Interactions between Milk Serum Proteins and Synthetic Fat Globule Membrane during Heating of Homogenized Whole Milk

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Interaction of serum proteins with the fat globule membranes (FGM) in homogenized whole milk affects the properties of a number of dairy products. The reactions of synthetic FGM in homogenized heated milk were investigated by quantitative analysis of the fat globules using electrophoresis and densitometry. The results indicated that only caseins constituted the newly formed surface of synthetic fat globules after homogenization. The serum proteins (β-lactoglobulin and α-lactalbumin) adsorbed to the FGM only when the fat globules had been heated above 70 °C. The α_{s2}- and κ-caseins and the serum proteins were only slightly dissociated when fat globules were washed in dissociating buffer solutions containing urea and EDTA, confirming that the denatured serum proteins attached to cysteine-containing caseins on FGM. The kinetics of deposition of β-lactoglobulin and α-lactalbumin on the FGM during heating of homogenized milk at 75 °C followed a pseudo-first-order reaction, when considered in terms of vacant "sites" on the fat globule surface. At lower temperatures, kinetics of deposition of the two proteins were similar.

INTRODUCTION

Homogenization of whole milk causes major physical changes in the size and structure of the fat globules. During homogenization, the natural fat globules (diameter 1–10 μm), are disrupted into small globules (diameter <1 μm) and therefore the surface area of the fat is increased by more than 10-fold. Although the original milk fat globule membrane remains on the fat globules (Keenan *et al.*, 1983), it is inadequate to cover the newly formed fat surface. The surface active proteins, especially caseins, either as semi-intact micelles or as micellar fragments, cover the newly formed surface of the fat globules, and this protects the fat from coalescing (Walstra and Oortwijn, 1982). This process alters the properties both of the fat globules and of the casein micelles; for example, the rennet coagulation of milk is considerably changed (Walstra, 1980; Peters, 1964). Heating of homogenized milk changes both physical and chemical properties in the milk fat globule membrane and serum proteins due to their interaction with each other (Van Boekel and Walstra, 1989). Both heating and homogenization affect the renneting properties of milk (Robson and Dalgleish, 1984; Sweetsur and Muir, 1983). Processing of such heated milk into products such as cheese may cause changes in such properties as meltability, flowability, and other textural characteristics (Banks, 1990).

The interactions between fat globule membranes (both natural and synthetic) and milk proteins (caseins and serum proteins) during heating have not yet been fully understood. In whole milk, it has been shown (Dalgleish and Banks, 1991; Houlihan *et al.*, 1992*a*) that β-lactoglobulin (β-lg) and α-lactalbumin (α-la) both interact with the fat globules when whole milk is heated. However, the details of changes in synthetic fat globule membranes (FGM) when homogenized milk is heated have not been intensively studied, although it has been assumed that interactions between synthetic fat globules and serum proteins occur, similar to those which are known to take place when skim milk is heated (Dalgleish, 1990; Dannenberg and Kessler, 1988a,b). These interactions occur via the formation of disulfide groups between the denatured whey proteins and the caseins, especially κ-casein (McKenzie *et al.*, 1971; Jang and Swaisgood, 1990; Parris *et al.*, 1990). We therefore have attempted to investigate the mechanism of interactions between milk serum proteins and synthetic FGM by (i) determining the types and amounts of milk proteins which adsorb to the surface of fat globules in homogenized milk before and after heating and (ii) measuring some kinetics of the interactions of β-lg and α-la with the synthetic FGM.

MATERIALS AND METHODS

Fresh whole cow's milk was obtained from the Elora Dairy Research Station of the University of Guelph. A small portion (200 mL) of whole milk was homogenized at a temperature of 40 °C using a Microfluidizer (Microfluidics Corp., Newton, MA) with an input pressure of 0.3 MPa, which corresponds to a homogenization pressure of 42 MPa. Microfluidization is analogous to homogenization but produces smaller particles (Pouliot *et al.*, 1991). This increases the surface area of the fat which is accessible to the milk proteins but does not otherwise greatly alter the properties of the homogenized milk, compared with products from a valve homogenizer. Because it maximizes the amount of protein available for reaction, the technique is ideal for the study of the interactions involving adsorbed proteins. The milk samples were passed through the homogenizer three times to ensure proper homogenization with no coalescence of fat globules. The milk was not pasteurized before homogenization, and this may explain some differences between this study and some others (see below).

The homogenized milk samples (12 mL) were immediately heated in glass test tubes immersed in a water bath at temperatures ranging from 65 to 90 °C for different times after specific temperature had been attained (coming-up time of 1.75 min). The milk samples were then cooled immediately to room temperature in an ice bath. To analyze the proteins associated with the fat globules, the milk was centrifuged at 60000*g* for 20 min at 25 °C. The serum portion was discarded carefully, and the cream layer was washed by resuspending in one of three solutions (see below) for 30 min at 32 °C. The resuspended cream was recentrifuged under the same conditions, and the fat layer was collected and spread on a filter paper (Whatman No. 1) to allow the serum portion to drain out completely. This method of collecting the fat globules ensured that as much as possible of the original protein coat was retained in the samples, thereby

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avoiding problems of trying to separate the adsorbed protein from the fat globules, which may not give quantitative results. The draining of the centrifuged cream layer ensures the removal of residual serum, allowing the accurate weighing of aliquots of the fat for subsequent treatment with SDS.

To preserve the original coating of casein micelles and micellar fragments on the fat globules, they were washed in a buffer containing 5 mM $CaCl_2$, 50 mM NaCl, and 20 mM imidazole, pH 7.0. To try to remove parts of the casein layer, the particles were washed in a solution of EDTA buffer that contained 10 mM EDTA, 50 mM NaCl, and 20 mM imidazole, pH 7.0, and to dissociate the adsorbed casein micellar layers as much as possible, the particles were washed in a buffer containing 6 M urea, 5 mM EDTA, and 20 mM imidazole.

Weighed quantities of the washed and drained fat samples (about 0.025 g) were suspended in 0.15 mL of a buffer (10 mM Tris, 1 mM EDTA, and 20 mM imidazole, pH 8.0). The samples were then heated for 5 min in a boiling water bath after 0.25 mL of a 20% solution of sodium dodecyl sulfate, 0.1 mL of 2-mercaptoethanol, and 0.1 mL of a 0.05% solution of bromophenol blue were added. This treatment achieves two objectives: the first is the displacement of the protein from the fat by the detergent, which causes the oil droplets to coalesce into a layer which has a much smaller surface area than the original emulsified fat. Several studies have demonstrated that the SDS displaces adsorbed proteins completely from the oil–water interface (de Feijter *et al.*, 1987; Dickinson and Tanai, 1992). Second, the SDS dissociates the desorbed protein complexes and binds to the proteins as is normal in SDS treatment. The treated samples therefore contained all of the protein which was associated with the fat, in a form suitable for SDS–PAGE electrophoresis. Aliquots (1 μL) of the treated samples were loaded onto a 20% homogeneous Phastgel (Pharmacia LKB Ltd., Baie d'Urfé, Québec, Canada), which was run as described elsewhere (Dalgleish and Banks, 1991). The protein bands were stained using Coomassie Blue indicator, and the stained gels were scanned the next day at 633 nm using an Ultroscan densitometer (Pharmacia LKB).

The quantification of the β-lg and α-la was carried out by using their respective standard curves. Standard samples of purified β-lg and α-la (Sigma Chemical Co., St. Louis, MO) were run on the electrophoresis gels in various amounts in the range 15–300 μg. A satisfactory linear plot (regression coefficient = 0.99) obtained between the integrated peak areas and the sample concentration was then used to quantify the serum proteins in the samples derived from the FGM.

The sizes of the fat globules and their specific surface area in homogenized milk were determined using a Mastersizer X (Malvern Instruments Ltd., Malvern, England). The presentation factor for the Mastersizer was 0303 (i.e., refractive index 1.41 and absorption 0.001). In all of the experiments the average diameters (d_{32}) of the fat globules as calculated according to this method were in the range 0.30–0.35 μm. The amounts of serum proteins deposited per unit area of the FGM were calculated by dividing the amount of protein per gram of fat by the specific surface area of the fat as calculated from the size distribution measured by the Mastersizer. The interfacial area was approximately constant in all of the experiments, since the milks were always homogenized under identical conditions.

The data were analyzed statistically using a General Linear Model and Duncan's means tests (SAS, 1988). The kinetic models for the deposition of β-lactoglobulin and α-lactalbumin on the fat globule membrane were fitted using a Gauss–Newton method of the NLIN procedure (SAS, 1988).

RESULTS AND DISCUSSION

Casein Composition of Synthetic Fat Globule Membrane. The scanning patterns of the electrophoresis gels of homogenized milk fat indicated that the milk protein, especially casein, covered the newly formed surface of the fat globules (Figure 1a). Neither of the serum proteins β-lg or α-la could be detected in the electrophoresis patterns, and it must, therefore, be assumed that they do not adsorb to the surfaces of the fat under the experimental conditions used. This result was true no matter which treatment was used to wash the fat globules from the unheated milk. These observations differ from those of Oortwijn and Walstra (1979) and Walstra and Jenness (1984), who claimed that the serum proteins form part of the synthetic FGM. McPherson *et al.* (1984) found small amounts of β-lg in the fat globule membranes of homogenized milks, but the milks had also been pasteurized, and the β-lg may therefore have been incorporated during the heating stage. Our results suggest that undenatured β-lg does not adsorb to the interface and interacts only when thermal denaturation has occurred (McKenzie et al., 1972). In addition, serum proteins are incapable of displacing casein from the fat–water interface (Dalgleish et al., 1991). On the contrary, caseins are most likely to displace serum proteins (Walstra and Oortwijn, 1982).

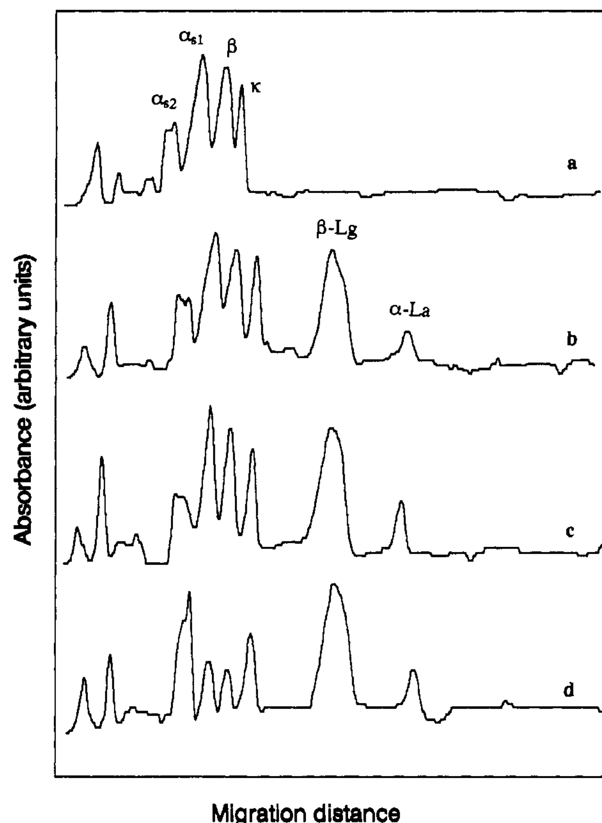

Figure 1. Scanning patterns of the protein bands on an SDS–PAGE Phastgel, for fat globules isolated from homogenized milk. The major casein and serum protein bands are identified. (a) Unheated homogenized milk; (b) homogenized milk heated at 90 °C for 2 min and washed in buffer containing calcium; (c) homogenized milk heated at 90 °C for 2 min and washed in buffer containing EDTA; (d) homogenized milk heated at 90 °C for 2 min and washed in buffer containing urea and EDTA.

When homogenized milk was heated at temperatures between 65 and 90 °C for 1–5 min, there was no further increase in the amount of casein deposited on the fat surfaces, although there was deposition of serum proteins (Figure 1b). McPherson and Kitchen (1983) have reported that a κ-casein/β-lg complex is formed on the natural fat globule surface during heating and that this will increase the amount of κ-casein associated with the fat globules, but our results did not support this hypothesis. The amounts of the different casein fractions (α_s, β, and κ) which remained when the fat samples were washed with EDTA and urea/EDTA buffers were unaffected by heating (Figure 2), although the amounts of the caseins were changed by the washing procedures (Figure 1c,d). This suggested that although homogenization caused the dis-

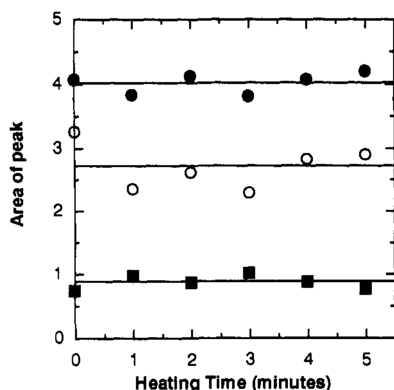

Figure 2. Amount of α_{s1}-casein adsorbed on the synthetic fat globule membrane in homogenized whole milk when heated at 90 °C for 1–5 min. The fat globules were washed in three types of buffers: containing either calcium chloride (solid circles), EDTA (open circles), or urea/EDTA (solid squares).

ruption of casein micelles, there seemed to be no further change in the composition of the adsorbed caseins in the temperature range of 65–90 °C. Although heating should increase the amount of colloidal calcium phosphate in casein micelles (Kannan and Jenness, 1961), this seemed to have no effect on the extent of dissociation of casein fractions by EDTA and urea buffers. Original membrane proteins remained associated with the fat globules: particularly after homogenization, an additional band was observed in the electrophoresis, overlapping with α_{s2}-casein, and there were also bands of lower mobility (Figure 1a). Quantification of the combined α_{s2}-casein band indicated that the material was not dissociated from the fat globules either by EDTA or by urea/EDTA treatment. For reasons which are at present unexplained, heating and urea or EDTA treatment caused changes in these minor bands, so that there was an increase in the band overlapping the α_{s2}-casein. Although this band made quantification of the α_{s2}-casein difficult, it was clear that the casein was still present in the composite band.

The dissociation of different casein fractions by washing of the homogenized fat globules was studied in detail. There was a significant dissociation ($P < 0.05$) of α_{s1}- and β-caseins when the fat layer was washed in EDTA and urea/EDTA buffers (Table I and Figure 1c,d). The amount of α_{s1}-casein decreased by between 10 and 27% when the fat samples were washed with EDTA buffer (Figure 2). With urea/EDTA treatment, the dissociation was greater, with about 80% of the α_{s1}-casein being lost from the fat globules (Figure 2). Similarly, between 18 and 42% of the β-casein was dissociated by EDTA buffer and about 78% by urea/EDTA buffer. The other fractions, α_{s2}- and κ-casein, dissociated very much less in either of the dissociating buffers (Table I). In EDTA, dissociation of κ-casein ranged from 6 to 11% and from 15 to 25% in urea/EDTA buffer. Similarly, dissociation of α_{s2}-casein was only 3–5% in EDTA buffer and nil in EDTA/urea buffer. For α_{s2}- and κ-caseins, the differences between dissociation in EDTA and urea/EDTA buffers were not statistically significant at 95% confidence level (Table I). Proteins adsorbed directly to the interface will not be removed when the globules are treated with either EDTA or urea/EDTA: only the proteins present in the residual micellar framework would be expected to dissociate. Therefore, the observation that the κ- and α_{s2}-caseins remain associated with the fat suggests that the micelles adsorb to the fat surface through these proteins. This conclusion is rather surprising and requires further study. Whatever the reason, it is clear that the cysteine-containing caseins make up much (although not all) of the adsorbed casein when the adsorbed micelles are dissociated.

Similar results were observed for unheated homogenized milk samples in dissociating buffers (Table I), where the dissociation of α_{s1}- and β-casein averaged about 40% in EDTA and about 75% in urea/EDTA buffer. Again, both α_{s2}- and κ-caseins resisted much dissociation in these two buffers. Thus, the heating of homogenized milk within the specified range does not affect the dissociation of the casein micelles adsorbed to FGM, although serum proteins bind to the caseins when the milk is heated (see next section).

Interaction with Serum Proteins. When homogenized milk was heated at 65 °C, neither β-lg nor α-la appeared to be sufficiently denatured to interact with the synthetic fat globules, since no bands corresponding to these proteins were seen on the electrophoresis gel. At 70 °C, deposition of β-lg was detected by the appearance of bands in the SDS–PAGE, as in Figure 1b, and it increased with heating time to about 3.7 mg/g fat or 0.17 mg/m^2 fat surface area (FSA) after 10 min of heating. Dalgleish and Banks (1991) also noted the deposition of β-lg on natural fat globules at 70 °C while heating whole milk. At 72.5 and 75 °C the amount of deposited β-lg increased steadily to 0.19 and 0.25 mg/m^2 FSA, respectively, in a 10-min heating period.

The amount of β-lg deposited on FGM was not significantly affected ($P > 0.05$) when the fat globules from homogenized milk were washed either in EDTA or in urea/EDTA buffer. For homogenized milk heated at 80, 85, and 90 °C and suspended in calcium or EDTA buffers, the differences in the amount of β-lg were +3.3, −3.9, and −12%, respectively. For milk heated at 90 °C, only 10% of the β-lg was lost from the fat globules when they were treated with urea/EDTA buffer. These overall results indicated that the amount of β-lg associated with the fat globules was only slightly affected when the fat was washed with buffer which dissociates the casein micelles (cf. Figure 1, parts b and d). It has been shown above that κ-casein also resists dissociation by these treatments. This strongly suggests that, as would be predicted from the behavior of the proteins in skim milk, the major binding site of the β-lg is on κ-casein. In addition, it is known that about 10% of the surface of homogenized fat globules is still covered by their natural membranes (Keenan *et al.*, 1983), and it is possible for serum proteins to interact with natural membrane proteins which may be denatured during heating but which are not dissociated from the fat surface by urea/EDTA buffer (Houlihan *et al.*, 1992b).

Although a thermal transition of α-la takes place at a lower temperature (55 °C) (Ruegg *et al.*, 1977) compared to β-lg (68 °C) (De Wit and Swinkels, 1980), the deposition of α-la onto the homogenized fat globules followed a similar kinetic curve to β-lg during heating at 70 or 75 °C. As observed in the case of β-lg, there was no significant difference in the amount of α-la ($P > 0.05$) when the fat was washed with EDTA or urea/EDTA buffer. At 70 and 75 °C, the kinetics of α-la deposition on FGM followed the same type of pseudo-first-order reaction as β-lg (Figure 3). The absolute amount of α-la deposited was less than the amount of β-lg, the ratio being about 0.6: this is in fact higher than the ratio of the two proteins in milk and suggests that α-la may bind more effectively than β-lg. This is clearly different from previously published results (Dalgleish and Banks, 1991), where it has been shown that with casein micelles and natural fat globules the α-la binds less well than β-lg.

Table I. Duncan's Means Test for Various Casein Fractions and Serum Proteins Obtained from Fat Globules Which Were Washed in Different Buffers[a]

buffer	α_{s1}	α_{s2}	β	κ	β-lg	α-la
homogenized heated milk						
calcium	4.38 a	2.20 a	3.56 a	2.00 a	3.91 a	0.92 a
EDTA	3.47 b	2.16 a	2.74 b	1.77 b	3.60 b	0.81 a
urea/EDTA	0.85 c	2.26 a	0.62 c	1.40 b		
homogenized unheated milk						
calcium	5.20 a	2.10 a	3.61 a	1.99 a		
EDTA	3.05 b	1.76 a	2.27 b	1.47 b		
EDTA/urea	1.18 c	2.28 a	0.98 c	1.97 a		

[a] The data are the averages of four temperature treatments (65, 80, 85, and 90 °C) except in samples washed in urea/EDTA buffer, where it was for two treatments (65 and 90 °C). The data are shown as the integrated area of the different peaks of proteins obtained by scanning of gel electrophoresis, absorbance units/g of fat. Means with the same letters are not significantly different ($P > 0.05$) within each column.

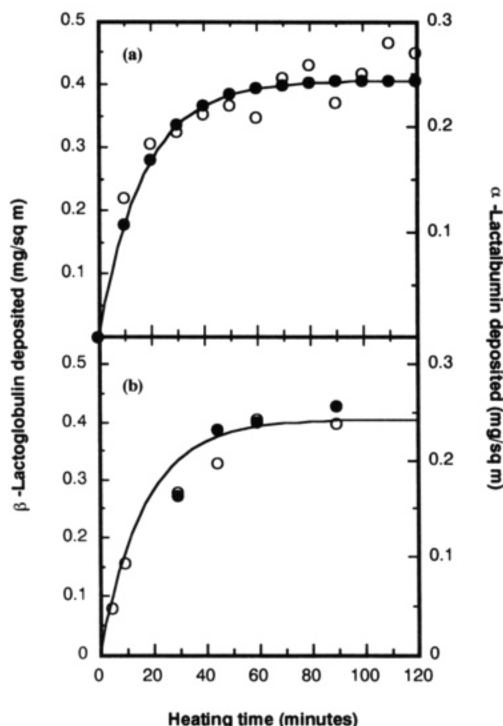

Figure 3. Kinetics of β-lg and α-la deposition on fat globule membranes in homogenized milk heated at 75 (a) and 70 °C (b) for 0–120 min. The kinetics followed a pseudo-first-order reaction as shown by the predicted lines which closely followed the experimental data; the predicted lines are the same in the two graphs. Note the scales for the two proteins differ. Solid points, α-la; open points, β-lg.

To confirm that the results were not the result of specific sampling of the fat globule distribution, an experiment was carried out to determine the difference in the amount of β-lg deposited on the fraction of fat globules obtained by centrifugation at 60000*g* and on the overall fat globules obtained by dissolving 10 mL of homogenized milk in 6 M urea. The results showed that there was no difference in the relative amounts of β-lg in the two sets of globules, i.e., that the samples from centrifugation were representative of the whole distribution.

Kinetics of the Reaction. The amount of fat collected during centrifugation was substantially higher in urea/EDTA buffer because of the high density difference between fat and 6 M urea buffer solution and because the dense casein micelles have been dissociated from the fat. Since urea/EDTA buffer does not dissociate the whey proteins associated with the fat globules, as shown in the preceding sections, we used this buffer to wash the fat samples when determining the kinetics of deposition of β-lg and α-la on FGM.

The kinetics of serum protein deposition on FGM at 70 and 75 °C is shown in Figure 3. The results could be fitted

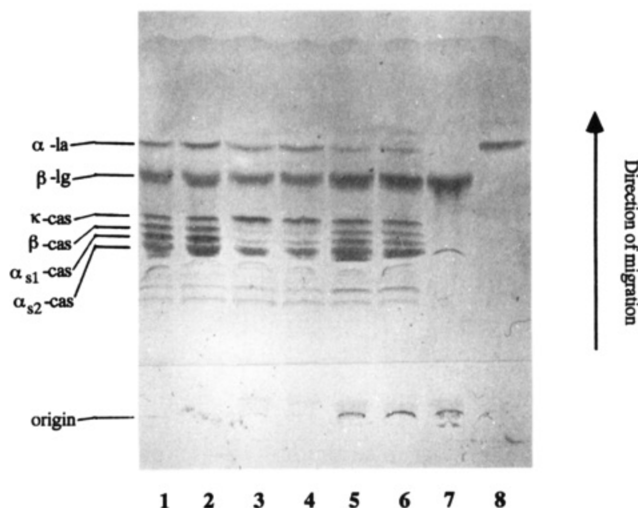

Figure 4. SDS–PAGE electrophoresis of the proteins from heated homogenized milks. (Lanes 1 and 2) Milk heated at 70 °C for 30 and 45 min, washed with Ca buffer; (lanes 3 and 4) milk heated at 75 °C for 20 and 30 min, washed with EDTA/urea; (lanes 5 and 6) milk heated at 90 °C for 1 and 2 min, washed with urea/EDTA; (lane 7) β-lactoglobulin; (lane 8) α-lactalbumin.

by a pseudo-first-order model for the filling of vacant sites on the homogenized fat surface

$$[\beta] = [\beta](\infty)\{1 - \exp(-kt)\} \quad (1)$$

where $[\beta]$ is the amount of β-lg deposited on FGM at time t, mg/m^2 FSA; $[\beta](\infty)$ is the saturating amount of β-lg on FGM, mg/m^2 FSA; and k is the apparent reaction rate constant. The calculated fit (Figure 3) gave an apparent reaction rate constant (k) of 0.058/min at 75 °C, and a similar value was found at 70 °C. No information is available in the literature on the apparent order of reaction (or of the rate) for deposition of serum proteins on homogenized FGM during heating. Published studies on heated milk model systems have shown no general agreement on the order of the denaturation reaction of β-lg. Many researchers found the reaction to be of first order (Dalgleish, 1990; Luf, 1988; De Wit and Swinkels, 1980), whereas others reported it to be of second order (Manji and Kakuda, 1986; Hillier and Lyster, 1979; Lyster, 1970). In another case, Dannenberg and Kessler (1988a,b) reported that a reaction order of 1.5 was best suited to their results. In the experiments described in this paper, the rate of deposition is being measured directly, and this is a process involving several reactions, not simply the denaturation of the serum proteins.

At 75 °C, the deposition of β-lg seems to be complete after 80 min of heating with 0.46–0.55 mg/m^2 FSA. As expected, the rate of β-lg deposition was faster above 75 °C. At 80 and 85 °C, the depositions reached 0.37 and 0.41 mg/m^2 FSA, respectively, after the milk was heated

for 5 min, and at 90 °C, the deposition reached its maximum within 2 min and gave value in the range 0.56–0.69 mg/m^2 FSA. These results indicated that the kinetics of the binding of β-lg to FGM were highly temperature dependent. Darling and Butcher (1978) obtained increased electrophoretic mobility of the fat globules when cream was heated, and this was attributed to the deposition of serum proteins on FGM. However, no further information is available on the load of serum proteins on synthetic fat globule membranes during heating.

Lyster (1970) has reported that the denaturation of α-la followed a first-order reaction but that it is slow at 70 and 75 °C compared with that of β-lg. As has just been shown, this is not true for the binding to synthetic fat globules at these low temperatures. However, at 90 °C, there appears to be a difference in the mechanism; there, the deposition of α-la became once again slower than that of β-lg. Figure 4 shows this clearly: the intensities of the bands of α-la are quite strong in the electrophoresis of globules heated at 70 and 75 °C, whereas the α-la band at 90 °C is much less heavy in comparison with the β-lg band. Using the present equipment, it was not possible to perform kinetic studies at 90 °C because the reaction was too fast, so it is not possible to quote rate constants for the binding. However, it seems evident that there are very different reactions at the higher and lower temperatures.

Conclusions. The results confirm much of what is expected when homogenized milk is heated, in that we have shown that the denatured whey proteins interact with the fat globules, as they do both with native fat globules and with casein micelles. Since the fat globule surfaces in homogenized milk are covered by partially disrupted casein micelles (Buchheim, 1985), it is only to be expected that β-lg and α-la will bind to them during heating. The observation that the serum proteins and the κ- and α_{s2}-caseins remain on the fat globule surface after the other caseins have been dissociated confirms that the serum proteins attach to the cysteine-containing caseins. There is clear evidence of the involvement of α_{s2}-casein, which presumably has interacted with κ-casein or whey proteins or both via the formation of disulfide bonds.

A major unresolved problem arises from the dissociation experiments. From electron microscopy (Oortwijn *et al.*, 1977), it looks as if much of the surface of the adsorbed casein micelles ought to be intact, in which case much of the κ-casein would not be associated directly with the fat surface. On this basis, it would be expected that the dissociation of the adsorbed micelles would cause a decrease in the amount of κ-casein associated with the fat globules. As has been shown, this is not the case, since nearly all of the κ- and α_{s2}-caseins, and serum proteins remain on the fat surface. The implications of this for the structure of the interfacial material are not clear, but one possibility is that heating and adsorption of denatured serum proteins cause a structural rearrangement in the adsorbed micellar fragments. This may possibly be used to create or change the functionality of the particles in homogenized milk.

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