Comparison of the Effects of Three Different Phosphatidylcholines on Casein-Stabilized Oil-in-Water Emulsions

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ABSTRACT: Soy oil-in-water emulsions, stabilized by casein, but incorporating one of three different phosphatidylcholines (PC), namely egg-PC, di-palmitoyl phosphatidylcholine (DPPC) and di-oleyl phosphatidylcholine (DOPC), have been studied by photon correlation spectroscopy, light scattering, fast protein liquid chromatography, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Egg-PC enhanced the stability of emulsions made with low casein concentration, and it competed for space with casein at the oil–water interface during the emulsification process, but no further displacement of protein was found. DPPC had little effect on emulsion stability nor did it show a detectable competition at the interface with casein during or after emulsification. DOPC, however, not only competed with casein at the interface during emulsification, it also removed casein from the interface during storage of the emulsion. The displacement of casein caused instability of the emulsions. Adding DOPC to emulsions also led to displacement of casein from the interface and caused instability of the emulsion, but the process was much slower and occurred to a smaller extent compared to emulsions prepared with DOPC. The different behavior of egg-PC, DPPC, and DOPC on the oil–water interface was in good agreement with their relative solubility in the oil phase as measured by spectrophotometry. All three lipids modified the hydrodynamic thickness of the adsorbed casein layer corresponding to their modification of the surface concentration of casein.

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KEY WORDS: Casein, lecithin, lipid protein interaction, oil–water interface.

Lecithin is a common ingredient in food products (1,2), and casein is often an important component in these products as well. There have been studies on the interaction between lecithin and casein, but it is far from well understood. It is generally agreed (3,4) that phospholipids displace casein from the interface but less efficiently than other small molecule surfactants. Phospholipids are a very special type of surfactant, possessing low critical micelle concentration (CMC) in aqueous solutions, so that they tend to form lamellar mesophases and vesicles in water (5), and their interaction with protein can be far more complicated. Lecithin from egg yolk has a synergistic effect on the emulsifying property of protein (6), and soy lecithin enhances the stability of homogenized concentrated milk (7,8). When surfactant coexists with protein in an emulsion, competitive adsorption often occurs (9–12), with some surfactants tending to displace protein from the interface independently (9) of the way they are introduced into the system (before or after emulsification). Protein displacement by small molecule emulsifiers also has been visualized (13) by confocal scanning laser microscopy. Egg-phosphatidylcholine (PC) was found (14) to displace β-casein from the droplets of *n*-tetradecane more efficiently than from the droplets of soy oil at quite high egg-PC/casein molar ratios. Besides competing for space with protein at an interface, surfactants may also form complexes with protein. Lecithin forms complexes with β-lactoglobulin (15,16), while sodium dodecyl sulfate (SDS) promotes (17) the self association of β-casein. It has been shown (18) that a weak protein–polysaccharide complex at the oil–water interface is formed between β-lactoglobulin and propylene glycol alginate.

The chemical characteristics have been proven important for the emulsifying properties of the phospholipids; for example, the composition of a commercial lecithin mixture has been shown (19) to correlate with the oil–water interfacial tension and with the frying property of margarine (spattering). In general, commercial lecithins are composed of different types of phospholipids, and the functionality of each component is not well defined.

In this report, we have compared the behavior of three different phospholipid molecules, egg-PC, di-palmitoyl phosphatidylcholine (DPPC), and di-oleyl phosphatidylcholine (DOPC), in oil–water emulsions stabilized by the casein protein complex. Although the three phospholipids share the same headgroup, they differ in their hydrocarbon chains. We have previously studied the stability of the emulsions during storage and have determined the hydrodynamic layer thickness of adsorbed casein by using light- scattering techniques. The hydrodynamic layer thicknesses of adsorbed casein at saturation on the surfaces of polystyrene latices and on oil droplets in an emulsion are about the same size, about 10 nm (20). In the absence of phospholipid, the casein layer thickness has a minimum value of 5 nm (21) at low casein surface concentration. In this paper, the effect of the three different

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PC on these properties of the emulsion have been studied in parallel. Fast protein liquid chromatography (FPLC) and SDS–polyacrylamide gel electrophoresis (PAGE) have been used to determine the amount of the casein adsorbed on the oil droplets.

EXPERIMENTAL PROCEDURES

DPPC, DOPC, and egg-PC, imidazole, soybean oil, and TPCK-trypsin were purchased from Sigma Chemicals (St. Louis, MO). Sodium caseinate was prepared in the laboratory by acid precipitation of skim milk to pH 4.6, and the precipitate was filtered and washed with abundant water before being redissolved at pH 7 in NaOH solution and freeze-dried.

Emulsions (10 mL) were prepared in a Microfluidizer 110S (Microfluidics Corp., Newton, MA). The concentration of oil was kept at 20 wt% for all samples, and the concentration of casein was varied from 0.3 to 2 wt%. Phospholipids were incorporated at 0.2% and 0.5 wt%. The aqueous phase of the emulsions was a buffer of 20 mM imidazole/HCl, pH 7.0. After a preliminary mixing of all the ingredients, the mixture was circulated through the homogenizing unit ten times before being collected; the inlet pressure was set at 0.3 MPa, which corresponds to a pressure drop of 42 MPa in the homogenizing chamber. The emulsions were studied immediately or were stored at 4°C for studies of stability and changes during storage.

The mean size of the emulsion droplets and their size distribution were measured by Mastersizer X (Malvern Instruments Inc., Southboro, MA). Stability of the emulsions was monitored by measuring the change of the droplet size and size distribution with time.

The hydrodynamic thickness of adsorbed casein layers was measured by studying the changes in diameter as the protein layer was digested with trypsin, by using photon correlation spectroscopy (PCS). A Malvern Instruments System 4700, linked to a Multi-8 correlator, was used for the measurement. Solutions of trypsin (1 mg · mL^{-1}) were prepared. Aliquots (1.5 μL) of the emulsion were diluted into 3 mL of buffer, which had been filtered through a 0.22 μm filter (Millipore Ltd., Mississauga, Ontario, Canada). The initial hydrodynamic diameters of the emulsion droplets were measured with a set of ten individual PCS runs, each lasting 1 min, and the results were averaged. The scattering angle was set at 90°, and the temperature of the sample was controlled at 25 ± 0.2°C with a circulating water bath. Then, 1 to 3 μL of trypsin solution was added to the same sample, and after a new equilibrium was reached, the diameter was measured again. The hydrodynamic thickness of the adsorbed layer was taken to be half of the decrease in the average particle diameter caused by trypsin treatment (20). Experiments were replicated to decrease the error in the measurement of layer thickness to less than 1 nm; the quoted values of the layer thickness are therefore ± 1 nm.

The amount of casein adsorbed to the oil–water interface was analyzed by FPLC [Pharmacia Biotech (Canada) Ltd., Baie d'Urfé, Québec, Canada] with a Mono-Q HR 5/5 ion exchange column and by SDS–PAGE electrophoresis in a Phast-System (Pharmacia Biotech). For FPLC analysis, the emulsion was centrifuged at 15,000 × *g* for 1 h, the serum phase was collected carefully with a syringe, and the cream phase (emulsion droplets) was discarded. The serum phase was filtered through a 0.22-μm filter before being applied to the column. The eluting buffer was 3.3 M urea/20 mM bis-tris propane at pH 7.0, and a linear gradient of NaCl between 0 and 0.4 M was run. Only the emulsions containing egg-PC were analyzed by this method (21), because the presence of small oil droplets (about 80 nm) in the serum tended to block the column and made cleaning difficult. Therefore, we also used quantitative SDS–PAGE (22) to analyze the adsorbed protein in the emulsions that contained DPPC and DOPC.

For this analysis, the emulsions were centrifuged at 15,000 × *g* for 1 h, and the cream phase was collected, resuspended in buffer, and centrifuged again at the same speed. The cream phase, collected after the second centrifugation, this time was used for SDS–PAGE analysis. An adequate amount of the cream, after drying on a filter paper to remove excess water, was weighed out, and water was added to make a dispersion containing 20% oil phase. This dispersion was allowed to equilibrate for several hours before analysis; then 150 μL was mixed with 250 μL of 20% SDS solution, 100 μL of 2-mercaptoethanol, and 100 μL of 0.05% bromophenol blue solution. This mixture was heated at 100°C for 5 min to allow denaturation of the protein. Samples of the whole emulsion were also treated in the same manner as the cream phase dispersion and run on the same gel as standards. The electrophoresis was run on homogeneous 20% polyacrylamide gels (Pharmacia Biotech).

The gels were scanned with a gel scanner (UltraScan XL; Pharmacia Biotech), and the amounts of protein adsorbed on the oil droplets were quantitated by using the whole emulsion as a standard. By combining the surface area of the oil droplets measured with Mastersizer and the amount of protein adsorbed, it was possible to calculate the surface concentration of adsorbed casein.

The relative solubility of egg-PC, DPPC, and DOPC in the soy oil was measured by spectrophotometry with a UV-1200 model (Shimadzu, Kyoto, Japan). Weights of 50 mg of each phospholipid were added to 10 mL soybean oil and stirred for 10 h before measurements. The wavelength used is 500 nm at which soy oil gives maximum transmittance. Decreased solubility was estimated from the turbidity of the oil/phospholipid suspensions.

RESULTS AND DISCUSSION

Emulsion stability. Stable emulsions can be made with casein concentrations as low as 0.3 wt% with 20% oil, as long as the surface concentration of casein (Γ) is above 1 mg · m^{-2} (21). The size distribution of droplets in an emulsion (20% oil, 0.5% casein) that contained no phospholipid, measured immediately and after a time interval of 7 d, is shown in Fig-

ure 1A. The overlapping of the two distributions demonstrates that the emulsion was stable at least for a week. Emulsions with a similar amount of casein but containing 0.5% of egg-PC and DPPC, respectively (added before the emulsion was formed), are shown in Figure 1 (B and C). These emulsions were also stable, and the average droplet sizes (around 320 nm) and size distributions were similar. We know that the presence of egg-PC enhances the stability of the emulsions with Γ below 1 mg · m^{-2} (23). DOPC had a different effect (Fig. 1D). The size distribution of the fresh emulsion at 0.5% casein and 0.5% DOPC, compared to the other emulsions shown in Figure 1 (A–C), showed a larger average size (350 nm), and after 3 d of storage, the size distribution had become bimodal, with the formation of a population of particles with sizes about 10 μm. This increase in size with time indicated that the emulsion was not stable during storage. Thus, we can conclude that DOPC destabilized the emulsions, whereas egg-PC and DPPC either enhanced or did not affect the stability of the emulsion.

To determine if the destabilizing effect of DOPC depended on the presence of DOPC during emulsion formation, we added 0.5% DOPC after the formation of emulsions made with 0.3 and 0.5% casein. The original emulsions and the emulsions with added DOPC were measured daily over the period of one week (Fig. 2, A and B). In the absence of DOPC, both emulsions were stable. Three days after DOPC was added, there was a small but detectable peak of larger particles developing in the size distribution, which was more evident for the emulsion with 0.3% casein. After one week,

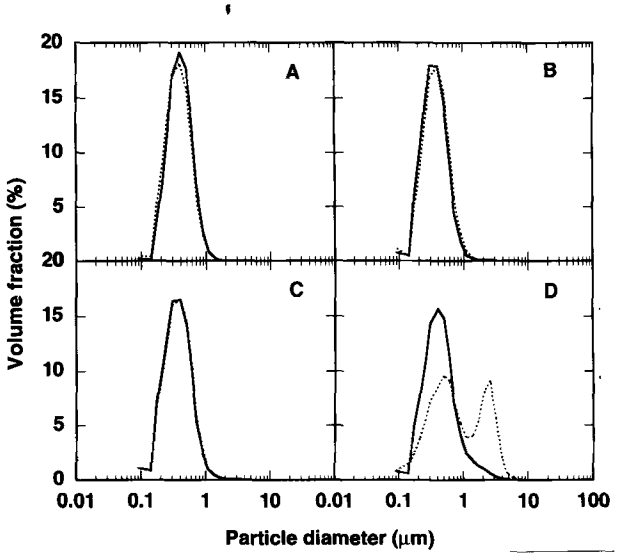

FIG. 1. Size distributions of droplets in fresh and stored emulsions (20 wt% soy oil), measured in the Malvern Instruments Mastersizer (Malvern Instruments, Inc., Southboro, MA): (A) emulsions containing 0.5% casein and no phospholipid; (B) emulsions containing 0.4% casein and 0.5% egg-phosphatidylcholine; (C) emulsions containing 0.5% casein and 0.5% di-palmitoyl phosphatidylcholine; (D) emulsions containing 0.5% casein and 0.5% di-oleyl phosphatidylcholine. Full lines are results obtained from fresh emulsions and broken lines the results obtained from the same emulsions stored for one week at 4°C.

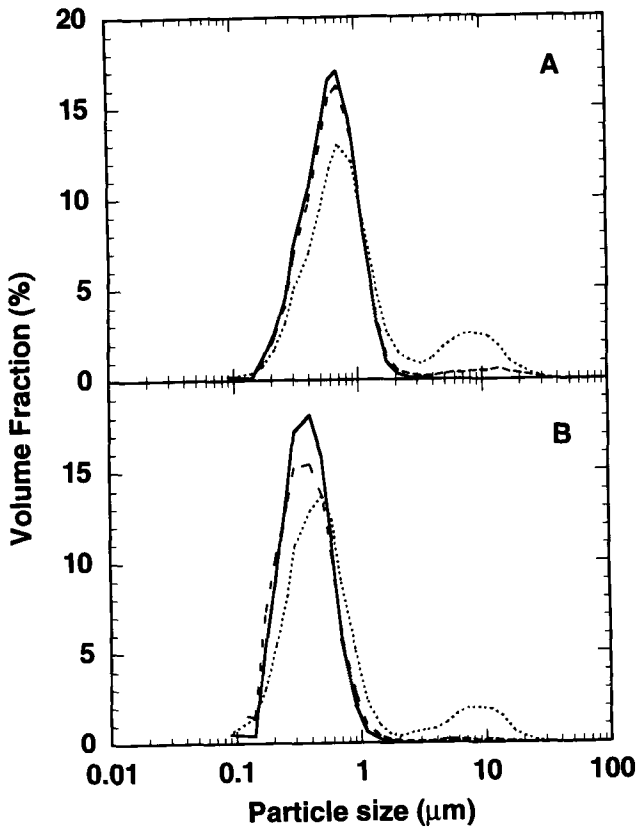

FIG. 2. Size distributions of emulsion droplets measured by Mastersizer, for emulsions (20 wt% soy oil) to which 0.5% di-oleyl phosphatidylcholine (DOPC) was added after the emulsion was formed: (A) emulsions containing 0.3% casein; (B) emulsions containing 0.5 % casein. In both diagrams, the full lines show the results from the emulsions that contained no DOPC, stored for 2 d before measurement; the broken and dotted lines are from emulsions with added DOPC measured after 2 and 6 d of storage, respectively. See Figure 1 for company source.

the amount of material in the larger aggregates had become more prominent, and again the effect was most pronounced in the emulsion that contained 0.3% casein. Unlike the egg-PC and DPPC, DOPC destabilized emulsions either when it was present during emulsion formation or was added to the emulsion. From the SDS–PAGE analysis (see below), we know that DOPC removed casein from the interface. The displacement of protein from the interface by egg-PC has been reported previously (14) but no destabilizing effect was found unless the emulsion was subjected to shear flow (24). Although DOPC added to an already formed emulsion caused destabilization (Fig. 2), the process was much faster and was carried to a greater extent (Fig. 1D) if DOPC was present during homogenization. Because the solubility of phospholipid in molecular form in the solution is small, it is likely that the concentration of dissolved phospholipid in both cases would be close; if the removal of adsorbed casein is only caused by DOPC in solution, then we might expect that the effect of having DOPC present during homogenization or added afterwards would be the same. The greater effect obtained while

DOPC was present during homogenization indicates that the coexistence of DOPC and casein on the interface facilitated the interaction between casein and DOPC, and the removal of casein from the interface might not be a simple process of casein being displaced by DOPC. It is more likely that the adsorbed casein and DOPC form a more hydrophilic molecular complex, which desorbs from the interface into the aqueous phase (25).

Surface concentration of casein. Like their influence on emulsion stability, egg-PC, DPPC, and DOPC also have a different effect on Γ in these emulsions. The dependence of Γ on the total casein concentration of emulsions that contained egg-PC is shown in Figure 3A. At low casein concentrations (<0.8%), the difference between emulsions with egg-PC and the control sample (stabilized by casein only), was small because at this low casein concentration, nearly all of the caseins were adsorbed to the interface despite the presence of egg-PC on the surface. At higher casein concentrations, the presence of egg-PC decreased the amount of casein adsorbed on the interface to a small extent. However, the differences between 0.2 and 0.5% egg-PC were small; even at 0.2% egg-PC, there was an excess of lipid remaining in the aqueous phase. In emulsions containing DPPC (Fig. 3B), neither 0.2 or 0.5% DPPC had a detectable influence on the concentration of adsorbed casein. The molecular weights of egg-PC (around 732) and DPPC (734) are similar, so the molar ratio of PC/protein is similar in both cases. Thus, the difference between Figure 3 (A and B) suggests that DPPC has a much weaker affinity for the oil droplets than egg-PC. The latter contains a mixture of hydrocarbon chains, and they are in a liquid crystalline state at room temperature, whereas DPPC is in a gel state until 40°C. The soybean oil is liquid at room temperature; therefore, under the experimental conditions, the DPPC chain and the triglycerides are in different states.

DOPC has two unsaturated chains and is in a liquid crystalline state at room temperature, and the effect of DOPC on the surface concentration of casein is the strongest among the three PC. The results obtained when the emulsions were analyzed shortly after they were made are shown in Figure 4A. A significantly lower surface coverage of casein was observed, even at the lowest casein concentration, and there was no significant difference between emulsions containing 0.2

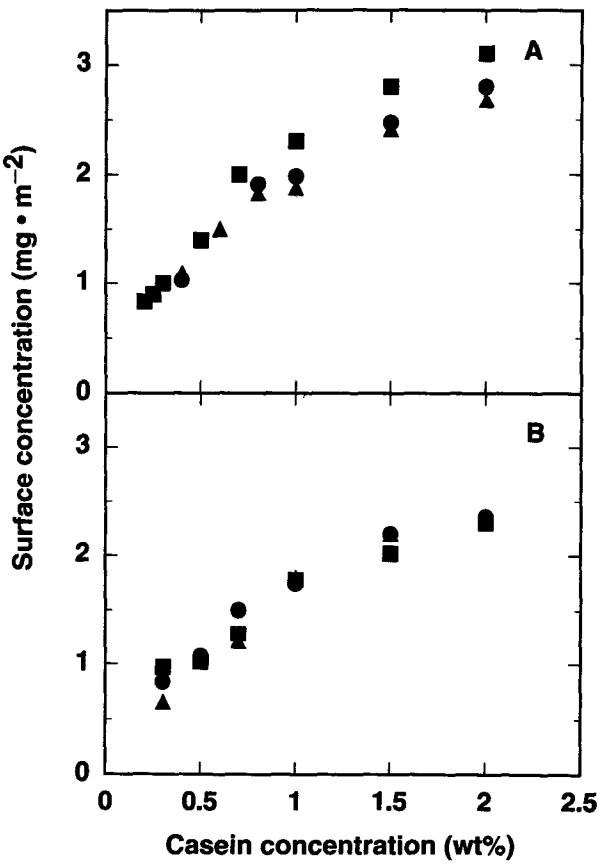

FIG. 3. Dependence of the surface concentrations of casein as a function of total casein concentration in the emulsions (20 wt% soy oil): (A) emulsions containing di-oleyl phosphatidylcholine; (B) emulsions containing di-palmitoyl phosphatidylcholine. In both diagrams, ■, ●, and ▲ denote emulsions containing 0, 0.2, and 0.5% phospholipid, respectively.

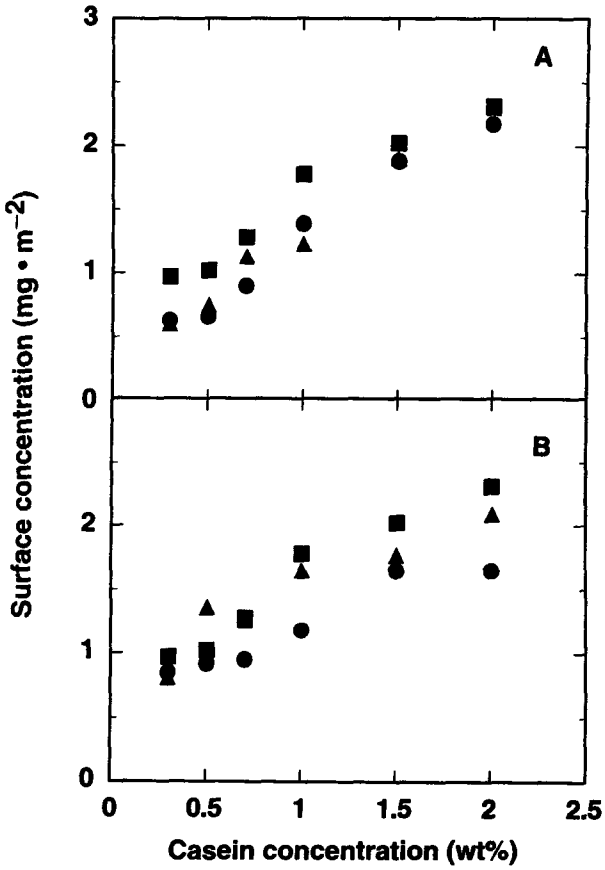

FIG. 4. Dependence of the surface concentrations of casein as a function of total casein concentration in the emulsions (20 wt % soy oil) containing di-oleyl phosphatidylcholine: (A) analyzed shortly after the emulsions were made; (B) emulsions stored for 2 d before analysis. In both diagrams, ■, ●, and ▲ denote emulsions containing 0, 0.2, and 0.5% phospholipid, respectively.

and 0.5% DOPC when the analysis was done shortly after the emulsions were made. However, the surface concentration of casein changed greatly (Fig. 4B) after the emulsions were stored for 48 h, when an increase was observed at low casein concentration and a decrease at high casein concentration, the change being greater with 0.5% DOPC. At low casein concentration, the apparent increase in Γ could be explained by the instability caused by DOPC, which led to an increase in droplet size of the emulsion and a decrease in total surface area of the emulsion droplets. The higher the DOPC concentration, the greater was the effect on emulsion stability and Γ. At high concentrations of casein, the emulsions were stable, and the decrease in Γ was a direct indication of the removal of casein from the interface by DOPC. Among the three PC, DOPC was the only one that removed casein from the interface during storage of the emulsion, and its presence on the surface destabilized the emulsions droplets, while egg-PC had a lesser influence on the surface concentration of casein, but its presence on the surface together with casein had a synergistic effect on the emulsion stability; DPPC had the least effect on the stability of emulsions and on Γ. Even though some of the PC changed the amounts of casein bound to the interface, they at no concentration ratio displaced the casein, as is known for other small molecule surfactants (3,4,9,10).

Hydrodynamic layer thickness of adsorbed casein. The hydrodynamic dimensions of the adsorbed casein measured by PCS provide information about the morphology of the adsorbed protein (23). The hydrodynamic thickness of casein in emulsions containing 0.2% phospholipids is shown in Figure 5A. The layer thickness of casein did not change with the age of the emulsion, except for emulsions containing DOPC. At this concentration, DPPC did not affect the layer thickness of the casein compared to the emulsions stabilized with casein only. The layers had the same thickness in the presence of egg-PC or DOPC (if measured on the first day), in good agreement with the similarity of the surface concentration of casein with these two phospholipids. Compared to the emulsions in the absence of casein, the presence of egg-PC or DOPC gives a thicker layer at low concentrations of casein (<1%) and a thinner layer when the casein concentration is greater than 1%. The increase in layer thickness at lower casein concentration can be interpreted as a change in the packing of the adsorbed casein molecules; with phospholipid co-adsorbed at the interface, it is no longer necessary for casein molecules to extend so far to cover the oil–water interface (23), and the adsorbed protein can adopt a more favored structure, projecting into solution. The thinner casein layer at high casein concentration (>1%) in the presence of egg-PC and DOPC is less easy to explain because the amount of casein displaced by the phospholipids is relatively small, and we cannot explain the decreased layer size as simply arising from the decrease in the surface concentration of casein. It is possible that the presence of the phospholipid induces changes in the conformation of the adsorbed casein molecules. In emulsions in the presence of DOPC during 48 h of storage, the surface concentration of casein is changed (Fig. 4B), and this is reflected in changes in the casein layer thickness as well (Fig. 5A).

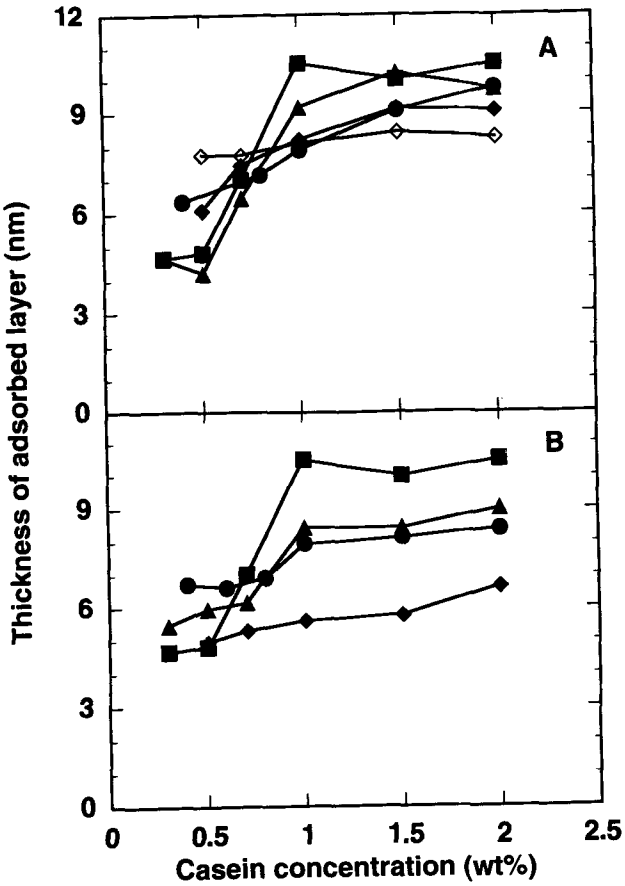

FIG. 5. Hydrodynamic thickness of adsorbed layers of casein as a function of the casein concentration: (A) emulsions containing 0.2% phospholipids; (B) emulsions containing 0.5 % phospholipids. In both diagrams, ■ denotes emulsions made containing no phospholipid; ● emulsions containing egg-phosphatidylcholine; ▲ emulsions containing di-palmitoyl phosphatidylcholine; ◆ emulsions containing di-oleyl phosphatidylcholine (DOPC) analyzed the same day as the emulsion was formed; ◇ emulsions containing DOPC analyzed after the emulsions had been stored for 2 d.

In the presence of 0.5% egg-PC and DPPC, the casein layer thickness has the same plateau value of about 8 nm at casein concentrations above 1%, and the layer thickness for emulsions in the absence of phospholipids had a plateau value of 10 nm in the same concentration range (Fig. 5B). From the previous section, we know that neither 0.2 or 0.5% DPPC significantly affected the surface concentration of casein, and 0.2% DPPC also had a negligible effect on the casein layer thickness. However, at a concentration of 0.5%, DPPC caused a decrease in the layer thickness to the same extent as 0.5% egg-PC, indicating that although the surface concentration of casein is an important factor for the adsorbed casein layer thickness, it is not the only one. The casein layer thickness in emulsions that contained 0.5% DOPC at all casein concentrations is at the lower plateau value (5 nm) of emulsions without PC, even though Γ remained relatively high. These emulsions were sensitive to the trypsin treatment, so that a smaller amount of trypsin had to be applied to ensure that the emulsion droplets remained stable for a reasonable length of time after the breakdown of the casein

layer. The coexistence of DOPC and casein at the interface must therefore modify the conformation of the protein adsorbed to the surfaces of the emulsion droplets, so that the emulsion becomes unstable to the hydrolysis of the adsorbed casein. Because the emulsions containing 0.5% DOPC were not stable, it was not possible to measure the layer thickness in emulsions stored for 48 h.

Solubility of egg-PC, DPPC, and DOPC in oil. Egg-PC, DPPC, and DOPC have different solubilities in soybean oil at room temperature. Each PC (50 mg) was added to 10 mL oil, and the mixtures were stirred for 10 h. The solutions containing egg-PC and DOPC looked transparent, as did the pure oil, but the solution with DPPC remained turbid. The transmittance values (at $\lambda = 500$ nm) of pure oil and the different dispersions of phospholipid in the oil showed that egg-PC and DOPC gave solutions almost as transparent as the pure oil (transmission of 98.3 and 99.8%, respectively), but the dispersion of DPPC gives a transmittance of only 11.2%, indicating that DPPC has low solubility in oil. The difference between egg-PC and DOPC was small, although it might be sufficient to explain the difference in Γ of emulsions that contained these two PC. As estimated by its effect on the emulsion, DOPC had a slightly higher affinity to the oil surface than egg-PC, and DPPC showed no significant influence on the casein surface concentration, even at a concentration of 0.5%.

It has been reported (14) that egg-PC has a larger effect on the droplet size of emulsions if the oil phase is *n*-tetradecane rather than soy oil, and also the amount of egg-PC associated with the nonaqueous phase (i.e., either dissolved or adsorbed) is much higher in *n*-tetradecane than on soy oil; indeed, the amount adsorbed to soy oil is small. From our results, we can conclude that the compatibility between the oil phase and the surfactant plays an important role in the ability of the surfactant to displace protein at the interface. Among the three PC, DPPC has the highest melting point and is in the gel state at the temperature of the experiments (25°C), while the soy oil is in the melted state; the hydrocarbon chains of both compounds are not compatible, and this could partially explain the considerable difference of the affinity on the oil surface between DPPC from egg-PC and DOPC. Because DPPC has low affinity to the oil surface, it is less likely for it to displace casein adsorbed on the interface.

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