

Changes in structure of the bovine milk fat globule membrane on heating whole milk

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(Received 15 April 1991 and accepted for publication 19 December 1991)

SUMMARY. The effects of heat-induced interactions between milk fat globule membrane components and skim milk proteins in whole milk on the structure of the membrane were examined by isopycnic sucrose density gradient centrifugation and by using Triton X-100 as a membrane probe. Skim milk components were incorporated into all the lipoprotein fractions separated by density gradient centrifugation. High density complexes, higher in density than those found in the natural milk fat globule membrane, were formed during the heat treatment. Losses of natural membrane polypeptides from the medium and low density lipoproteins were observed on heating. Heating whole milk also altered the rate of release of membrane components by detergent, with decreases in protein released and an increase in phospholipid constituents released. Studies on washed cream indicated that some of the changes in the membrane on heating whole milk occurred due to the heat treatment alone, independent of the interactions with skim milk proteins.

Heating whole milk (80 °C for 2.5–20 min) has been reported to cause interactions between skim milk proteins (β -lactoglobulin and κ -casein) and milk fat globule membrane (MFGM) components. Such heat treatments result in compositional changes to the membrane system, with the most extensive changes observed after 20 min (Houlihan *et al.* 1992). Isopycnic sucrose density gradient centrifugation and techniques using membrane probes such as detergents have been used to study the structure of the natural MFGM (Kobylka & Carraway, 1972; Kitchen, 1977; Nielsen & Bjerrum, 1977; Freudenstein *et al.* 1979; Kanno & Yamauchi, 1979). In the present investigation, these procedures have been used to examine structural changes induced in the membrane by heating whole milk at 80 °C for 20 min.

MATERIALS AND METHODS

Materials

Fresh raw milk was obtained from a local Friesian herd on three occasions. The milk was cooled (10–15 °C) on the farm and transported in an insulated container to the laboratory.

Sucrose (Aristar grade for density gradients), acrylamide and SDS (especially pure) were purchased from BDH Chemicals Ltd (Poole, Dorset, UK). Triton X-100, Coomassie brilliant blue R (R250) and Tris(hydroxymethyl)aminomethane (Trizma Base grade) (Tris) were obtained from the Sigma Chemical Co. (St Louis, MO 63178, USA). *N,N*-methylenebisacrylamide was purchased from the Eastman Kodak Co. (Rochester, NY, USA). Bovine serum albumin (fraction V), used as the protein standard, was supplied by Calbiochem Corporation Australia Pty Ltd (Sydney, NSW). Reagents for triacylglycerol determination were purchased from Worthington Diagnostic Systems Inc. (Freehold, NJ, USA). All other reagents were of the highest grade available.

Sample treatment

The changes induced in the MFGM by heating whole milk and resuspended washed cream were investigated using one heat treatment of 80 °C for 20 min. Washed cream, prepared as described by Houlihan *et al.* (1992), was resuspended in buffer (10 mM-Tris-HCl buffer, pH 7.5, containing 0.25 M-sucrose and 1 mM-Mg²⁺) to yield a suspension with a fat content similar to that of the original milk. Control samples that had not been heated were also examined. The heating procedure and the method of membrane isolation were described by Houlihan *et al.* (1992).

Isopycnic sucrose density gradient centrifugation

Linear density gradients (18 ml volume) with a density range of 1.018–1.258 g/ml were prepared from 5 and 55% (w/w) sucrose solutions in 10 mM-Tris-HCl buffer, pH 7.5, using an MSE gradient mixer and a peristaltic pump (Pharmacia P3). Membrane material (2 ml, containing 10–15 mg protein in 10 mM-Tris-HCl buffer, pH 7.5) from whole milk or resuspended washed cream was layered carefully on to the gradient surface and the tube was centrifuged (75 000 g_{av} , 3 h, 5 °C) in an MSE PrepSpin 50 centrifuge using a 3 × 25 ml swinging-bucket rotor.

Fractions (1 ml) were recovered using an MSE density gradient displacement stand and a Pharmacia peristaltic pump at a flow rate of 1 ml/min. The fractions were analysed for protein contents and sucrose concentrations using an Abbé-type refractometer. Turbidity readings, which indicated the presence of lipid-containing material in the fractions, were obtained by measuring the absorbance at 450 nm. The fractions were pooled according to the protein and A_{450} profiles.

Detergent release studies

Membrane preparations (2 ml, containing 10–20 mg protein in 10 mM-Tris-HCl buffer, pH 7.5) from whole milk and resuspended washed cream were incubated with a range of Triton X-100 concentrations (0, 0.5, 1.0, 2.0 mg detergent/mg membrane protein) for 1 h at 25 °C. One ml was removed from the individual incubation mixtures for analysis of protein, phospholipid and triacylglycerol contents. Material released by the detergent was isolated in the supernatant by centrifugation (100 000 g_{av} , 1 h, 5 °C; MSE PrepSpin 50 centrifuge) of the incubation mixtures. The supernatants were analysed for protein, phospholipid and triacylglycerol levels and by SDS-PAGE. The level of each component in the supernatant was expressed as a percentage of the level in the individual incubation mixtures.

Analytical methods

Methods for SDS-PAGE and determination of protein, phospholipid and triacylglycerol contents were described by Houlihan *et al.* (1992).

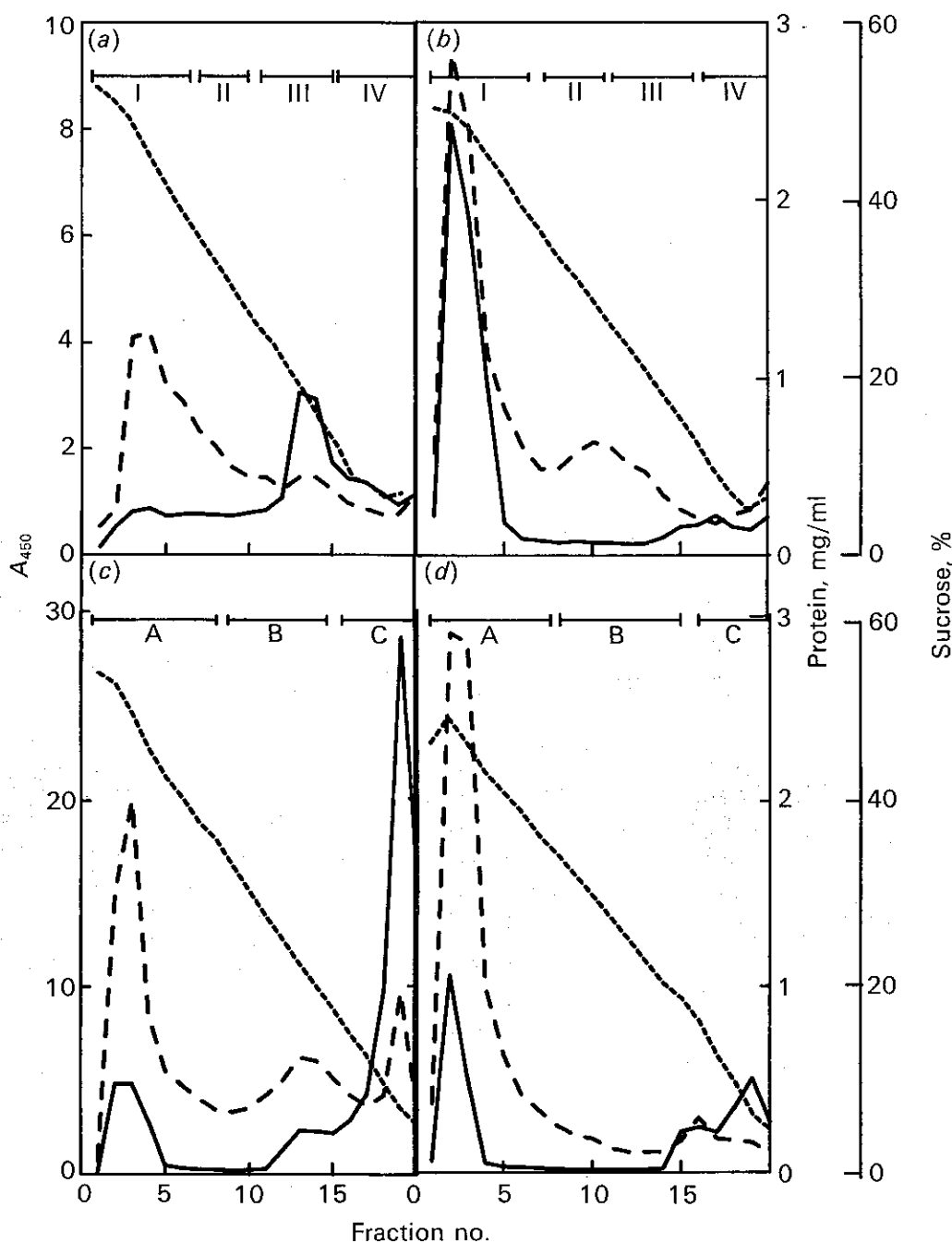


Fig. 1. Isopycnic sucrose density gradient patterns of membrane material from (a) unheated whole milk, (b) whole milk heated at 80 °C for 20 min, (c) unheated resuspended washed cream, (d) resuspended washed cream heated at 80 °C for 20 min. —, Absorbance at 450 nm; ---, mg protein/ml; ----, % sucrose. Fractions were pooled as indicated by bars.

RESULTS

Isopycnic sucrose density gradient centrifugation

Four fractions were obtained from membrane material from whole milk covering the following density ranges: I, 1.156–1.258 g/ml; II, 1.104–1.152 g/ml; III, 1.050–1.100 g/ml; IV, 1.018–1.048 g/ml. Three fractions were obtained from membrane material from resuspended washed cream: A, 1.150–1.258 g/ml; B, 1.074–1.143 g/ml; C, 1.018–1.060 g/ml.

Density gradient profiles of membrane material isolated from unheated milk and milk heated at 80 °C for 20 min are shown in Fig. 1 (a, b). Major changes in the high-

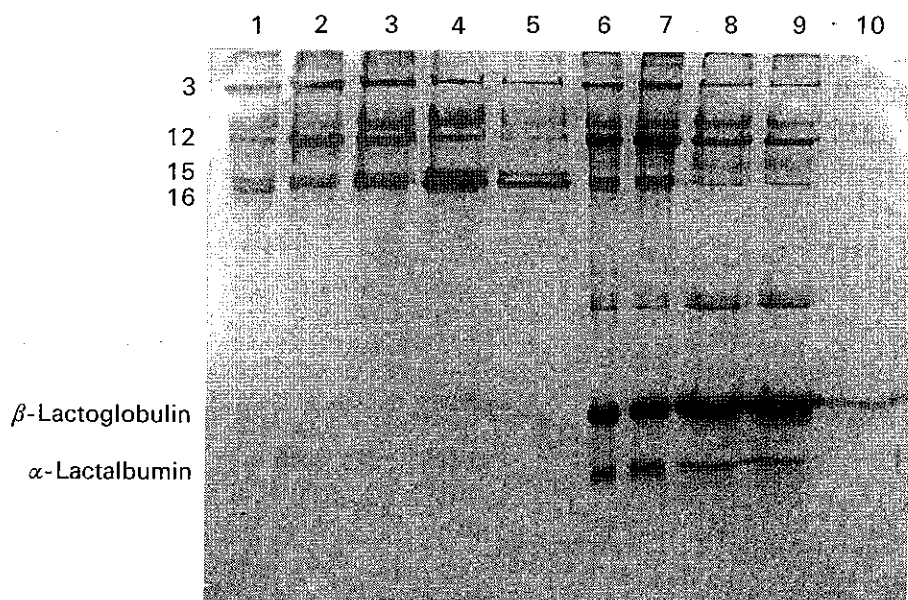


Fig. 2. SDS-PAGE patterns (15% acrylamide gel) of pooled density gradient fractions. Slots: 1, membrane material from unheated whole milk; 2-5, fractions I-IV from unheated milk membrane material; 6, membrane material from whole milk heated at 80 °C for 20 min; 7-10, fractions I-IV from heated milk membrane material. Membrane polypeptides are numbered according to the nomenclature system of Mather & Keenan (1975). For fractions, see Fig. 1. Protein loads, 20-30 μ g.

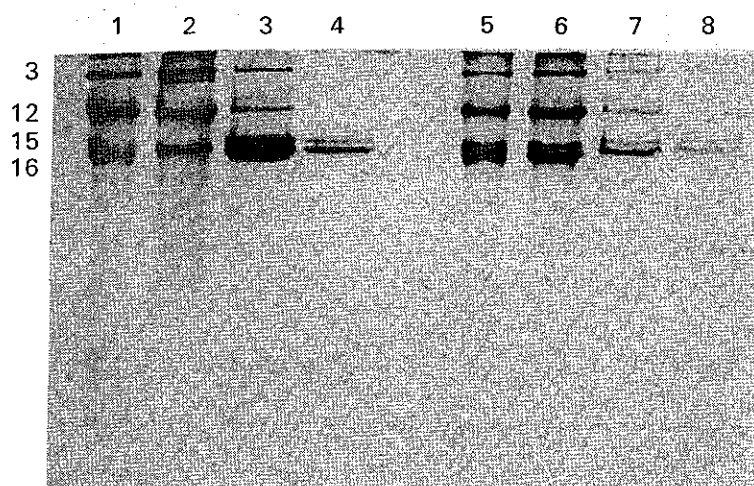


Fig. 3. SDS-PAGE patterns (15% acrylamide gel) of pooled density gradient fractions. Slots: 1, membrane material from unheated resuspended washed cream; 2-4, fractions A-C from unheated washed cream; 5, membrane material from resuspended washed cream heated at 80 °C for 20 min; 6-8, fractions A-C from heated washed cream. Membrane polypeptides are numbered according to the nomenclature system of Mather & Keenan (1975). For fractions, see Fig. 1. Protein loads, 20-30 μ g.

density lipoprotein complexes (fraction I) in membranes from heated milk were apparent, with an increase in density towards the higher density region of this fraction, and much higher protein levels and turbidity readings (A_{450}) than in fraction I from unheated milk. SDS-PAGE patterns (Fig. 2) showed that corresponding density gradient fractions from heated and unheated milk membranes had markedly different protein compositions. The membrane polypeptide composition of the high density fraction (fraction I) of heated milk membranes appeared similar to that of a medium density fraction (fraction II) of the control, suggesting that the main complexes in heated milk membranes may have originated in part

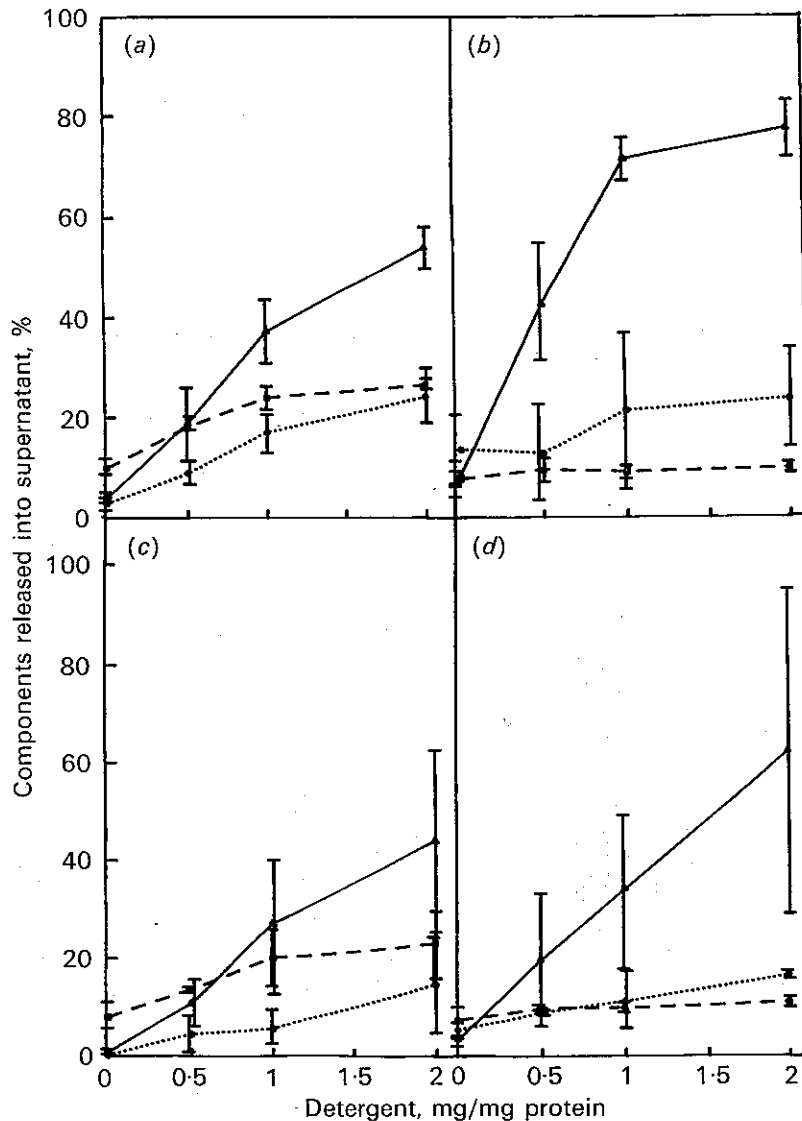


Fig. 4. Release of components by Triton X-100 from membrane material from (a) unheated whole milk, (b) whole milk heated at 80 °C for 20 min, (c) unheated resuspended washed cream, (d) resuspended washed cream heated at 80 °C for 20 min (results expressed as % of level in individual incubation mixtures). —, Protein; ···, triacylglycerols; —, phospholipid. Values are means with their SE shown by vertical bars.

from medium density lipoproteins. The levels of polypeptides 15 and 16 were reduced in all fractions of heated milk, but particularly in the low density fractions III and IV, compared with the levels in the corresponding fractions of the control. Skim milk components were associated with all the lipoprotein fractions present in membrane material from heated milk.

The density gradient profiles of membrane material from unheated washed cream and washed cream heated at 80 °C for 20 min are shown in Fig. 1(c, d). Changes similar to those observed in heated milk membranes occurred in the lipoprotein fractions on heating washed cream, with an increase in high density lipoprotein complexes (fraction A) and a reduction in low density lipoproteins (fraction C). The polypeptide compositions of corresponding lipoprotein fractions were different (Fig. 3), with increases in polypeptides 15 and 16 in fraction A and decreases in these components in fraction B on heating.

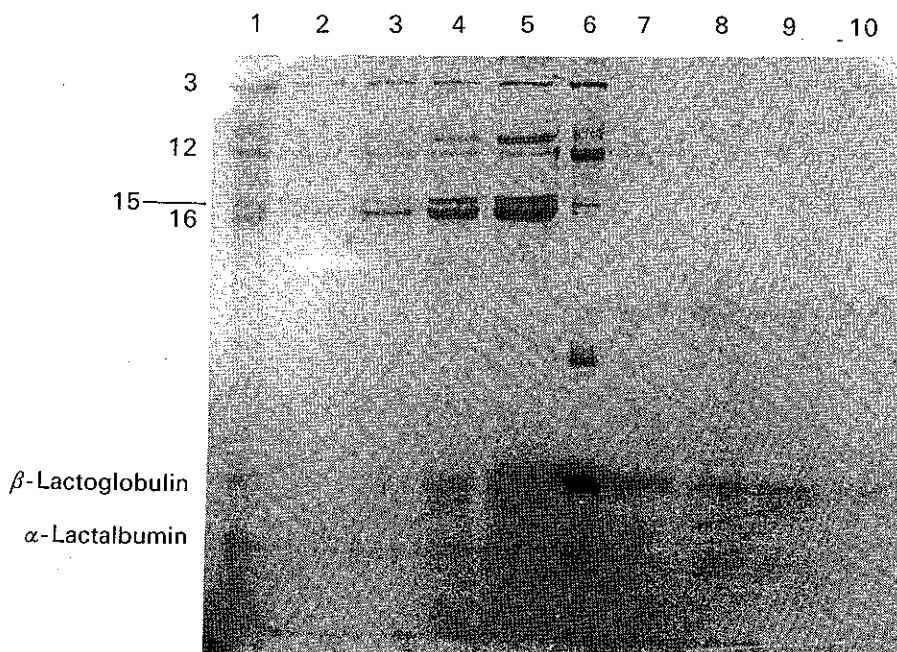


Fig. 5. SDS-PAGE patterns (15% acrylamide gel) of polypeptide components released by Triton X-100. Slots: 1, membrane material from unheated whole milk (protein load, 25 μ g); 2-5, components released at 0, 0.5, 1.0, 2.0 mg detergent/mg membrane protein (sample loads, 120 μ l); 6, membrane material from whole milk heated at 80 $^{\circ}$ C for 20 min (protein load, 25 μ g); 7-10, components released at 0, 0.5, 1.0, 2.0 mg detergent/mg membrane protein (sample loads, 150 μ l). Membrane polypeptides are numbered according to the nomenclature system of Mather & Keenan (1975).

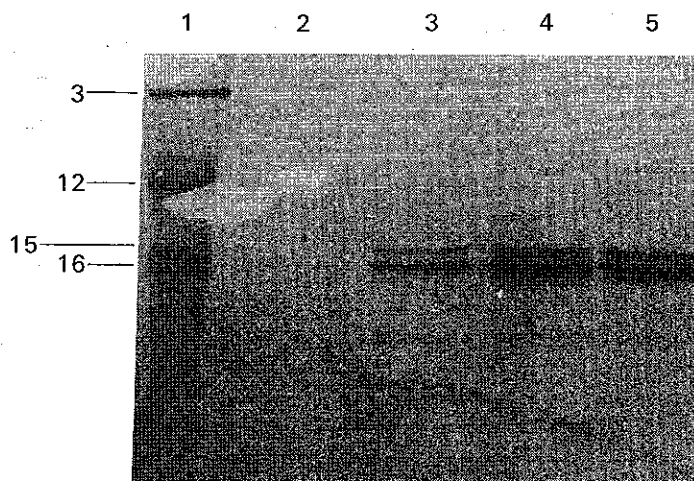


Fig. 6. SDS-PAGE patterns (12.5% acrylamide gels) of polypeptide components released by Triton X-100 from membrane material from unheated resuspended washed cream. Slots: 1, membrane material from unheated washed cream (protein load, 25 μ g); 2-5, components released at 0, 0.5, 1.0, 2.0 mg detergent/mg membrane protein (sample loads, 170 μ l). Membrane polypeptides are numbered according to the nomenclature system of Mather & Keenan (1975).

Detergent release studies

Lower levels of protein components and a higher proportion of phospholipids were released by Triton X-100 from heated milk membranes than from the unheated control (Fig. 4*a, b*). Electrophoretic analysis (Fig. 5) showed components 15 and 16 were the major polypeptides released by Triton X-100 from the natural membrane system, although other membrane polypeptides were also released in substantial

amounts. The membrane polypeptides in heated milk membrane material appeared resistant to Triton X-100, while low levels of skim milk components were released.

The release of components from unheated and heated washed cream membranes by Triton X-100 is shown in Fig. 4(c, d). Heating resulted in a reduction in protein released. Electrophoretic analysis (Fig. 6) showed that polypeptides 15 and 16 were released from unheated washed cream membranes. Owing to the low concentration of protein material released from heated washed cream membrane material, the individual polypeptides could not be detected by electrophoresis.

DISCUSSION

It is apparent that there are differences in the density gradient profiles of unheated milk membranes and unheated cream membranes, even though these preparations would be expected to be similar. It appears that washing the cream has affected the densities of the lipoprotein complexes. The variation in the composition of the MFGM has been well documented (McPherson & Kitchen, 1983), and can be influenced by subtle changes in the isolation procedure. Anderson & Brooker (1975) have shown that washing cream prior to the isolation of the MFGM caused losses of some membrane components. From a practical point of view, it was necessary to use different procedures for the separation of the cream phases in the whole milk experiments (no washing steps) and in the washed cream experiments (three washing steps) (Houlihan *et al.* 1992). This, no doubt, has contributed to the compositional differences observed. Despite these differences, the effects of heating on the lipoproteins appeared similar in both membrane systems, in that heating resulted in an increase in high density material and a decrease in low density lipoproteins.

The high density fraction (fraction I) of heated milk membranes contains the high density lipoproteins (fraction I) of unheated milk membranes, as a loss of lipid or an increase in protein components during heating would result in complexes of higher, not lower, density being formed. However, from the electrophoretic profiles (Fig. 3), the composition of the high density fraction (fraction I) of heated milk membranes more closely resembles that of the medium density fraction (fraction II) of unheated milk membranes, indicating that the medium density lipoproteins could be forming high density complexes during heating, through interactions with skim milk components or loss of lipid components. Similarly, the high density lipoproteins (fraction A) in heated cream membranes are similar in composition to the medium density complexes (fraction B) in unheated cream membranes, indicating that heating alone caused changes in the membrane which resulted in complexes of higher density being formed.

The low density lipoproteins were also affected by heating, with reductions in the levels of polypeptides (15 and 16) in both milk and cream membranes, indicating that these components were heat-labile with losses occurring independently of the interactions with skim milk proteins. These components have been reported to be located on the outer surface of the membrane (Mather & Keenan, 1975). Lipoproteins situated in the outer region of the MFGM may be more affected by heat treatments than those in an internal membrane environment. A major difference between the compositions of the lipoprotein fractions in heated milk and heated cream was the presence of skim milk components in heated milk membrane material. These components may have bound to the lipoproteins, replacing those polypeptides (15 and 16) lost during heating. Alternatively, the skim milk components may have formed a layer of denatured protein over the entire MFGM surface during heating.

Similar mechanisms for the formation of complexes between skim milk proteins and MFGM components on heating whole milk were proposed by Dalgleish & Banks (1991).

The results indicate that heating alone had a profound effect on the natural components of the MFGM lipoproteins, with changes occurring independently of the interactions with skim milk components.

The release of components from unheated milk membrane material by Triton X-100 was similar to that reported by other workers (Nielsen & Bjerrum, 1977; Freudenstein *et al.* 1979; Kanno & Yamauchi, 1979), although there has previously been little information reported on the release of lipid components. The release of phospholipid and protein components from unheated milk membranes and unheated cream membranes appeared similar. A lower proportion of the total triacylglycerol components was released from unheated washed cream membranes, which may reflect compositional differences due to the different isolation conditions. Triacylglycerol components appeared largely unaffected by heating or by the incorporation of skim milk components. Heating milk and washed cream resulted in similar changes to the percentage of the total protein released, indicating that the heat treatment alone may have altered the solubilization characteristics of these components. The incorporation of skim milk components into heated milk membranes appeared to result in the phospholipids becoming more accessible to the detergent. Heating alone had little or no effect on the phospholipids.

The combination of the heat treatment and the incorporation of skim milk proteins into the MFGM altered the permeability of the MFGM to a membrane probe such as Triton X-100. The increased accessibility of the phospholipids, a major structural component of the MFGM, could result in reduced membrane stability under similar processing conditions.

These studies have shown that heating affected the structure of the MFGM by changing the density and composition of MFGM lipoprotein complexes and altering the solubilization characteristics of membrane components by detergents. These changes appear to have resulted from a combination of the effects of the heat treatment alone and the incorporation of skim milk components into the MFGM.

The authors acknowledge the technical assistance of Mr A. Wood and Ms S. Veldhoven. This research was supported by a grant from the Australian Dairy Research and Development Corporation.

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