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OBSERVATIONS ON THE AIR-SERUM INTERFACE OF MILK FOAMS

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Abstract

A new rapid method for the preparation of milk foams for transmission electron microscopy is described. The air-serum interface of foams made from skimmed milk consists of a uniform electron dense layer (5 nm thick) to which casein micelles become secondarily attached. Changes in bubble volume lead to the formation of folds of excess interfacial material which project into the aqueous phase. Using collapsed bubble ghosts to study the attachment of micelles to the air-serum interface it was concluded that neither disulphide bridge formation nor hydrophobic interactions were of major importance. Similar preparations of interfacial material but without casein micelles attached were prepared from milk plasma and solutions of β- lactoglobulin. The former fragmented slowly into small particles at room temperature but very rapidly when heated to 55° C whereas material derived from β- lactoglobulin was quite stable. The destruction of bubble ghosts in skimmed milk by heating is attributed to interface breakdown rather than to micelle detachment. The air-serum interface, of which casein micelles do not form an integral part, probably consists of a mixture of globular whey proteins and some soluble caseins. Thus, using high pressure liquid chromatography, foamed milk plasma from which bubble ghosts had been removed was shown to be depleted in both α- lactalbumin and β- lactoglobulin.

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Introduction

It is well established that solutions of many different types of proteins readily foam when they are whipped or shaken. During the formation of a foam, protein molecules become adsorbed at the air-liquid interface of each bubble where they may suffer conformational changes, undergo intermolecular rearrangement and form a stabilising film (Alexander and Johnson, 1949). Foamability of a solution is related to the rapidity with which this protein film is formed at the surface of each air bubble whereas foam stability is determined very largely by the rheological properties of the interface (Graham and Phillips, 1976). Although individual milk proteins have been used to elucidate the mechanisms by which adsorbed protein layers at air-water interfaces are able to stabilize foams (Graham and Phillips, 1976; Halling, 1981), the very existence of such an interfacial layer in the case of milk foams has never been demonstrated directly using electron microscopy. The structure and properties of this interface are of importance in determining the foamability and stability not only of milk foams but also perhaps of fat-rich foams such as whipped cream and ice cream in the early stages of their formation. Thus, the observations of Schmidt and vanHooydonk (1980) on whipped cream suggest that for fat globules to stabilize air bubbles they must penetrate and partially replace a film-like air-serum interface which is probably formed by the adsorption of proteins early in the whipping process.

The virtues of using freeze fracturing methods with scanning and transmission electron microscopy in the study of foams, especially fat-rich foams such as ice cream and whipped cream, have been extolled by several authors (Berger et al., 1972; Buchheim, 1978; Schmidt and vanHooydonk, 1980; Kalab, 1983). Whilst it is true that such methods are very valuable in preventing the extraction of fat during processing, their advantage over conventional sectioning methods for the examination of protein foams is less convincing; the resolution of the scanning electron microscope can be too limiting and the featureless but distinct interfacial layers between air and aqueous phases can be difficult to detect in the

metal replicas produced by freeze fracturing methods for transmission electron microscopy. However, when using conventional sectioning methods the initial fixation of foams is a problem. With robust protein foams containing a relatively large concentration of protein e.g., egg albumen foam, fixation prior to embedding can be performed by direct addition to an aqueous fixative (Meyer and Potter, 1975); in the case of the more fragile milk foams, even fat-rich foams, more care is required. In the present study, the vapour fixation method of Graf and Muller (1965) for whipped cream was adapted for fat-free milk foams to give a relatively rapid procedure.

Materials and Methods

The skimmed milk used was: a) bulk tank milk from the Institute's herd of Friesian cows. The milk had been separated by centrifugation at 3,000xg for 10min in the laboratory without any heat treatment and b) separated bulk tank milk which had been pasteurized in the Institute's dairy. All milks were stored at 6°C prior to use.

Milk serum was obtained from pasteurized or unpasteurized skimmed milk by centrifugation at 100,000xg for 2h using a Beckman L8-80 ultracentrifuge fitted with an SW 25 rotor. The milk serum was removed by piercing the side of the centrifuge tube with a syringe.

Aqueous solutions of purified β- lactoglobulin (Sigma Chemical Co.) contained 3 mg/ml.

Foam production

Two different controlled methods were used to produce foams from pasteurized and unpasteurized skimmed milk. In one of these, 10 ml of milk were placed in a 50 ml conical flask held in a Stuart flask shaker and shaken horizontally for 1-2h with a frequency of 40Hz and an amplitude of 10mm.

In another method, 5ml of milk were placed in a filter funnel fitted with a sintered glass filter of diameter 30mm. Air was then passed through the filter at a rate of 0.5 ml/sec for 1-2h to produce the foam.

Foam collection and processing

Samples of foam for electron microscopy were collected 0.5, 1 and 2h after the initiation of foaming. The foam was supported on a wire loop (3.5 mm diameter) consisting of 2 coils of 0.2 mm diameter platinum wire which had been set into the cap of a 5 ml bottle. The wire loop was dipped into the foam, quickly removed and then fixed for 10 min at room temperature in glutaraldehyde vapour. This was achieved by sealing the loop into a 5 ml glass bottle containing 0.5 ml of 25% glutaraldehyde. The foam was transferred on the loop to osmium tetroxide vapour for 0.5-1h at room temperature and then placed in acetone vapour for a further 0.5h to harden the foam. It was then briefly dipped into molten 2% agar at 50°C, allowed to solidify and dipped a second time. From this stage the foams could withstand immersion in liquids. If additional electron density was required, the specimen could be further treated by immersion in buffered 1% osmium tetroxide and aqueous 1% uranyl acetate for 0.5h before being dehydrated in a series of acetone-water mixtures and 100% acetone. The wire loop was removed from the bottle top and the foam was embedded in Araldite. In the final embedding moulds, the foam was carefully dislodged from the wire loop and the resin allowed to polymerize. In order to obtain the maximum number of bubble profiles in any one section, the disc of foam was flat embedded so that sections could be cut parallel to the plane of the original loop.

Thin sections were cut on a Reichert OmU3 ultramicrotome, stained in lead citrate and examined at an accelerating voltage of 100kV in a Hitachi 600 transmission electron microscope.

Preparation of bubble ghosts

The association of casein micelles with the air-water interface of air bubbles was most easily studied using the ghosts resulting from foam collapse. In these studies, foams were produced from pasteurized skimmed milk; a) at room temperature, b) at 6°C and c) at room temperature, but to which 0.2mM p-chloromercuribenzoate (PCMB) or 5mM N-ethylmaleimide (NEM) had been added to block free sulphydryl groups; in treatments b) and c) a control foam was made at room temperature without any additives. The milk containing bubble ghosts together with the collapsing foam was fixed for 1h by addition of sufficient 25% glutaraldehyde to give a final concentration of 3%; milk which had been foamed at 6°C was fixed in the cold using pre-cooled fixative. The milk was mixed in a ratio of 1:1 with 2% agar at 50°C, allowed to solidify and chopped into 0.5mm wide strips with a razor blade. After washing in 0.2M cacodylate-HCl buffer (pH 7.2) for 1h, specimens were postfixed in 0.1M cacodylate buffered 1% osmium tetroxide for a further hour. Specimens were washed in distilled water and stained *en bloc* for 0.5h in aqueous 1% uranyl acetate. They were then dehydrated, embedded and sectioned as described above.

In another study, the stability of the association of casein micelles with the air-serum interface was examined. Bubble ghosts obtained by foaming pasteurized skimmed milk at room temperature were kept at ambient temperature and fixed for electron microscopy at hourly intervals for 4h after foaming had ceased. The stability of the air-serum interface itself was investigated by electron microscopy using bubble ghosts prepared from milk plasma and from solutions of β- lactoglobulin. Collapsed foams were fixed and embedded as described above; a) immediately after preparation, b) after heating in a water bath at 55°C for 10min and c) after storage at room temperature for 24h in the presence of 0.05% sodium azide to stop bacterial growth. Changes in light absorption (at 490nm) of the milk sera were followed using a Cecil CE272 spectrophotometer.

Analysis

The involvement of globular whey proteins in the formation of the air-serum interface was determined indirectly in a depletion study. After the collapse of a milk serum foam, bubble ghosts were removed by centrifugation at 3,000xg for 10 min and the levels of the major whey proteins in the supernatant and in an untreated control serum were compared using high pressure liquid chromatography (HPLC). This was performed using a

TSKG 3000 SW column with a Pye PU 4020 UV detector (operating at 280nm wavelength) and an Altex 100A dual piston pump. 20 μl injection of the samples was used and the column was eluted with 0.1M phosphate buffer (pH 6.8) at a rate of 1 ml/min.

Results and Discussion

Foams

In all foams made from pasteurized and raw skimmed milk, the interface between the air and aqueous phases of each bubble was marked by a uniform layer of electron dense material 5 nm thick (Fig. 1). Casein micelles were attached to the milk serum side of this interface, usually as a discontinuous layer. Since the milk-filled space between two air bubbles (lamella) contained numerous casein micelles it was difficult at first sight to decide whether micelles in contact with the interface were firmly attached to it or whether the contact was transitory. However, during the preparation of some foams for electron microscopy, the milk occupying a proportion of the lamellae drained away and was lost. Because in such cases the micelles remained associated with the interface (Fig. 1 inset), it was concluded that they were truly attached. There was no evidence to suggest that attached micelles were in any way deformed or spread over the air-serum boundary and there was no suggestion that casein submicelles form an integral part of the interfacial layer. Because casein micelles were not in direct contact with air and do not form part of the interface sensu stricto, it is not surprising that their behaviour during foaming differed from that seen in situations where they are truly surface active e.g. , in homogenized milk (Henstra and Schmidt, 1970).

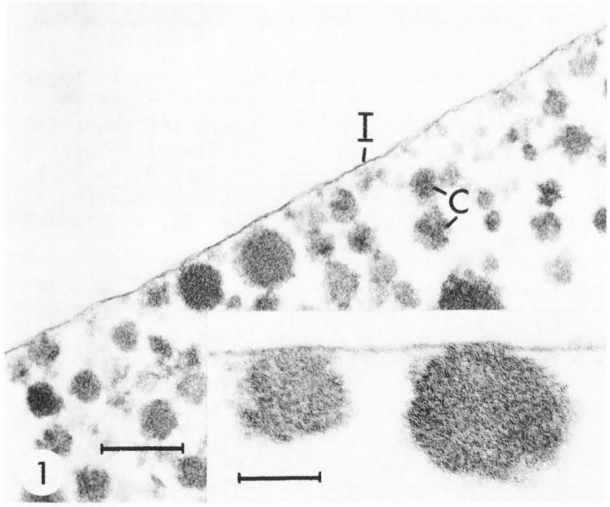


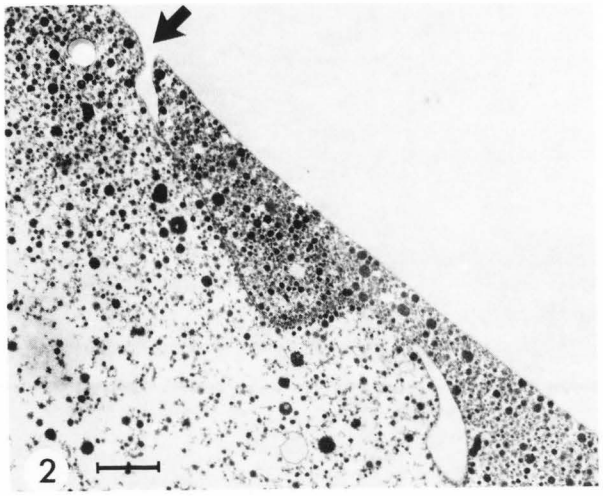
Fig. 1 Air-serum interface (I) of a bubble in pasteurized milk foam. C = casein micelle. Bar = 0.2 μm. **Inset:** Interfacial layer with two attached casein micelles. Free micelles have drained from the lamella. Bar = 0.1 μm.

The presence of a continuous interfacial boundary layer described here poses a difficult problem when considering the changes in bubble surface area that occur during coalescence and the loss of air by diffusion especially from small bubbles. Both of these events result in an excess of the interfacial layer relative to the new surface area of the bubble and it is usually considered necessary to postulate either loss of protein surfactant by desorption or an increase in its surface concentration i.e., the interface thickening. That an entirely different mechanism operates in milk foams is suggested by observations in the present study which showed that the air-serum interface possessed folds projecting beyond the curved surface of the bubble into the aqueous phase, sometimes for several micrometres (Figs 2, 3). Serial sections showed that the folds were sheet-like in form and that although the two interfacial layers were closely apposed over much of their area, there were also dilated zones which still contained some air. The large numbers of such appendages projecting from the surface of air bubbles in milk foams suggest that they accumulate as the foam ages and that they are therefore relatively stable structures. It seems highly likely that the presence of such appendages affects the properties of the foam by influencing both the rate of water drainage from the lamellae and the forces of cohesion between the interfaces of adjacent bubbles. There is every reason to suppose that the same phenomenon of interface folding occurs in other protein foams; thus, micrographs of protein foams obtained by Meyer (1974) show similar folds in the air-protein interface of whipped egg albumen.

Bubble Ghosts

When the air bubbles in milk foams burst, no matter how they were produced, the spherical interfacial layer together with its single layer of attached casein micelles collapsed and generally formed a concavo-convex structure that will be referred to here as a bubble ghost(Fig. 4). These bodies were first described in detail from whole milk by Hekma and Brouwer (1923) who realised that they resulted from the foaming of milk. Indeed, they concluded that separator slime was composed very largely of collapsed foam cells. Mulder and Walstra (1974) demonstrated by electron microscopy that micelles are associated with the surface of collapsed air bubbles and suggested that in skimmed milk foams micelles aggregate into a 2-dimensional network in the air-serum interface but they did not detect the true non-micellar interface. More recently, Hill et al.(1982) showed that ghosts could give rise to falsely high electronic cell counts in milk and concluded that the wall of each ghost consisted solely of several layers of casein micelles. Because the interfacial layer is difficult to detect unless it is sectioned perpendicular to its plane (Fig. 5) or unless the specimen is tilted, it is not surprising that Hill et al. (1982) did not observe it. However, Fig. 4 demonstrates how a single layer of casein micelles can appear to consist of several layers if the section is tangential to the ghost surface.

At least two mechanisms for the attachment



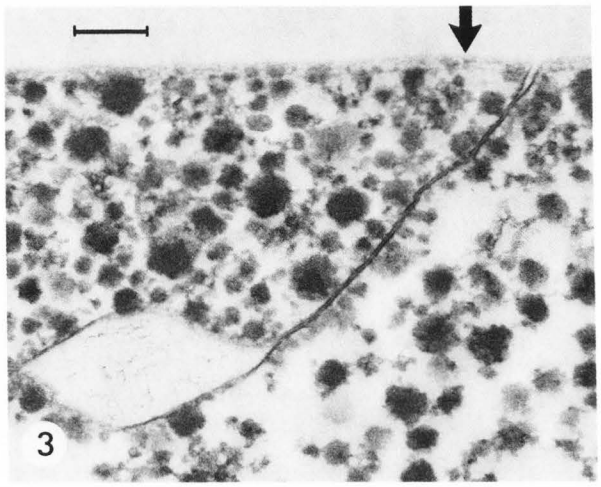
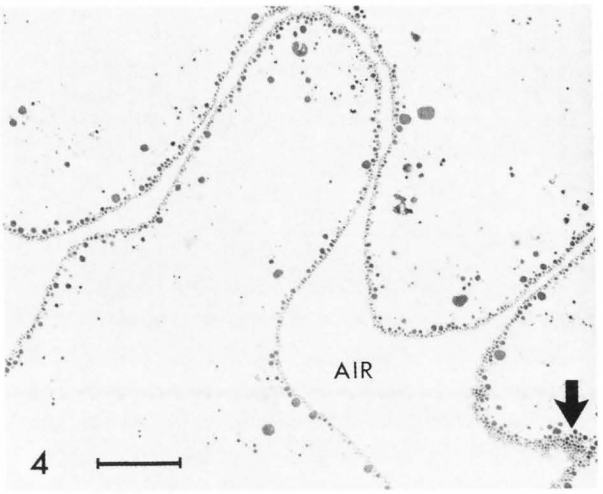


Fig. 2 A fold of the air-serum interface in skimmed milk foam. The origin of the fold is shown by an arrow; the interface of the bubble is sectioned tangentially and so does not appear as a clearly defined structure. Bar = 1 μm.
Fig. 3 Part of a fold in the air-serum interface showing the two closely apposed interfacial layers. The interface proper (arrow) is sectioned tangentially. Bar = 0.2 μm.



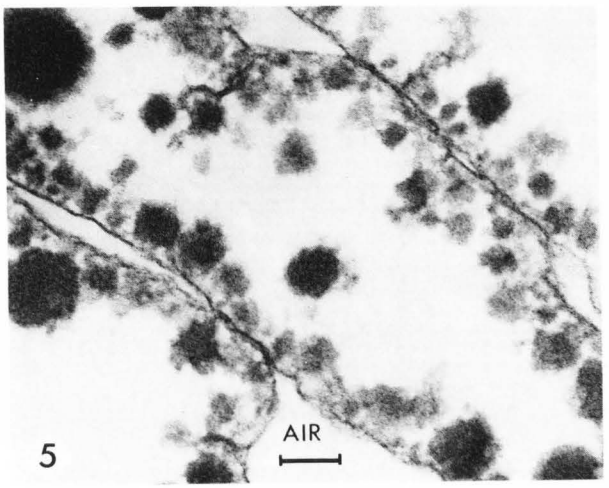
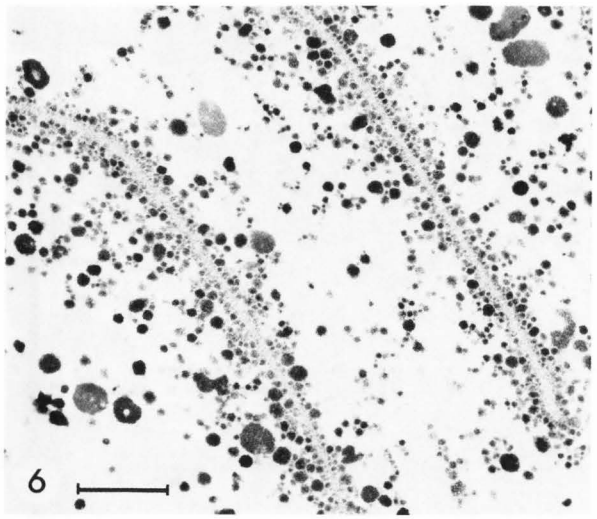


Fig. 4 Bubble ghost consisting of a single layer of casein micelles. Several layers of micelles appear to be present in areas where the ghost is cut tangentially (arrow). The interfacial layer cannot be seen at this magnification. Bar = 2 μm.
Fig. 5 Bubble ghosts showing the attachment of micelles to the interfacial layer. Bar = 0.1 μm.

of casein micelles to the air-serum interface can be postulated. In one of these, attachment is mediated through an intermolecular disulphide bridge arising from the interaction of sulphydryl groups on the κ- casein of the micelle and β- lactoglobulin (see below) in the interface. Although in undenatured β- lactoglobulin the sulphydryl groups are masked (Lyster, 1964), it is possible that they become exposed and therefore highly reactive as a result of the conformational changes that take place during adsorption at the interface (Phillips, 1977; 1981). In the present experiment, milk was foamed in the presence of PCMB or NEM to block sulphydryl groups (Sawyer, 1968; Sawyer et al., 1963) as and when they became exposed at the interface. However, bubble ghosts fixed immediately after foaming were studded with micelles in the normal way and were identical in appearance to those formed from control milk. It appears therefore, that intermolecular disulphide bridging is not of great importance in micelle attachment.

According to unsubstantiated reports by Mulder and Walstra (1974) and Walstra and Jenness (1984), 'ghost membranes' are not found in milk that is foamed at refrigerator temperature. Such a clear temperature dependence for the association of micelles with the interface might indicate an underlying hydrophobic interaction between the two. In the present study however, foams were produced at 6^{o}C on several occasions without



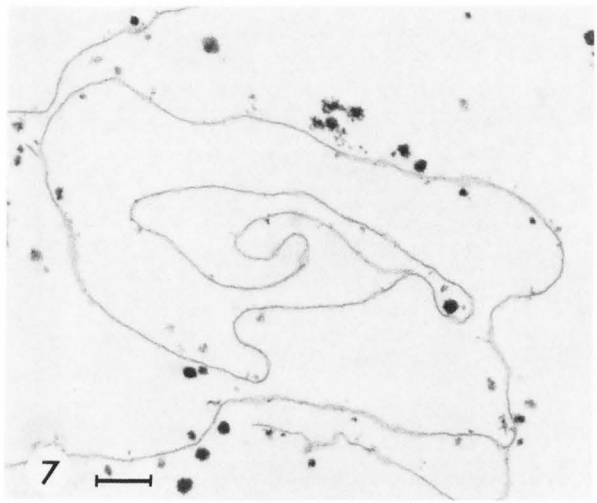
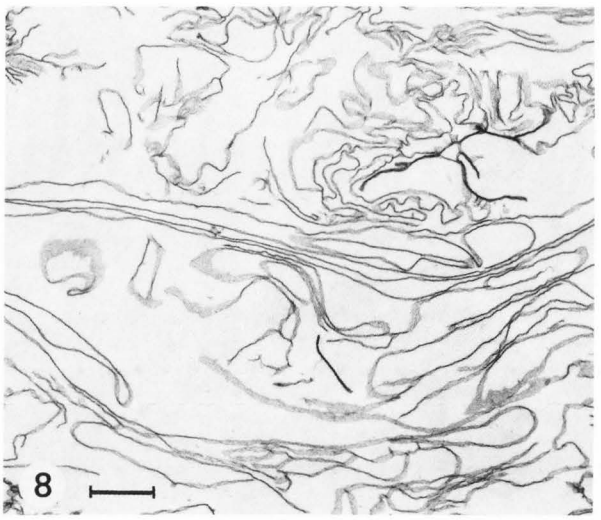


Fig. 6 Bubble ghosts from pasteurized milk foamed at 6°C. Bar = 1 μm.
Fig. 7 Bubble ghost in milk 1h after cessation of foaming showing almost complete dissociation of micelles from the interfacial layer. Bar = 0.5 μm.

observing any decrease in the number of micelles associated with the collapsed interfacial material (Fig. 6). Similarly, in some of the so-called 'adsorptive bubble separation techniques ' where the attachment of non-protein colloids to air bubbles is used to commercial advantage, neither hydrophobic interactions nor electrostatic attraction to the bubble surface appears totally to explain the observed behaviour of the colloid (Melville and Matijevic, 1976). The possible involvement of London-van der Waals forces of attraction in such phenomena (Melville and Matijevic, 1976) remains to be investigated in the case of casein micelles and the air-serum interface. It is interesting to note, moreover, that foaming at low temperature had no effect on the formation or structure of bubble ghosts



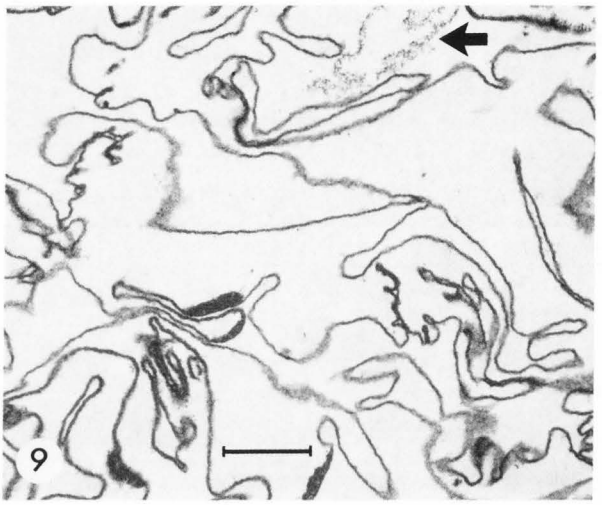


Fig. 8 Centrifugation (3,000xg, 10min) pellet of collapsed bubble ghosts from a foamed solution of β- lactoglobulin. Each dark line consists of two layers of closely apposed interfacial material. Bar = 1 μm.
Fig. 9 Centrifugation (3,000xg, 10min) pellet of bubble ghosts from foamed milk plasma. In some areas (arrow) there is evidence of interface fragmentation. Bar = 1 μm.

produced from milk plasma or solutions of β- lactoglobulin.

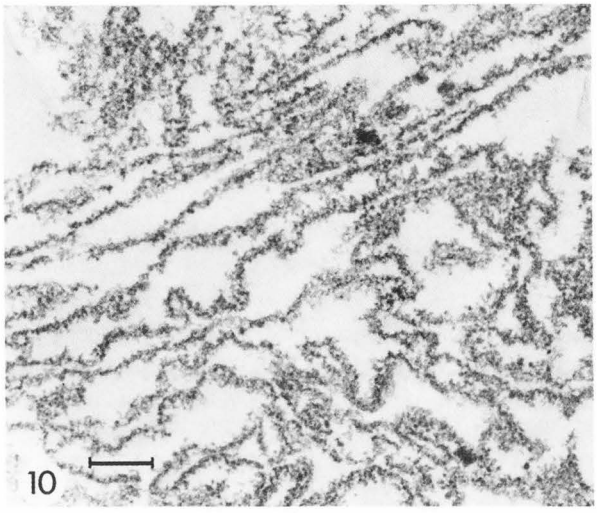
The association of casein micelles with the interfacial layer of bubble ghosts was reversible. Experiments in which bubble ghosts, from pasteurized and unpasteurized skimmed milk, were allowed to age for up to 4h before fixing for electron microscopy showed that the micelles gradually became detached when aged at room temperature (Fig. 7). The percentage of ghosts without micelles or with reduced numbers increased steadily after 1h from 1.4% to 21% at 4h; it is possible therefore that the collapsed interface would

became completely denuded of micelles if stored long enough. Although the loss of micelles can be explained in terms of a simple reversal of the attachment mechanism, the desorption of protein molecules in the interfacial layer to which micelles are irreversibly attached could lead to the same result.

Interfacial layer

Except for the complete absence of casein micelles, the air-serum interface in foams produced from milk plasma is identical to that seen in skimmed milk foams and the collapsing foam produces bubble ghosts. Similarly, when a solution of a single, purified plasma protein, β-lactoglobulin, was foamed by shaking, collapsed bubble ghosts were produced (Fig. 8) and in such profusion that the solution became turbid within a matter of minutes. Whilst it is widely accepted that soluble proteins can be quantitatively precipitated by bubbling air through their solutions (e.g., Alexander and Johnson, 1949), the form of the insoluble protein as collapsed bubbles, as demonstrated here, is less well known.

Since casein micelles in milk foams do not appear to form an integral part of the air-serum interface, milk plasma was used to produce purified preparations of interfacial material in the form of bubble ghosts for experimental purposes and for electron microscopy. The appearance of interfacial material from foamed milk plasma after centrifugation is shown in Fig. 9. Such preparations consisted of numerous compacted bubble ghosts and there were always clear signs that interfacial membranes break down naturally into loose chains of particles (20 - 30 nm diameter) (Fig. 10). Although evidence of interface instability was always found, fragmentation is evidently a slow process at room temperature for even after storage for 24h no more than 10% of the ghosts showed signs of disruption. At higher, but still moderate, temperatures (55°C) however, the interfacial material was very unstable and within minutes broke down into particulate aggregates of protein of very variable size (Fig. 11). It is interesting to note that ghost membranes obtained from β- lactoglobulin foams were stable at this temperature even after extended treatment (1.5h). Interface degradation could be followed by spectrophotometry, for the light scattering properties of the numerous small protein aggregates caused a rapid increase in light absorption measurements (fig. 12) and the suspensions turned visibly more turbid. These observations are relevant to the report by Hill et al. (1982) that bubble ghosts in whole milk are dispersed by heating to 55°C for 10min. The authors supposed that this was because the casein micelles undergo increased Brownian movement as the temperature rises but the present work suggests ,in addition, that the dispersion of casein micelles may be only secondary to the breakdown of the air-serum interface. The temperature instability of milk plasma bubble ghosts suggests that hydrophobic forces between molecules are not predominant in stabilizing the interface and that at least some of the intermolecular forces between proteins are weaker than in interfacial material composed entirely of β- lactoglobulin.



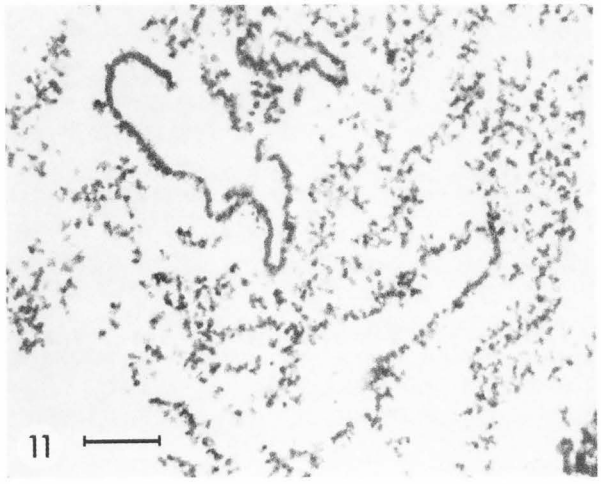
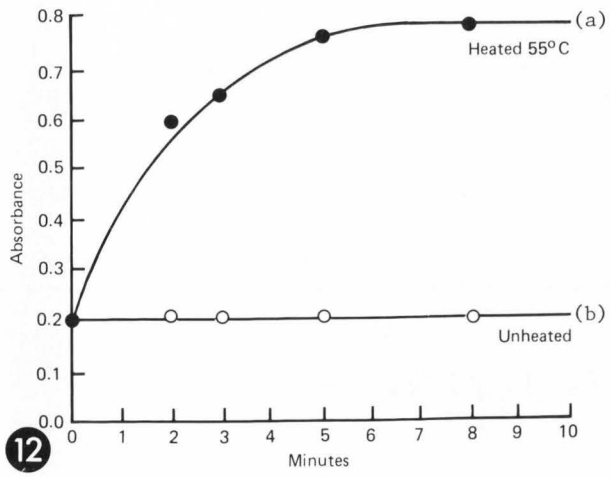


Fig. 10 Same pellet as in Fig. 9 showing a collection of interfacial membranes in the process of fragmentation. Bar = 0.5 μm.
Fig. 11 Same material shown in Fig. 10 which has been heated at 55°C for 3min before fixation. Most of the interfacial material is in the form of small fragments. Bar = 0.5 μm.

Interface Composition

Sharp et al., (1936) found that milk proteins accumulated in skimmed milk foams in approximately the same proportions as they occur in milk. Because of this, they supposed that at the instant of bubble formation, all milk proteins that happened to be at the bubble surface, including casein micelles, were fixed in position to form an interface. However, it is clear from the present study that micellar casein is only secondarily associated with the air-serum interface and that it cannot be regarded as a component of the interface proper. It follows from these observations that the interface may consist of a mixture either solely of the globular whey proteins or together with the soluble caseins. This is consistent with the



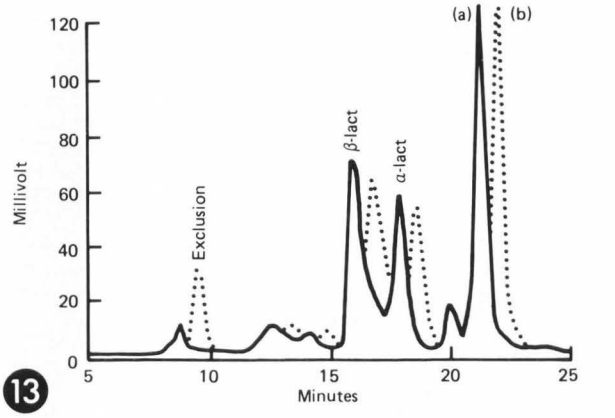


Fig. 12 Change in light absorption (490 nm) of a suspension of bubble ghosts a) heated to 55°C and b) held at room temperature.
Fig. 13 Part of elution profile (at 280 nm) of a) normal milk plasma and b) foamed milk plasma from which bubble ghosts have been removed. The two curves are slightly offset for the sake of clarity. Results obtained by HPLC.

results obtained from a depletion experiment in which levels of α- lactalbumin and β- lactoglobulin were compared in milk plasma and in foamed milk plasma from which interfacial material in the form of collapsed bubble ghosts had been removed by centrifugation. In foamed samples, the levels of both these proteins were consistently diminished by proportionately the same amount (Fig. 13). The increase in size of the exclusion peak (material of M.W. > 120,000) after foaming can be attributed to protein aggregates resulting from the spontaneous breakdown of interfacial material. However, a more complete and direct analysis of interfacial material is required to clarify the possible involvement of the caseins. It should be noted, for example, that since part of the casein, especially the β- casein, is lost from casein micelles at lower temperatures, its opportunity for involvement in interface formation and for influencing the properties of a foam produced in the cold, increases. Although other factors are undoubtedly involved, it is interesting to note that the foamability and stability of milk foams are considerably affected by temperature (Sanmann and Ruehe, 1930; Mohr and Brockmann, 1931).

Acknowledgement

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Discussion with Reviewers

W. Buchheim: Do you have experience with regard to the applicability of this preparation method to fat-rich foams such as dairy or non-dairy whipped creams?

Author: Yes. The method produces equally good results with fat-rich foams but because of the danger of lipid extraction by processing solvents, it is necessary to use osmium tetroxide buffered with imidazole (Allan-Wojtas P, Kalab M. 1984 Michwissenschaft, 39, 323-327) if the fat is to be preserved in situ.

W. Buchheim: On Fig. 6 short chains of associated casein micelles can be seen very frequently. Could this represent a special artifact?

Author: I think that these may represent the remains of disrupted bubble ghosts in which micelles are still held together by remnants of interfacial material.

D. G. Schmidt: Bubble ghosts from milk serum are subject to break down during storage, particularly at higher temperature, whereas ghost membranes from β - lactoglobulin are stable. Should this be ascribed to the incorporation of soluble casein, particularly of β - casein, into the membranes? This would result in "weak" sites in the membrane, possibly due to weaker binding between β - lactoglobulin and β - casein. Please comment.

Author: I very much agree with you. We have experiments in progress to test ideas such as these. Certainly, our preliminary results do suggest the presence of β- casein in the interface. In a single protein solution it is possible to imagine chemical interaction between sulphydryl groups of adjacent β- lactoglobulin molecules on a scale that would not be possible by the addition of β-casein to the system.

D. E. Carpenter: It would seem that direct compositional analyses of the bubble ghosts themselves (or even of the protein in the excluded volume of the chromatogram) would be preferred, rather than the less sensitive difference method which you used. It is interesting to speculate about the nature of the interfacial membranes, especially in terms of the nature of interaction with themselves and degree of denaturation. Would you care to comment?

Author: Direct analysis of interfacial material is now in progress using polyacrylamide gel electrophoresis. Initial results suggest that β- casein is a major component of the interface with smaller amounts of α - casein, β - lactoglobulin and α - lactalbumin. Furthermore, much of the protein is undenatured. The thermal lability of bubble ghosts from skimmed milk suggests that hydrophobic interactions between molecules are not predominant in stabilizing the interface. It is possible that because of changes in the conformation of molecules at the interface reactive groups become exposed and take part in chemical reactions in an entirely unknown way. Such events would be expected to produce stable structures that may be represented by the particulate material remaining at the end of heating to 55°C.

D. E. Carpenter: The similarity of these ghosts to nonnative milk fat globule membranes is interesting, especially in terms of integral and peripheral proteins (micelles).

Author: Although there is a strong morphological similarity between the two, I think it is worth emphasizing that in bubble ghosts produced from skimmed milk the casein micelles do not form an integral part of the interface. This is quite unlike fat/water emulsions where micelles are surface active and contribute to the structure of the interface.

Reviewer I: The author mentioned that the collapsing foam was fixed by addition of glutaraldehyde. Did the author have any idea how fast the foam collapsed? If so, at what decay stage should the foam be fixed in order to obtain the bubble ghosts with best representation?

Author: The rate of foam collapse after the addition of glutaraldehyde was not measured. The foam can of course be fixed and forced to collapse at any stage in its formation. One might, for instance,wish to compare the structure of the interface produced in the very earliest stage of foaming with that in later stages. The foam can be collected at both stages and collapsed by addition of the fixative. My experience has been that structure of the interface is identical no matter when it is collected. This is not to say of course that interface composition will be found to be identical.