Competitive Adsorption of Hen's Egg Yolk Granule Lipoproteins and Phosvitin in Oil-in-Water Emulsions

Rotimi E. Aluko and Yoshinori Mine*

Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Competitive adsorption of egg yolk granule lipoproteins and phosvitin in oil-in-water emulsions was investigated at pH 4.0, 7.0, and 9.0. Protein solutions contained different ratios of granule proteins to pure phosvitin. The droplet size of the pH 4.0 emulsions was higher than the values obtained for the pH 7.0 and 9.0 emulsions at all of the different combinations of granule and pure phosvitin. Unlike the pH 7.0 and 9.0 emulsions, the amount of phosvitin bound to the oil−water interface at pH 4.0 increased with increase in weight ratio of added pure phosvitin. Time-dependent exchange experiments showed that displacement of phosvitin from the interface by granule lipoproteins was higher and more rapid at pH 7.0 than at pH 4.0, suggesting that the reduction in negative charges of phosvitin molecules at pH 4.0 increases its affinity to the interface. There was an initial increase in droplet size of the phosvitin emulsions upon addition of a granule preparation, which was probably as a result of bridging flocculation of the emulsions by the adsorbing lipoproteins. The results suggest that granule lipoproteins are more surface active than phosvitin and that protein mixtures containing lipoproteins and pure phosvitin would stabilize food emulsions better at pH 7.0 and 9.0 than at pH 4.0.

Keywords: *Egg yolk granule; competitive adsorption; emulsion; phosvitin; lipoproteins*

INTRODUCTION

Hen's egg yolk, which consists of a soluble plasma and particulate granules, is known to be an excellent emulsifier and is an active ingredient in various food products such as mayonnaise, salad dressings, and cakes (Kamat et al., 1973; Kiosseoglou and Sherman, 1983). Egg yolk contains various emulsifying agents such as hydrophobic and hydrophilic proteins, phospholipids, and cholesterol. These compounds are believed to interact during emulsion formation, though the nature of such interactions has not been fully elucidated (Kiosseoglou and Sherman, 1983; Carrillo and Kokini, 1988).

The granule fraction can be isolated by diluting whole egg yolk with water or dilute saline solution followed by centrifugation to give a supernatant made up mainly of the plasma fraction and the precipitate that contains the granule fraction (Itoh et al., 1986; Causeret et al., 1991). The granule contains ∼70% lipovitellins, ∼16% phosvitin, and ∼12% low-density lipoprotein (LDL), all of which are believed to contain phosphocalcic bridges between and within their structures (Burley and Cook, 1961; Causeret et al., 1991). The phosvitin residue, by virtue of its high content (10%) of phosphorus, is more hydrophilic than the lipovitellin and LDL molecules, which contain up to 2% phosphorus (Itoh et al., 1986; Causeret et al., 1991). Moreover, the lipovitellin and LDL molecules contain up to 22 and 89% lipid residues, respectively (Kamat et al., 1972; Causeret et al., 1991) when compared to phosvitin. Therefore, during emulsion formation involving granule components, interaction of the hydrophobic proteins (lipovitellin and LDL) with oil droplets should be stronger than that between the hydrophilic protein (phosvitin) and oil droplets (Halling, 1981; Parker, 1987).

* Author to whom correspondence should be addressed [telephone (519) 824-4120, ext. 2901; fax (519) 824-6631; e-mail ymine@uoguelph.ca].

Granules exist as insoluble particles in egg yolk, primarily as a result of strong interactions between lipoproteins and phosvitin molecules mediated by phosphocalcic bridges (Chang et al., 1977; Causeret et al., 1991). In acidic or basic pH conditions, there is disruption of the granule structure as a result of breakup of the phosphocalcic ionic bridges that join phosvitin to lipoproteins, resulting in increased solubilization of the granule (Causeret et al., 1991). Similarly, increase in ionic strength has been shown to disrupt the granule structure, and at 1.7 M NaCl concentration there is complete dissociation of the granule leading to release of lipoproteins and phosvitin into the salt solution (Chang et al., 1977; Ternes, 1989; Causeret et al., 1991). Sodium chloride-induced dissociation of granule is believed to be as a result of substitution of monovalent sodium for bivalent cations, which leads to breakup of the phosphocalcic bridges and release of calcium (Causeret et al., 1991). Therefore, there is no substantial affinity between phosvitin and lipoproteins at acidic or basic pH and at 1.7 M NaCl concentration. In fact, the effect of 1.7 M NaCl on granule structure was used as the basis for isolation of phosvitin by Losso and Nakai (1994), since at this ionic strength phosvitin exists as single protein entities that are not involved in complex asssociation with granule lipoproteins.

The phosvitin molecule has a total of 220 amino acid residues, half of which are phosphoserines, and while the remaining half is composed of a large number of acidic amino acids (Taborsky, 1974). The basic amino acids that are present are insufficient to balance all of the negative charges, and thus the protein is a polyelectrolyte (Vogel, 1983). The phosphoserine residues have been shown to be in the dianionic form at physiological pH and can be protonated and deprotonated with decrease or increase in pH, respectively (Taborsky, 1974; Vogel, 1983). It has been shown that when a granule that has been destructured by addition of NaCl was dialyzed against water, only the phosvitin molecules retained their solubility, apparently as a result

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of the large number of negative charges associated with the phosphoserine residues (Causeret et al., 1991). In addition, phosvitin contains only one residue each of tyrosine and tryptophan and, together with all of the serine phosphate residues and the backbone amide protons, are exposed to the solvent, resulting in a highly unfolded and flexible protein (Vogel, 1983).

Adsorption of protein emulsifiers involves competition between peptide residues on the same molecule as well as between those on different molecules; therefore, competitive adsorption is believed to be central to understanding of emulsifying properties of food proteins (Dickinson, 1986). There are various studies in the literature on the competitive adsorption of various mixtures of milk proteins. For example, it has been shown that β-casein will displace α_{s1}-casein from the interface of oil-in-water emulsions to a greater extent than the displacement of β-casein by α_{s1}-casein (Dickinson et al., 1988). Recently, caseins have also been reported to displace whey proteins from emulsions made in the presence of potassium chloride, though displacement of β-lactoglobulin decreased with increase in age of the emulsions (Hunt and Dalgleish, 1996). In another study, it was shown that β-casein and β-lactoglobulin readily displaced phosvitin from an *n*-tetradecane−water interface, a result attributed to the lower surface activity of phosvitin when compared to the milk proteins (Dickinson et al., 1991). It was hypothesized that the low surface activity of phosvitin is due to the fact that only a small proportion of its amino acid residues becomes directly attached to the interface after adsorption, the vast majority interacting with the aqueous environment (Dickinson et al., 1991).

There are reports detailing the emulsifying properties of egg yolk granule as well as those of pure phosvitin. Addition of NaCl to egg yolk has been shown to improve the rheological properties of oil-in-water emulsions and mayonnaise (Kiosseoglou and Sherman, 1983; Carrillo and Kokini, 1988), and phosvitin emulsions had better properties than those made from bovine serum albumin (Chung and Ferrier, 1991, 1992). At 80% solubility, the granule has been reported to stabilize emulsions better than whole egg yolk or yolk plasma (Anton and Gandemer, 1997). However, there is the need to better understand the competitive nature of the interfacial adsorption of granule lipoproteins and phosvitin. The aim of this work is to evaluate the emulsifying properties of binary mixtures of pure phosvitin and granule preparation with a view to determining their preferential adsorption at the oil−water interface.

MATERIALS AND METHODS

Fresh eggs were obtained from the University of Guelph's Arkell poultry farm. Crude triolein (65% purity) was a product of Sigma Chemical Co. (St. Louis, MO). Phosvitin was purchased from Sigma (St. Louis, MO) and used without further purification for determination of emulsifying properties of binary protein mixtures. Due to the large quantities required, phosvitin was also purified from egg yolk according to the method of Losso and Nakai (1994) and used for the exchange experiment.

Preparation of Egg Yolk Granule. Fresh eggs were separated into white and yolk, and the yolk was rolled on a filter paper to remove adhering fragments of egg white. The yolk membrane was punctured and the liquid yolk allowed to flow into a clean beaker. The liquid yolk was diluted with 4 volumes of 0.16 M NaCl, mixed on a magnetic stirrer, and centrifuged at 8000*g* and 4 °C for 30 min. The precipitate was washed twice with 3 volumes of 0.05 M NaCl and centrifuged as before, and the final precipitate was dispersed in 1.7 M NaCl solution containing 0.05% sodium azide. The granule preparation was stored at 4 °C and its protein content determined according to the method of Markwell et al. (1978).

Purification of Triolein. Crude triolein (65% purity) was purified by silica gel column chromatography, using a solvent mixture composed of hexane/diethyl ether (97:3, v:v). The solvent in the eluate was evaporated on a rotary evaporator, and the residual oil was transferred into an amber bottle and saturated with nitrogen gas before storage at 4 °C. Purity of the triolein preparation was >98% after analysis by thin-layer chromatography with flame ionization detection (TLC-FID) using the Iatroscan system (Iatroscan MK-5, Iatron Laboratories Inc., Tokyo, Japan).

Determination of Emulsifying Properties of Binary Mixtures. The granule preparation was diluted with each of the following buffers to give a final protein concentration of 0.5% (w/v), i.e., 0.0 weight fraction of pure phosvitin: 50 mM acetate, imidazole, and borate buffers containing 10% NaCl, pH 4.0, 7.0, and 9.0, respectively. Phosvitin (0.5%, w/v), i.e., 1.0 weight fraction of pure phosvitin, was also prepared with each of the above buffers. The granule (G) and phosvitin (PV) solutions were then mixed at various proportions to give 0.5% (w/v) protein mixtures with the following compositions. The corresponding weight fractions of pure phosvitin are shown in parentheses: 0.1% PV + 0.4% G (0.2); 0.2% PV + 0.3% G (0.4); 0.3% PV + 0.2% G (0.6); 0.4% PV + 0.1% G (0.8). Emulsions were prepared by homogenizing 2.0 mL of each protein solution with 0.5 mL of pure triolein for 1 min at a speed of 22 000 rpm using a Polytron PT 2000 homogenizer (Kinematica AG, Switzerland). The mean droplet size ($d_{3,2}$) of the emulsions was determined using Milli-Q water as dispersant on a Mastersizer X (Malvern Instruments Ltd., Malvern, U.K.) with optical parameters defined by the manufacturer's presentation code 0303. The emulsions were then centrifuged at 8500*g* and 20 °C for 30 min, and the serum was withdrawn with syringe and stored at 4 °C. To remove unbound proteins, the cream phase was washed twice by dispersing it in the appropriate buffer followed by centrifugation at 8500*g* and 20 °C for 30 min. The sera were pooled together for each sample and filtered through 0.2 μm filters, and the protein content of the filtrates was determined according to the method of Markwell et al. (1978). Since the particle size distribution of the emulsions (from Mastersizer results) showed that <0.5% of the emulsion droplets were ≤0.3 μm, the filtrate should not contain emulsified particles to the extent that the protein content of the sera will be overestimated. Protein concentration of sera containing only phosvitin could not be determined according to the modified Lowry method of Markwell et al. (1978) and was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Itoh et al., 1986). A known volume of serum was mixed with half its volume of 10% (w/v) SDS in 0.1 M Tris buffer, pH 8.0, containing 0.01% bromophenol blue. The samples were heated in boiling water for 5 min, cooled to room temperature, and centrifuged at 16000*g* for 5 min. Standard phosvitin solution was also prepared accordingly. The serum and phosvitin standard (0.5−15 μg/lane) samples were run on 4−15% gradient gels using the Bio-Rad Mini Protean II electrophoresis cell at a constant current of 20 mA/gel. The gels were stained using the modified Coomassie blue method for phosphoproteins (Hegenauer et al., 1977). After destaining in a solution containing acetic acid/methanol/water (1:8:12, v/v/v), gels were scanned on a Sharp JX-330 scanner (Sharp Electronics, Tokyo, Japan) and the raw volume of each band was determined using Pharmacia ImageMaster 1D software, version 2.0 (Pharmacia Biotech, Mississauga, Canada). The concentration of protein in the serum was calculated from a standard curve of phosvitin concentration versus raw volume. Protein content of the cream was estimated as a difference between protein concentration of the serum and the total protein used to make the emulsion. The amount of protein present in the cream was divided by the specific surface area (from Mastersizer results) of its emulsion to give the amount of protein bound per square meter.

Protein Composition of Creams. The washed creams were analyzed for protein composition by SDS−PAGE as

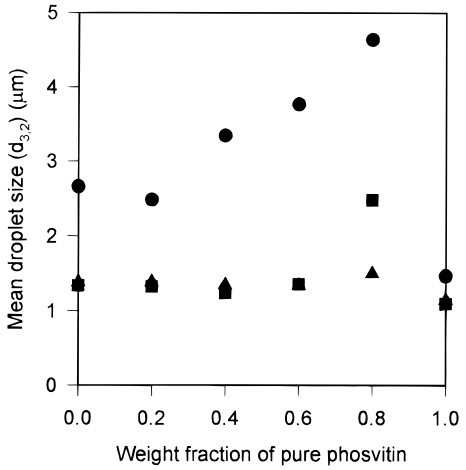

Figure 1. Mean droplet size of emulsions (20% oil, v/v) stabilized individually by 0.5% (w/v) granule proteins (0.0 weight fraction of phosvitin), 0.5% (w/v) protein mixtures containing granule protein and pure phosvitin (0.2−0.8 weight fraction of phosvitin), and 0.5% (w/v) pure phosvitin (1.0 weight fraction of phosvitin): (●) pH 4.0; (■) pH 7.0; (▲) pH 9.0.

follows. Creams were treated with 10% (w/v) SDS in 0.1 M Tris buffer, pH 8.0, containing 5% (v/v) 2-mercaptoethanol and 0.01% bromophenol blue, followed by heating at 95 °C with simultaneous shaking at 1400 rpm in an Eppendorf thermomixer 5436 (Fisher Scientific, Unionville, Canada) for 10 min. The samples were cooled to room temperature and centrifuged at 16000*g* for 5 min, and the protein composition of the supernatant was determined using 4−15% gradient gels as described above. The gels were first stained using the modified Coomassie blue method for phosphoproteins (Hegenauer et al., 1977) to stain the phosvitin molecules. The second staining used the normal Coomassie blue procedure to stain the lipovitellins followed by destaining with a solution containing acetic acid/methanol/water (1:8:12, v/v/v). The gels were scanned as described above, and the percentage ratio of each band was determined using Pharmacia ImageMaster 1D software, version 2.0 (Pharmacia Biotech).

Exchange Experiments. To determine the time-dependent behavior of interfacial protein exchange between granule lipoproteins and pure phosvitin, emulsions stabilized by 0.5% phosvitin were prepared, centrifuged, and washed to remove unbound proteins as described above. Mean particle size and protein surface coverage were also determined. The isolated cream was then dispersed in a buffered granule solution containing 0.5% (w/v) protein, pH 4.0 and 7.0, followed by mixing for 18 h. Samples were taken from the mixture at 0.5, 1, 2, 6, 12, and 18 h, and mean particle size was determined on the Mastersizer. The samples were also centrifuged and washed to determine surface protein concentration. The resultant creams were analyzed for protein composition by SDS−PAGE and gel scanning as described above.

RESULTS AND DISCUSSION

The mean droplet sizes of the emulsions containing protein mixtures are shown in Figure 1 as a function of weight fraction of pure phosvitin. At pH 4.0, droplet size of the emulsions was greater than were those obtained at pH 7.0 and 9.0, irrespective of the ratio of granule protein to phosvitin in the solution. However, when the protein solution contained only phosvitin, the emulsions had similar particle sizes ranging from 1.1 μm for pH 7.0 and 9.0 to 1.3 μm for pH 4.0. Dickinson et al. (1991) have also shown that emulsions made from protein solutions containing only phosvitin contained small droplets comparable to those found in emulsions stabilized by more hydrophobic milk proteins such as β-casein and β-lactoglobulin. It can be inferred that the presence of granule proteins was responsible for the increase in particle size of the emulsions containing protein mixtures, especially at pH 4.0 and at 0.8 weight fraction of phosvitin for pH 7.0 and 9.0. Therefore, at pH 4.0 phosvitin is a better emulsifier than granule proteins, whereas at pH 7.0 and 9.0 the granule proteins either alone or in combination with pure phosvitin behaved similarly to 100% phosvitin with respect to droplet size formation. The increase in particle size of granule-stabilized emulsions at pH 4.0 when compared to pH 7.0 and 9.0 may have been due to the reported dimerization of granule lipovitellins (major protein in granule) at pH values <7.0 (Burley and Cook, 1962; Kamat et al., 1973). It is probable that such a dimerization at pH 4.0 reduces their ability to unfold and effectively cover the oil droplets; thus, larger size emulsified droplets are formed. On the other hand, dissociation of the lipovitellins into monomeric units at pH 7.0 and 9.0 (Burley and Cook, 1962; Kamat et al., 1973) enhances their ability to unfold at the interface and interact with the oil phase. Since dimerization of lipovitellins would involve hydrophobic interactions, it is conceivable that there will be a reduction in the number of hydrophobic groups available to interact effectively with apolar oil droplets and thus a reduction in emulsifying capability at pH 4.0.

The better emulsifying properties of the emulsions stabilized by binary mixtures at pH 7.0 and 9.0 may also be due to increased solubility of the proteins since the granule is dissociated at pH >6.3 as a result of increased electrostatic repulsions (Causeret et al., 1991). Phosvitin has also been shown to have better emulsifying properties at high pH than at low pH (Chung and Ferrier, 1992) and, as such, could have contributed to the better emulsifying properties of the binary protein mixtures obtained at the high pH values. Even though pure phosvitin is a good emulsifier (Chung and Ferrier, 1992), the present result showed that in the presence of granule proteins and at pH 4.0, increasing the ratio of phosvitin in the protein mixture actually produced poor emulsions with larger droplet sizes. The result would suggest that the granule proteins are preferentially adsorbed to the interface even in the presence of high concentrations of phosvitin. Therefore, increasing the phosvitin ratio is similar to decreasing the amount of granule proteins available for adsorption at the interface,, and since the granule proteins produce poor emulsions at pH 4.0, a gradual reduction in their concentration resulted in increased droplet size formation. At pH 7.0, an increase in phosvitin concentration did not have any negative effect on droplet size formation until the weight ratio reached 0.8, suggesting that at lower weight ratios the effectiveness of the granule proteins as an emulsifier was still better than what was obtained at pH 4.0. Apart from the pH effect, the presence of salt has also been reported to reduce the molecular charge on lipovitellins, a condition that could enhance protein−protein interactions (Kiosseoglou and Sherman, 1983) and promote dimerization at pH 4.0.

The amount of protein present at the oil−water interface of emulsions containing granule proteins and pure phosvitin is shown in Figure 2. There was an increase in surface protein concentration at pH 4.0 with increase in weight fraction of phosvitin, unlike at pH 7.0 and 9.0. For emulsions made with phosvitin only, the surface protein concentration decreased with increase in pH from 1.0 mg/m^2 for the pH 4.0 emulsion to 0.5 mg/m^2 for the pH 9.0 emulsion. The result is consistent with the fact that at pH 4.0 the protein covers

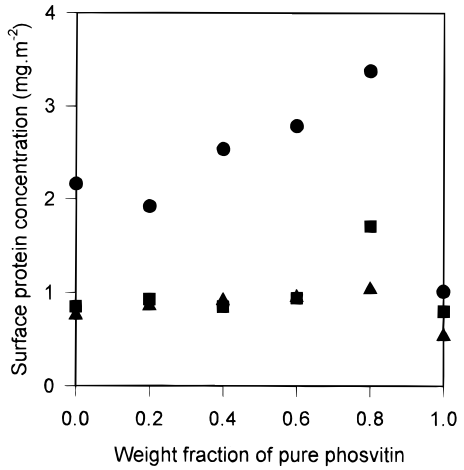

Figure 2. Surface protein coverage of emulsions (20% oil, v/v) stabilized individually by 0.5% (w/v) granule protein (0.0 weight fraction of phosvitin), 0.5% (w/v) protein mixtures containing granule protein and pure phosvitin (0.2−0.8 weight fraction of phosvitin), and 0.5% (w/v) pure phosvitin (1.0 weight fraction of phosvitin): (●) pH 4.0; (■) pH 7.0; (▲) pH 9.0.

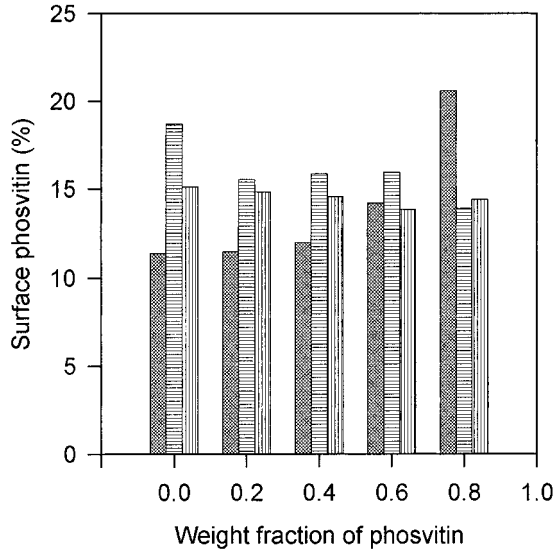

Figure 3. Amount of phosvitin present at the interface of emulsions (20% oil, v/v) stabilized by 0.5% (w/v) granule protein (0.0 weight fraction of phosvitin) and by protein mixtures (0.5%, w/v) containing granule protein and pure phosvitin (0.2−0.8 weight fraction of phosvitin): (cross-hatched bars) pH 4.0; (horizontally lined bars) pH 7.0; (vertically lined bars) pH 9.0.

less surface area, i.e., large droplet size, whereas at pH 7.0 and 9.0 there is an increase in surface area to give less protein coverage per unit area. Moreover, for each pH value there is a parallel between the change in droplet size of the emulsions and the amount of protein present per unit area (Figures 1 and 2), which supports the argument that for the same amount of protein the bigger the droplet size of the emulsion, the higher the concentration of protein at the interface per unit area. As the droplet size decreases, the specific surface area increases, resulting in a decrease in concentration of protein per unit area. The observed decrease in surface protein coverage for emulsions containing only phosvitin molecules as pH increased would suggest that binding of phosvitin to the interface is reduced by electrostatic repulsions. This may be due to the fact that as the pH increased from 4.0 to 9.0 the negative charge on the phosvitin molecules is higher due to increased ionization of the phosphate groups (Taborsky, 1974), presenting an energy barrier as a result of electrostatic repulsions between the charged groups in the adsorbed protein and similarly charged groups in the protein in solution (Parker, 1987). Kato et al. (1987) have also shown that the electrostatic repulsive force of phosphate in phosvitin significantly affects its emulsifying properties.

Phosvitin contents of the interfacial protein films from binary mixtures of granule and pure phosvitin solutions are shown in Figure 3. According to Burley and Cook (1961) proteins in the granule can be divided into lipoproteins (82%) and phosvitin (16%); therefore, the phosvitin contents shown in Figure 3 for the binary mixtures comes from both the granule and added pure phosvitin. One of the aims of this work was to determine whether the addition of pure phosvitin to the granule preparation before emulsification would allow for increase in the ratio of phosvitin bound to the oil−water interface. It is evident from Figure 3 that there was an increase in phosvitin binding to the interface with increase in phosvitin weight ratio at pH 4.0. On the other hand, the presence of additional phosvitin in the protein solution did not lead to any increase in interfacial phosvitin binding at pH 7.0 and 9.0. In fact, at pH 7.0, more phosvitin was bound to the interface when the emulsion was stabilized only by granule proteins than when pure phosvitin was added to the granule preparation. It is possible that at pH 7.0 the charge density in the granule is less because of the lower weight ratio of phosvitin, a condition which would allow greater binding of phosvitin to the interface due to reduced electrostatic repulsions when compared to the protein mixtures containing added pure phosvitin (higher weight ratios of phosvitin) and hence greater electrostatic repulsions. The results are consistent with the fact that because phosvitin molecules can be protonated and, therefore, are less charged at pH 4.0 (Taborsky, 1974; Vogel, 1983), electrostatic repulsions between the bound phosvitin and phosvitin molecules in solution decreae, allowing additional binding of phosvitin to the interface. However, at pH 7.0 and 9.0, at which the phosvitin carries more net negative charges (Taborsky, 1974), electrostatic repulsions at the interface reduce additional binding of phosvitin molecules. In other words, the energy barrier created by negatively charged phosvitin molecules at the interface is greater at pH 7.0 and 9.0 than at pH 4.0, a concept similar to that outlined by Parker (1987). It is also apparent in Figure 3 that for the granule-stabilized emulsions as well as 0.2 and 0.4 weight fractions of added pure phosvitin, more phosvitin was bound to the interface at pH 7.0 and 9.0 than at pH 4.0. This result could be due to the fact that at pH 4.0 dimerization of the lipoproteins coupled with the reduction in their net charge (Burley and Cook, 1962; Kamat et al., 1973) would increase protein−protein interactions between the lipoproteins to form dense films on the oil droplets. On the other hand, at pH 7.0 and 9.0 the lipoproteins exist as monomers and there is an increase in their net charge; therefore, the lipoprotein films would not be as dense as at pH 4.0. Since the more surface active lipoproteins are adsorbed preferentially before the more hydrophilic proteins, it would be harder for phosvitin to displace the more dense lipoprotein film present at pH 4.0 that at pH 7.0 and 9.0, especially at low phosvitin concentrations. However, as the concentration of phosvitin increased and that of lipoproteins decreased, packing of lipoproteins at the pH 4.0 interface will decrease, allowing more phosvitin molecules to bind to the interface and, hence, the high percentage of phosvitin present at 0.6 and 0.8

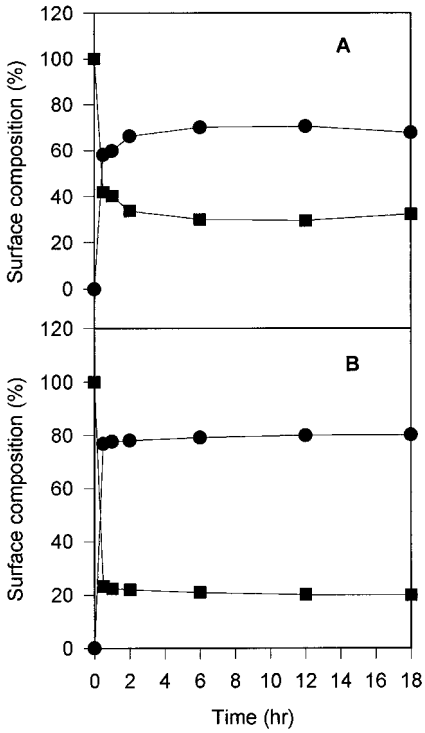

Figure 4. Proportions of protein exchange upon mixing a washed phosvitin stabilized emulsion (20% oil, v/v) at pH 4.0 (A) and 7.0 (B) with granule proteins: (●) lipoproteins; (■) phosvitin. Emulsions were first prepared with 0.5% (w/v) phosvitin (time zero), washed free of unadsorbed protein, and then mixed for 18 h with a buffered solution containing 0.5% (w/v) granule proteins; emulsion samples were taken at 0.5, 1, 2, 6, 12, and 18 h and analyzed.

weight ratios when compared to lower weight ratios of added pure phosvitin.

Figure 4 shows the protein composition of interfacial films from exchange experiments as a function of time. At pH 7.0, displacement of the interfacial phosvitin molecules was more rapid and higher than at pH 4.0 throughout the duration of the experiment (Figure 4). After 30 min of addition of granule proteins, ~80% of the interfacial phosvitin (including phosvitin from the granule) was displaced from the pH 7.0 emulsion by lipoproteins compared to ~60% for the pH 4.0 emulsion. It has also been reported that β-casein displaces >70% of phosvitin molecules from the interfacial protein film during the first 5 min of mixing of a phosvitin-stabilized emulsion with a solution containing β-casein (Dickinson et al., 1991). The present results suggest that the phosvitin molecules were weakly bound to the interface at pH 7.0, probably as a result of the higher net charge at this pH value, which would favor phosvitin−water interactions more than phosvitin−oil interactions. On the other hand, at pH 4.0, a decrease in the net charge of phosvitin would favor stronger binding to the interface as a result of increased interaction with the oil phase, thus reducing the amount displaced by granule lipoproteins. In a similar work, it was reported that reduction in the net charge on phosvitin molecules through addition of calcium ions resulted in increased binding of phosvitin to the oil−water interface and reduced displacement of the phosvitin molecules by β-casein (Hunt et al., 1993). Apart from increased interaction with the interface, it is possible that at pH 4.0 the phosvitin molecules form stronger protein films because the decreased net charge (Taborsky, 1974) allows them to interact more with each other through electrostatic attractions and hydrogen bond formation.

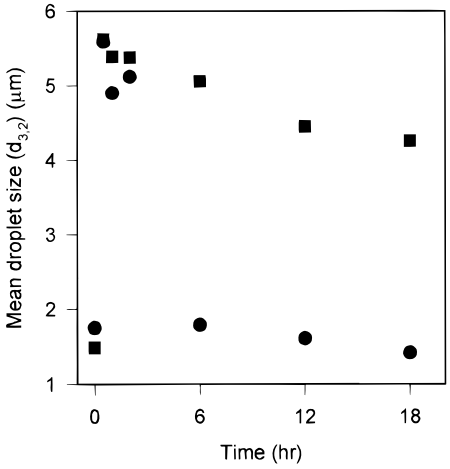

Figure 5. Changes in the mean droplet size of emulsions (20% oil, v/v) stabilized by a mixture of 0.5% (w/v) pure phosvitin and 0.5% (w/v) granule proteins: (●) pH 4.0; (■) pH 7.0. Emulsions were first prepared with 0.5% (w/v) phosvitin (time zero), washed free of unadsorbed protein, and then mixed for 18 h with a buffered solution containing 0.5% (w/v) granule proteins; emulsion samples were taken at 0.5, 1, 2, 6, 12, and 18 h and analyzed.

Such electrostatic interactions and hydrogen bond formation between the basic amino acids and phosphoryl moieties in phosvitin have been previously demonstrated (Vogel, 1983). The increased strength of the interfacial phosvitin film at pH 4.0 reduces its displacement by granule lipoproteins when compared to the pH 7.0 emulsion, in which electrostatic repulsions reduce interactions between the phosvitin molecules to give less cohesive protein films that are more readily displaced by granule lipoproteins. Differences in physical states of the competing lipoproteins at pH 4.0 and 7.0 could also have contributed to the results presented in Figure 4. For example, it has been established that at pH values <7.0, egg yolk lipoproteins occur as protein dimers, while at pH ≥7.0 they exist as monomers (Burley and Cook, 1962). It is possible, therefore, that the high molecular weight dimeric form of the lipoproteins at pH 4.0 has reduced ability to penetrate the oil−water interface and displace bound phosvitin when compared to the smaller monomeric units present at pH 7.0.

Figure 5 shows the time-dependent change in droplet size of 0.5% phosvitin emulsions, pH 4.0 and 7.0, after mixing with 0.5% granule proteins. There was an initial increase in droplet size of the emulsions from ~1.5 μm for the phosvitin emulsion to ~5.0−5.5 μm after 2 h of mixing with granule proteins. The result suggests that addition of granule proteins initially resulted in disruption of the interfacial protein films that stabilize the emulsions, making the droplets more susceptible to coalescence and/or flocculation to form bigger emulsion droplets. The disruption of the interfacial protein films could have arisen as the more hydrophobic and surface active granule lipoproteins try to displace the less surface active phosvitin molecules. A likely mechanism for the initial increase in particle size would be the so-called bridging flocculation, during which adsorbing proteins attach to more than one droplet at a time to form aggregated droplets (Dickinson, 1992). After 6 h of mixing, the droplet size of the pH 7.0 emulsion decreased slightly, while that of the pH 4.0 emulsion decreased substantially to a value similar to that of the 100% phosvitin emulsion. The result indicates that the strength of the protein bridges in the flocculated emul-

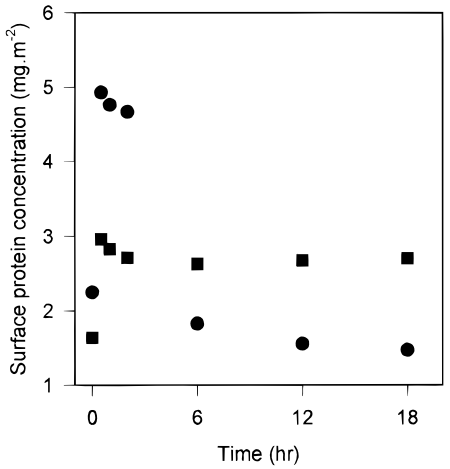

Figure 6. Changes in the surface protein coverage of emulsions (20% oil, v/v) stabilized by a mixture of 0.5% (w/v) pure phosvitin and 0.5% (w/v) granule proteins: (●) pH 4.0; (■) pH 7.0. Emulsions were first prepared with 0.5% (w/v) phosvitin (time zero), washed free of unadsorbed protein, and then mixed for 18 h with a buffered solution containing 0.5% (w/v) granule proteins; emulsion samples were taken at 0.5, 1, 2, 6, 12, and 18 h and analyzed.

sions was less at pH 4.0 than at pH 7.0, possibly because at acidic pH the lipoproteins exist as dimers and would be less flexible and effective in binding to two oil droplets, when compared to the more flexible monomeric units that are present at neutral and basic pH values. This is consistent with the results in Figure 4, which show that displacement of phosvitin from the pH 4.0 emulsion was less than at pH 7.0, indicating that interaction of the lipoproteins with the oil droplets was greater at pH 7.0 than at pH 4.0. This may explain why prolonged stirring of the granule into the phosvitin emulsion enabled conversion of the flocculated emulsion back to a dispersed emulsion at pH 4.0, while only a minimal decrease in particle size was obtained at pH 7.00, at which the strength of the lipoprotein bridges was stronger.

The concentration of protein at the interface as a function of time is shown in Figure 6. Initially, the phosvitin emulsions had similar surface protein coverage. However, upon addition of granule proteins and mixing for 0.5−2 h, the surface coverage was higher for the pH 4.0 emulsion than for the pH 7.0 emulsion, even though the particle sizes of the emulsions (Figure 5) were similar. The higher surface protein coverage at pH 4.0 could have been due to dimerization as well as increased protein−protein interactions that occur between the granule proteins at pH values <7.0 (Burley and Cook, 1962; Kamat et al., 1973). After 6 h of mixing, the surface protein coverage of the pH 4.0 emulsion was less than that of the pH 7.0 emulsion, a result which may be due to the higher surface area (lower droplet size, Figure 5) of the pH 4.0 emulsion. It has been pointed out above that the initial increase in particle size is probably as a result of bridging flocculation by the adsorbing granule lipoproteins; therefore, the decrease in surface area increases the protein concentration per unit area. However, as time progressed and the flocculated emulsion at pH 4.0 was converted into a dispersed emulsion, the surface area increased. Since there was no net increment in the ratio of lipoproteins adsorbed after 2 h (Figure 4), the increased surface area at pH 4.0 would result in a decrease in the amount of protein bound per unit area when compared to pH 7.0, at which there was little change in surface protein coverage because of minimal change in surface area of the emulsion.

CONCLUSION

Protein solutions containing mixtures of granule proteins and added pure phosvitin had better emulsifying properties at pH 7.0 and 9.0 than at pH 4.0. Emulsions containing 100% pure phosvitin had more proteins at the interface at pH 4.0 than at pH 7.0 and 9.0, suggesting that the lower charge density at pH 4.0 allows for greater protein packing at the interface. Electrophoretic analysis of the interfacial protein film showed that even at high weight ratios of added pure phosvitin, the granule lipoproteins were adsorbed more than the phosvitin, which indicates that the more hydrophilic phosvitin was less surface active than the more hydrophobic lipoproteins. In time-dependent studies of the competitive adsorption, more phosvitin was displaced at pH 7.0 by lipoproteins from the interface of emulsions containing only phosvitin than at pH 4.0. This shows that the reduced charge density on phosvitin molecules at pH 4.0 enabled formation of a stronger protein film that interacts more with the oil droplets than at pH 7.0, at which increased electrostatic repulsions reduce the strength of the adsorbed protein films.

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