid mixed systems was determined by the component present at higher concentrations and (ii) the existence of protein–lipid interactions controls the interfacial characteristics of the mixed films at the intermediate region. However, some specific patterns exist that depend on the aqueous phase composition.

On aqueous ethanol solutions (Figs. 4, 5, and 6), the BSA concentration at which the surface activity of the BSA–lipid mixed system was determined by the protein decreased with the ethanol concentration in the aqueous phase. That is, in

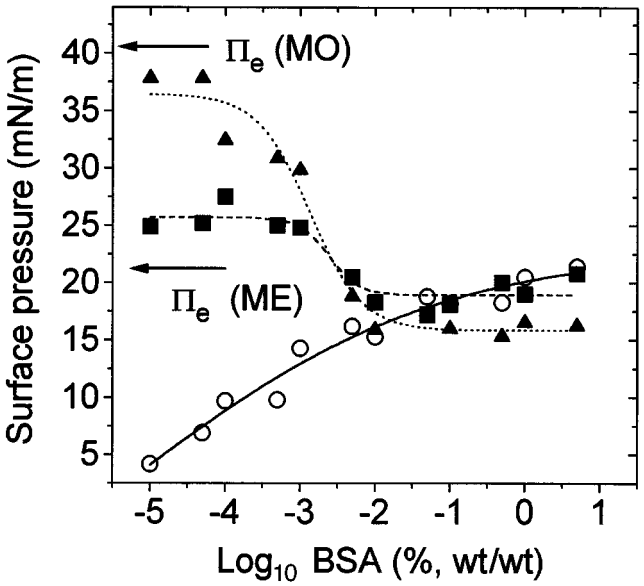

FIG. 4. The effect of spreading monostearin, ME (■), and monoolein, MO (▲), on a film of BSA, (○), previously adsorbed on the air–aqueous ethanol subphase interface. Temperature: 20°C. Lipid superficial density (molecule · nm^{-2}): monostearin (11.9), monoolein (8.9). Ethanol concentration in the subphase: 0.5 mol/L. The arrows indicate the equilibrium spreading pressure for monostearin, Πe (ME), and monoolein, Πe (MO). See Figure 1 for other abbreviation.

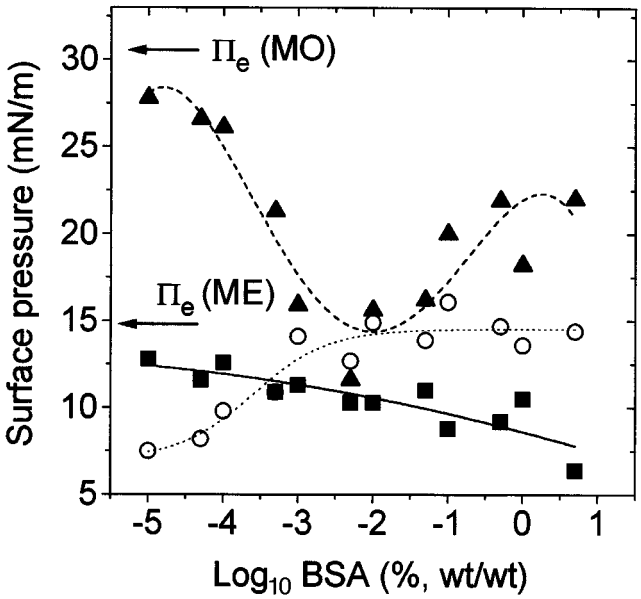

FIG. 5. The effect of spreading monostearin, ME (■), and monoolein, MO (▲), on a film of BSA, (○), previously adsorbed on the air–aqueous ethanol subphase interface. Temperature: 20°C. Lipid superficial density (molecule · nm^{-2}): monostearin (11.9), monoolein (8.9). Ethanol concentration in the subphase: 1.0 mol/L. The arrows indicate the equilibrium spreading pressure for monostearin, Πe (ME), and monoolein, Πe (MO). See Figure 1 for other abbreviation.

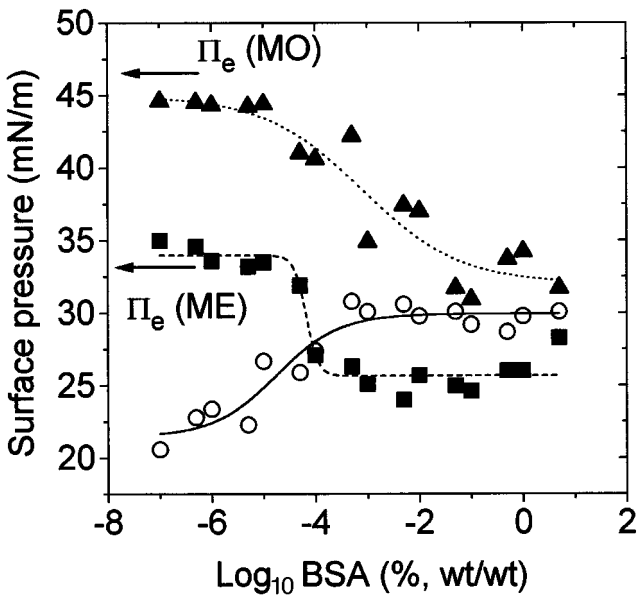

FIG. 7. The effect of spreading monostearin, ME (■), and monoolein, MO (▲), on a film of BSA, (○), previously adsorbed on the air–aqueous sucrose subphase interface. Sucrose concentration in subphase: 0.5 mol/L. Temperature: 20°C. Lipid superficial density (molecule · nm^{-2}): monostearin (11.9), monoolein (8.9). The arrows indicate the equilibrium spreading pressure for monostearin, Πe (ME), and monoolein, Πe (MO). See Figure 1 for other abbreviation.

aqueous ethanol solutions, lower BSA concentrations in the bulk phase than those in water were necessary to attain a film with the superficial characteristics of a pure BSA film. Over the range of the BSA–lipid ratio studied here, the surface activity of the mixed systems on ethanol aqueous solutions was lower than that on water (Fig. 3). Moreover, the surface activity of the mixed systems decreased as the ethanol concentration increased (Table 1). This phenomenon could be due to

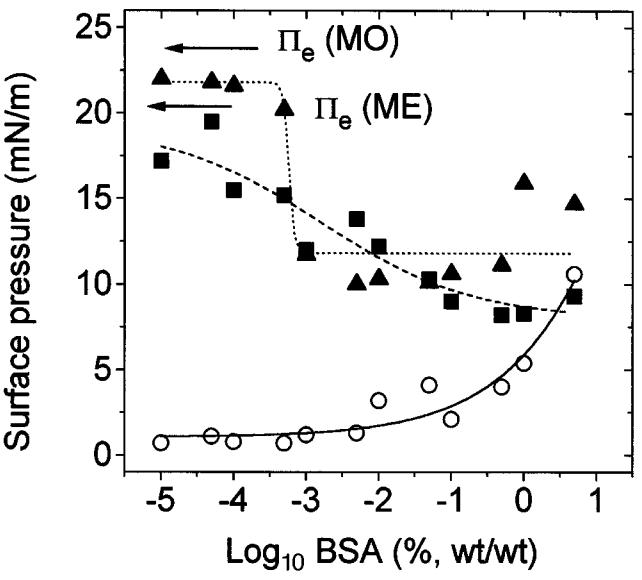

FIG. 6. The effect of spreading monostearin, ME (■), and monoolein, MO (▲), on a film of BSA, (○), previously adsorbed on the air–aqueous ethanol subphase interface. Temperature: 20°C. Lipid superficial density (molecule · nm^{-2}): monostearin (11.9), monoolein (8.9). Ethanol concentration in the subphase: 2.0 mol/L. The arrows indicate the equilibrium spreading pressure for monostearin, Πe (ME), and monoolein, Πe (MO). See Figure 1 for other abbreviation.

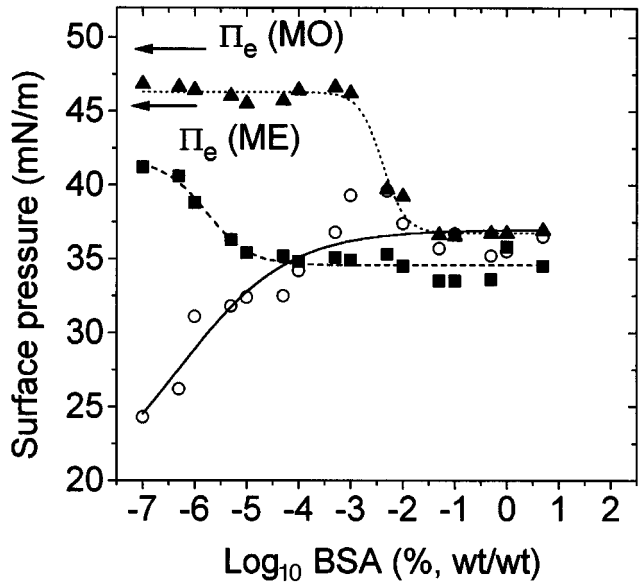

FIG. 8. The effect of the spreading of monostearin, ME (■), and monoolein, MO (▲), on a film of BSA, (○), previously adsorbed on the air–aqueous sucrose subphase interface. Sucrose concentration in subphase: 1.0 mol/L. Temperature: 20°C. Lipid superficial density (molecule · nm^{-2}): monostearin (11.9), monoolein (8.9). The arrows indicate the equilibrium spreading pressure for monostearin, Πe (ME), and monoolein, Πe (MO). See Figure 1 for other abbreviation.

the fact that ethanol competes with emulsifiers (protein and lipids) at the interface. In addition, ethanol can disrupt protein–lipid interactions and competes for lipid hydrophobic binding sites on the protein molecule to create a complex with different interfacial activity. Finally, ethanol can interact with monoglycerides at the interface, and as a consequence of these interactions, a monoglyceride molecular loss from the interface could take place (38).

On aqueous sucrose solutions (Figs. 7 and 8), the BSA concentration at which the surface activity of the BSA–lipid mixed systems was determined by the protein also decreased with sucrose concentration in the aqueous phase. At the higher sucrose concentration in the bulk phase, the surface activity of the protein in the mixed film prevails at lower BSA concentrations (Fig. 8). These results strengthen the hypothesis that, in the presence of sucrose, the protein is preferentially adsorbed in the interface, so higher relative lipid concentrations are necessary to attain a mixed BSA–lipid film with the surface activity of a pure lipid film. On the other hand, the surface activity of BSA–lipid mixed systems was higher than that on water and aqueous ethanol solutions, in that order (Table 1).

The removal of protein by surfactants is well studied in the literature (14). The degree of protein removal by a surfactant is affected by factors that are known to influence the binding strength of a protein to a surface. Thus, the removal of protein by surfactants decreases with conditions that favor conformational changes. However, removal of protein by surfactants will not only be influenced by protein properties but also by the type of surfactant (14) and the aqueous phase composition. Factors that favor conformational changes in protein—such as a decrease in protein concentration (Fig. 3) and the presence of ethanol (Figs. 4, 5, and 6) in the aqueous phase (39)—could increase the degree of BSA removal by monostearin or monoolein. In contrast, sucrose limits protein unfolding and protein-protein interactions, which correlates with a decreasing degree of BSA removal by lipids at the interface (Figs. 7 and 8). No regular differences between monostearin and monoolein were observed on the BSA–lipid relative concentration at which the removal of BSA by the lipid takes place. This behavior is different from that observed by Heertje *et al.* (37) at the oil–water interface. They found that, at high lipid concentrations in the oil phase, the amount of monostearin adsorbed at the interface was larger than that of monoolein, which also led to a more extensive displacement of caseinate from the interface.

The existence of BSA–monostearin and BSA–monoolein interactions on the same aqueous solutions has been proved by dynamic surface tension data (40,41) and by surface dilational measurements (20,21). So it can be concluded that surface tension measurement is an easier complementary experimental technique to provide information about the interfacial characteristics of pure protein and lipid films and about the existence of protein–lipid interactions at the interface. The surface activity of BSA–monostearin and BSA–monoolein mixed films, spread on water and aqueous solutions of ethanol and sucrose, is determined by the lipid (either monostearin or monoolein) at lower BSA concentrations in the aqueous phase than that of monolayer saturation because the surface density of the lipid is the same as that corresponding to its equilibrium spreading pressure. In contrast, the surface activity of the mixed film is determined by BSA at protein concentrations in the aqueous phase higher than that of monolayer saturation although at a lipid concentration similar to that corresponding to its equilibrium spreading pressure. That is, the equilibrium spreading pressure of an insoluble lipid and the adsorption isotherm of a protein–lipid mixed film are two important interfacial parameters that delimit the behavior of the system at the air–aqueous phase interface.

However, surface pressure-concentration experiments are not sufficient to allow final conclusions about the nature of protein–lipid interactions at the interface in more complex systems where solutes are present in the aqueous phase. In recent studies (20,21), we have observed that surface dilational rheology gives additional information about the nature of interactions between BSA and monoglycerides at the interface. From these experiments we concluded that, for BSA–monostearin mixed films, surface dilational modulus increased as either the concentration of monostearin spread on the interface was increased or as the content of ethanol or sucrose in the bulk phase was decreased (20,21). However, BSA–monoolein interactions at the interface have a negative synergistic effect on the surface dilational modulus (20,21). The effect of these interactions on the surface activity and surface rheological characteristics of the mixed films could have important consequences on the stability of emulsions and foams (1,14,42–44).

ACKNOWLEDGMENT

This research was supported in part by DGICYT through grants PB93-0923 and PB94-1459.

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[Received June 25, 1997; accepted May 5, 1998]