Biochimica et Biophysica Acta, 688 (1982) 727–734
Elsevier Biomedical Press

BBA 71208

RELEASE OF REMNANT PLASMA MEMBRANE FROM MILK FAT GLOBULES BY TRITON X-100

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(Received October 7th, 1981)
(Revised manuscript received January 15th, 1982)

Key words: Membrane fragment release; Triton X-100; Milk fat globule

The nonionic detergent, Triton X-100, was investigated as an agent for releasing plasma membrane from milk fat globules. The sedimentable material (50000 × *g*, 1 h) derived by treating washed goat globules with the detergent (0.2%) was compared to membrane made by the classical globule churning procedure. Characterization included lipid and protein analyses, gel electrophoresis of peptide components, determination of enzymatic activities, and examination with the electron microscope. The results established that the detergent-released material is membrane with similarities to the product by churning. Evaluation of variables revealed that a detergent concentration of 0.1 to 0.2% and reaction temperature of 20–22°C appear optimum with respect to membrane yield when a reaction time of 2 min is employed. At higher detergent concentrations or temperatures removal of phospholipid from the membrane was maximized. Triton X-100 was observed to release membrane from milk fat globules of the goat, human and cow, the latter with a minor procedural modification. The detergent based method is a convenient procedure for obtaining plasma membrane material in good yield for biochemical studies. It also should aid investigations of milk fat globule structure.

Introduction

Secretion of the milk fat globule is accomplished by its envelopment in apical plasma membrane of the lactating cell. (For reviews of the milk fat globule membrane, see Refs. 1 and 2). The value of this membrane for research purposes would be enhanced if there were better methods for its separation from the globule. The classical procedure of churning washed globules to obtain the membrane involves physical beating and aeration. Whether and when butter formation will occur and yields of membrane are unpredictable. The method is not conveniently applied to small samples (a few ml). The other established technique employs freezing and thawing. It requires holding frozen globules overnight or longer and repetition of freezing-thawing cycles when de-emulsification is inefficient. For most biochemical purposes these limitations are intolerable. Smith et al. [3] have characterized the lipids in a membrane fraction released from milk fat globules by 1% deoxycholate. In this connection, the procedure [4] of removing plasma membrane from cells with the non-ionic detergent., Triton X-100, is of interest. I report here a study of membrane release from milk fat globules with this detergent. A preliminary communication of this work has been abstracted [5].

Materials and Methods

Milk

Most of this research involved milks from indi-

vidual goats in mid-lactation either from small herds in San Diego County, CA or from the experimental herd at the Pennsylvania State University. Samples of individual cow and human milk were also evaluated to test applicability to them of the detergent procedure for releasing membrane from milk fat globules. Bovine milks were from large herds of Holsteins. The human milks were donated by two women in the early months (first and third) of their lactations. Milk samples were used in experiments within an hour of their collection. A few of the cow milks were maintained at 37°C during this period but generally the samples were allowed to adjust toward ambient temperature (20–25°C).

Membrane preparation

For comparisons, milk fat globule membrane was prepared by the classical churning procedure (reviewed in Ref. 1) as well as by release with detergent. In order to avoid contamination of membrane preparations with non-globule constituents the globules were first washed. The batch was then split and the two procedures applied.

Fat globules were isolated from milk and washed as follows: Polycarbonate tubes (38-ml) were filled with milk and centrifuged (Sorvall Model RC2b) at room temperature (20–25°C) and $2000 \times g$ for 15 min. The skim milk was decanted from under the compacted layers of globules, the latter being restrained with a spatula. Fifteen ml of 0.85% saline solution (20–25°C) was added to each tube. The cream layers were resuspended with the spatula and then by gentle agitation for about 30 s with a vortex mixer. The tubes were filled with saline, mixed with a spatula and centrifuged as before. The washing centrifuging-decanting-process was done three times after the initial removal of skim milk. After the second washing the cream layers were transferred by spatula to clean tubes. This rejects any pelleted casein and cell debris. After the final centrifuging, the lower wash solution was decanted from each tube and the globule layers were transferred to suitable flasks for dilution and membrane production. The yield of globules by this method, based on total lipids of the milk and the washed globule preparations, was $87 \pm 5\%$ measured in three trials. The yield of phospholipid, comparing that in 1 g of washed globule lipid with that in 1 g of total milk lipid was 63 and 65% in two trials. This is consistent with observations [1] that 30 to 40% of the phospholipid of milk is in the skim phase. In the preparation of membrane with the aid of Triton X-100, the washed globules were reconstituted in saline to their original milk volumes and to contain 0.2% of the detergent. Depending on sample size and experimental requirements this formulation was accomplished in flasks or centrifuge tubes using saline and 10% Triton in saline all at 20–25°C. The mixture was then gently agitated by either swirling the flask or inverting the capped tubes for 2 min to allow the detergent to act. The detergent-treated material was immediately cooled to 5°C or lower in an ice bath, transferred to polycarbonate tubes and centrifuged at 2–6°C and $50000 \times g$ for 1 h. As a result of this centrifuging the tubes contained a hardened fatty layer on top, a small, translucent, brown pellet of membrane at the bottom and a water-clear infranatant. The fat layer was dislodged from the tube wall and the infranatant decanted. Remaining fatty layer was removed with a spatula and the tube was wiped clean with tissue down to the membrane pellet. This membrane was suspended in a suitable volume of buffer, saline or distilled water by a few minutes of agitation on a vortex mixer. Depending on the experimental need the membrane preparation was promptly analyzed, further fractionated or placed in frozen storage for subsequent analysis.

Membrane was prepared from the washed globules also by churning in which process the physical agitation ultimately produces a stripping of membrane from globules. The latter then adhere as butter granules and the membrane, along with unaggregated globules, remain suspended in the buttermilk. To optimize this method the washed globule layers were adjusted to 20 to 25% lipid in saline and to a temperature of 12–14°C. This cream then was churned with the aid of a Waring blender. The degree of agitation was kept at a gentle level by a voltage regulator. The process required 15 to 25 min. The buttermilk was filtered through coarse mesh paper tissue to remove butter grains and then centrifuged as for the detergent-treated globule preparations. Isolation, suspension, and use or storage of the membrane pellets was as for those derived with detergent.

Membrane characterization

Membrane preparations were submitted to the following analyses in duplicate: Total lipids were

obtained by the extraction procedure of Folch et al. [6] and quantitated gravimetrically. For this purpose and that of defining lipid phosphorus, lipid was specified as the Folch-extractable material soluble in 1 to 2 ml of chloroform/methanol (19:1, v/v). Phospholipid was derived as lipid phosphorus ×25. The proportions of individual phospholipids were determined from two-dimensional thin-layer chromatographic (TLC) separations of membrane lipids on silica gel plates (Merck) as by Parsons and Patton [7]. The same separations were used to isolate cerebrosides. After confirming that the glucosyl- and lactosylceramides ran in the previously observed positions [7,8] by spraying the plates with orcinol reagent, we located the two components with light iodine staining, scraped the spots from the plates and analyzed them for carbohydrate using anthrone reagent [9]. The monohexoside was assayed using a glucose standard and the dihexoside with lactose as the standard. The total cerebrosides then were calculated using molecular weight of 728 and 890 and mean glucose and lactose contents of 24.7 and 38.4%, respectively, for the two species. Triacylglycerols in membrane lipids were first isolated on one-dimensional TLC plates of silic gel using hexane/ethyl ether/acetic acid (70:30:1, v/v) as mobile phase. Areas scraped from plates were assayed by the colorimetric method of Stern and Shapiro [10] using triolein as a standard. Total cholesterol in membrane lipids was determined by the method of Searcy et al. [11].

Triton X-100 in membrane lipids was cleanly resolved from the other lipids by TLC on silica gel plates with the solvent system: chloroform/methanol/water/ammonia (28%) (130 : 70 : 8 : 0.8, v/v). The areas of the plate containing the detergent (R_F 0.83), together with control areas, were scraped and extracted into absolute ethanol. Triton X-100 in these extracts was determined spectrophotometrically at its absorption maximum of 277 nm. The authentic material (Sigma, St. Louis, MO. U.S.A.) was used as a standard.

Protein was determined by the method of Lowry et al. [12] with bovine serum albumin (Sigma) as standard. In applying this procedure to washed globule preparations the samples (5–25 μl) were first dried in the assay tubes with a stream of nitrogen and neutral lipid was removed by two 0.5 ml extractions with ethyl ether. Turbidity due to lipids is a problem in analyzing fat globules directly by this method.

Excessive triacylglycerol is also a problem in analyzing total milk lipids or globule lipids for phosphorus. There is too much organic matter to be oxidized in these materials for the amount of phosphorus present. For these analyses neutral lipids were eliminated by TLC (see preceding system for triacylglycerol analysis). The phospholipids remaining at the origin could then be scraped from the plate and used satisfactorily, together with suitable controls, in the phosphorus determination. The patterns of peptides contained in membranes prepared by the two procedures were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Slab gels, 10 to 15 cm in running length and 1.5 mm thick were prepared according to Weber and Osborn [13]. Membrane suspensions or washed globules equivalent to 25 to 50 μg of protein were solubilized with SDS and mercaptoethanol as prescribed by Laemmli [14] and then heated in a boiling-water bath 2 to 3 min. Samples were applied to the gels and electrophoresed (CBS Scientific, San Diego, CA, U.S.A.) using a buffer system of SDS/Tris/glycine, pH 8.6 and an applied current of 30 mAmp. Coomassie blue staining [15] was used to reveal the peptide bands in gels.

Further comparison of membranes derived by the two procedures were made in the electron microscope. Suspensions of membrane were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4). Post fixation was with 1% osmium tetroxide in the same buffer. Further steps in processing the materials were according to Warchol et al. [16] as applied by us to mammary tissue [17]. Sections were examined and photographed in an Hitachi HU-11E electron microscope.

Variables in membrane release

To define more precisely optimum conditions for releasing membrane from milk fat globules with Triton X-100, detergent concentration and temperature of the reaction were evaluated as variables. The procedure was also tested on a number of bovine (six) and human (two) milk samples. In these trials 2.8 ml quantities of washed globules

were used in duplicate in polycarbonate centrifuge tubes of that capacity. Appropriate levels of detergent (10% solution) were added and mixed in the samples for 2 min by inversions of the capped tubes. Regarding evaluation of temperature, bulk samples of washed globules were warmed to 38°C and 2.8 ml samples were withdrawn into tubes at that temperature and other temperatures achieved during subsequent progressive cooling in an ice-water bath. Triton X-100 was added and mixed with each of these samples. After reaction with the detergent all samples were immediately cooled to 2 to 4°C in order to halt further membrane release. Samples were then centrifuged at 2 to 6°C and membrane pellets obtained for analysis as previously described.

Results

Data on composition of membranes prepared by churning milk fat globules or by treating them with Triton X-100 are given in Tables I and II. These show higher concentrations of triacylglycerol in the membrane by churning and more cerebroside in the detergent derived membrane (Table I). Triton X-100 formed part of the extractable lipids in the detergent processed samples but according to our estimates a substantial part of this could have been due to occlusion since the membrane preparations were not washed or dialyzed to remove unbound detergent. The Triton X-100 membrane contained more sphingomyelin and less of the other phospholipids, especially phosphatidylethanolamine, when compared to the membrane by churning (Table II). We have also compared some enzymatic activities of the two preparations from the same lot of globules (Trams, E.G. and Patton, S., unpublished data). The Triton X-100 derived membrane showed 114, 112 and 170% of the activities of the membrane by churning for Mg-ATPase, xanthine oxidase and 5'-nucleotidase, respectively.

TABLE I

MEMBRANE LIPID COMPOSITION

Means and ranges for two preparations of each membrane.

	%	
	Churned	Triton X-100
Phospholipid	42.6 (±6.8)	39.7 (±6.0)
Cholesterol	6.1 (±0.4)	7.2 (±0.1)
Cerebroside	6.7 (±0.7)	13.6 (±0.7)
Triacylglycerol	37.3 (±9.0)	19.3 (±0.5)
Triton X-100	–	14.2 (±0.7)
Unaccounted	7.4 (±1.9)	6.2 (±4.1)
Lipid/protein	46/54 (41–51/49–59)	52/48 (52–53/47–48)

TABLE II

MEMBRANE PHOSPHOLIPID COMPOSITION

Means and ranges for two preparations of each membrane.

	%	
	Churned	Triton X-100
PE	28.9 (±0.8)	19.2 (±2.2)
PC	29.2 (±2.7)	24.6 (±1.4)
SP	22.3 (±0.8)	42.0 (±3.0)
PS	12.9 (±0.9)	10.6 (±0.3)
PI	6.9 (±1.9)	3.8 (±0.3)

Typical peptide patterns by SDS-polyacrylamide gel electrophoresis for products of the two methods are presented in Fig. 1. Although the similarities are substantial, there are two rather diffuse bands (arrows) present in the churned membrane which are weak in the Triton membrane pattern. The two bands were clearly evident in patterns for intact globules (see arrows, Fig. 3).

Ultrastructural aspects of membranes prepared by the two methods were similar (Fig. 2) although the membrane via churning had somewhat thicker profiles than that by detergent treatment. Trilaminar structure was only occasionally observed in either, possibly due to the rather drastic physical manipulations of globule washing and churning, Fig. 2, upper, closely resembles other electron micrographs of milk fat globule membrane produced by churning [18–20].

Fig. 3 presents gel electrophoresis patterns of peptides in material sedimented after treating washed globules (goat) with varying concentrations of Triton X-100. The patterns are very nearly identical except in overall intensity. This indicates

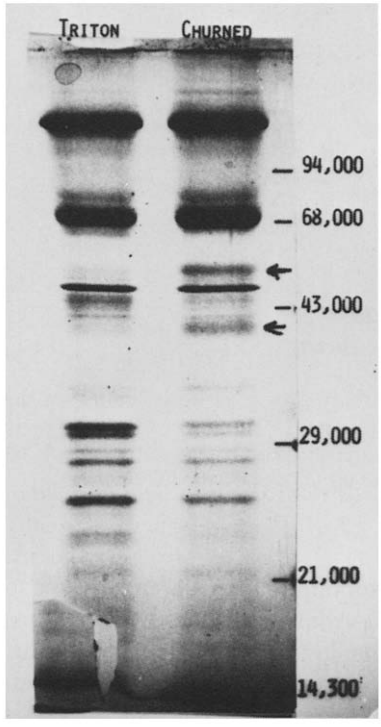

Fig. 1. Electrophoretic separation of peptides in membranes (goat) prepared by churning washed milk fat globules (right) and by treating washed globules with 0.2% Triton X-100 (left). Reference molecular weight proteins, run on the same gel, were positioned as indicated. Samples containing 50 μg of protein, treated with sodium dodecyl sulfate and mercaptoethanol were electrophoresed in 12.5% polyacrylamide. Staining was with Coomassie blue.

that membrane fragments of uniform peptide composition are being released with respect to varying detergent concentration. However, as previously mentioned, two components of intact globules are substantially reduced in the Triton-derived membrane (arrows, Figs. 1 and 3). An effect of Triton X-100 concentration very similar to that in Fig. 3 was seen for gel patterns of peptides in material released from human milk fat globules (data not shown).

To better define conditions for freeing membrane from globules with Triton X-100, additional experiments were performed on effects of detergent concentration and reaction temperature. To measure yields, amounts of protein and phospholipid sedimenting with respect to these varia-

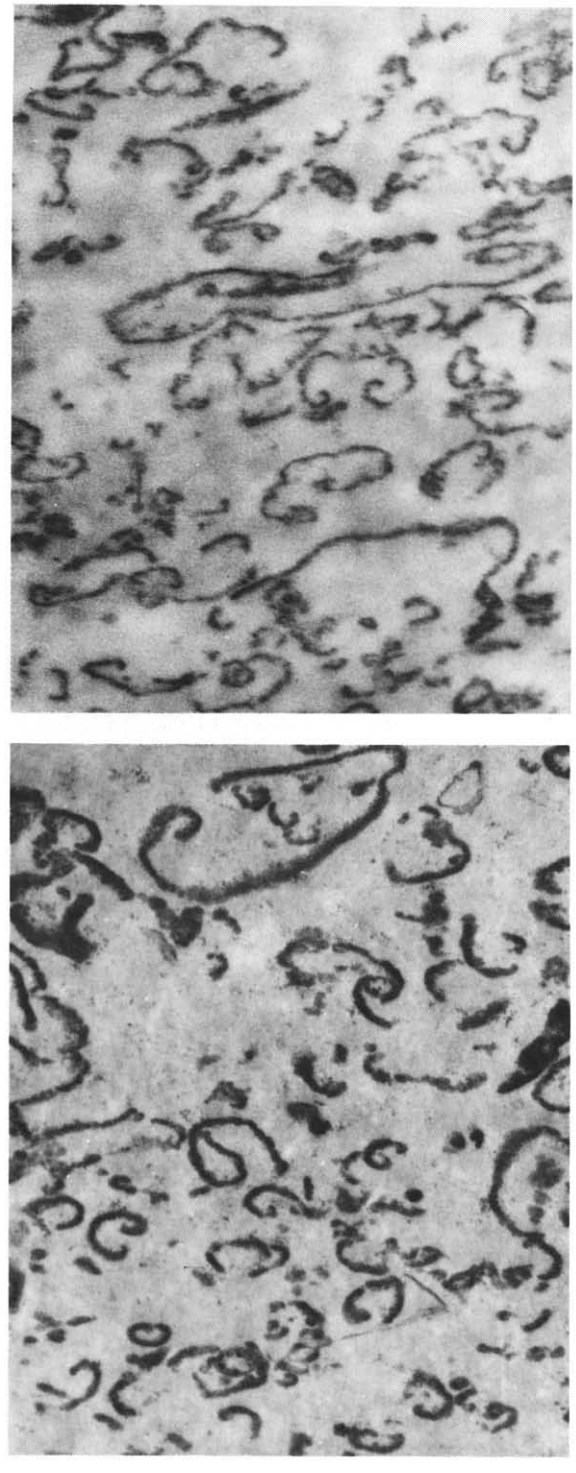

Fig. 2. Electron photomicrographs of membranes (goat) prepared by churning washed milk fat globules (upper) and by treating washed globules with Triton X-100 (lower), ×30700.

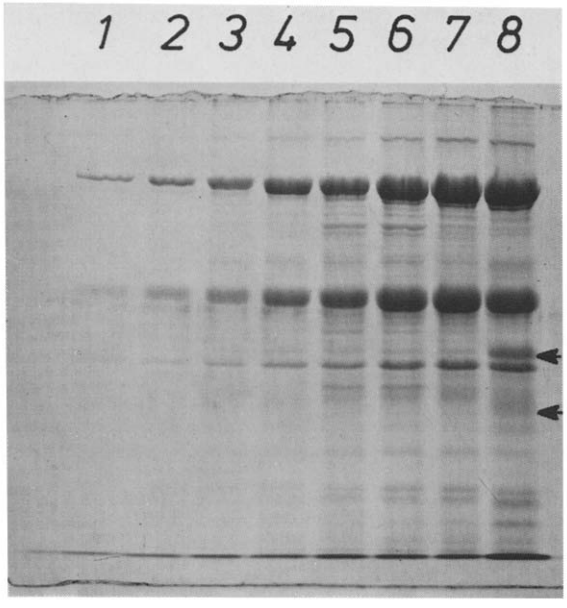

Fig. 3. Electrophoretic separation of peptides in membranes (Goat) sedimenting from washed milk fat globule samples after treatment with various Triton X-100 concentrations as follows: 1, 0%; 2, 0.01%; 3, 0.025%; 4, 0.05%; 5, 0.1%; 6, 0.25%; 7, 0.5%; 8, washed globules (not centrifuged). Membrane pellets were dispersed in a standard volume of phosphate buffer (pH 7.2) and 6 μl of each dispersion was used for electrophoresis. The gel was 7.5% polyacrylamide with other conditions as for Fig. 1. The protein applied in sample 8 was 80 μg.

bles were analyzed. Fig. 4 is representative of several trials on the relationship of detergent concentration to membrane yield. Proportionality of

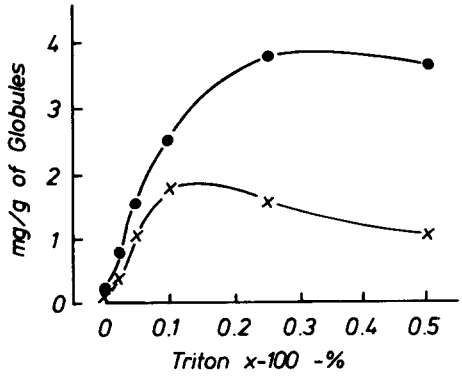

Fig. 4. The effects of varying Triton X-100 concentration on the protein (●———●) and phospholipid (×———×) contents of membrane (goat) released from washed milk fat globules. The reactions were for 2 min at 22°C.

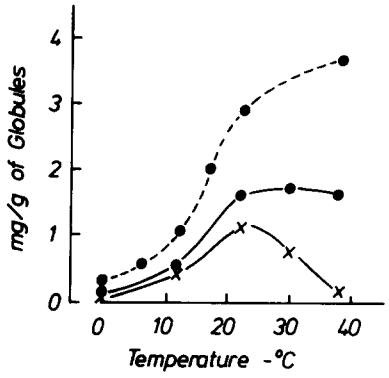

Fig. 5. The effects of varying reaction temperatures on the protein (●) and phospholipid (×) contents of cow (———) and goat (- - - - -) membrane released from washed milk fat globules by exposure to 0.2% Triton X-100 for 2 min.

protein and phospholipid yield is shown up to 0.1% Triton X-100. Above that concentration, protein is increasing while phospholipid is decreasing. An implication is that at higher detergent concentrations phospholipid in released membrane is being extracted to a greater degree into detergent micelles than at lower concentrations. Temperature produces similar effects on membrane recovery, Fig. 5. Regarding the data on cow globules (Fig. 5), phospholipid yield is maximal at about 22°C above which temperature it decreases. Similar results (not shown) were obtained for goat globules. The main break in the temperature curve for membrane yield is between 12° and 20°C. In my preliminary work [5], I used 4°C in accord with applications to cells in culture [4]. This temperature is too low for consistent and acceptable yields of milk fat globule membane. Sufficient numbers of cow globule samples have not yet been tested for membrane yield to reveal whether the difference between cow and goat globules (Fig. 5) is consistent. As discussed following, release of membrane from cow globules should receive further research.

Using data for protein and phospholipid in membrane preparations, yields were determined as a function of totals for the two constituents in washed goat globules. The mean yields plus ranges for three trials were: protein 44 ± 13%, phospholipid 18 ± 7%. One interpretation of these data is that the native surface of the fat globule, prior

to secretion, is relatively devoid of protein and rich in phospholipid.

An investigation is planned of what remains associated with the detergent-extracted globules and in the extracting solution from which membrane was removed by sedimentation. The latter is a clear solution and its gel electrophoresis pattern (not shown) is comprised of a few relatively weak bands and one strong one which corresponds to a globule component (approx. mol. wt 54000) much diminished in the detergent prepared membrane (upper arrow Figs. 1 and 3).

Discussion

The material released from milk fat globules by Triton X-100 is membrane. This is evident from composition, enzymatic activities, sedimentation characteristics, morphology and similarities to milk fat globule membrane prepared by churning. By comparison to the latter, membrane released by the detergent is richer in sphingomyelin, cerebroside and 5′-nucleotidase activity. These enrichments in plasma membrane markers, together with lower triacylglycerol levels, might suggests that the detergent method yields a less contaminated preparation. They may also be explained as a selective extraction of membrane components or regions. Unfortunately composition of the native milk fat globule surface; i.e., the surface it had prior to envelopment in membrane at secretion, is not known. The possibility that components of this surface may equilibrate with the plasma membrane overlaying it makes it difficult to evaluate composition of membranes isolated by the two methods.

We propose two principal effects of the detergent on milk fat globules. One is release of membrane which predominates at detergent concentrations below 0.2% and temperatures below 25°C. At higher concentrations and temperatures there is also solubilizing of membrane constituents as evidence by lower phospholipid content in the membranes obtained under such conditions (Fig. 4 and 5). Further it is not precluded that Triton X-100 removes some components from membranes at detergent concentrations below 0.1%.

The removal of phospholipid molecules into micells and insertion of detergent molecules into the membrane could create regions in which water carrying detergent can penetrate and release the membrane. It is evident that the forces holding large areas of the membrane together must be somewhat stronger than those which bind it to the globule. It is notable from Fig. 2 that Triton X-100 does not cause vesiculation or myelin configuration of the released membrane. Rather, a structure like that of churned membrane is retained. Properties of detergents, including Triton X-100, and their effects on membrane have been reviewed [21].

The findings of this study show the considerable promise of a procedure using Triton X-100 to release membrane from milk fat globules, although additional research, both biochemical and ultrastructural, will be needed. From the data it is evident that gentle mixing of washed globules containing 0.1% Triton X-100 at 20–25°C for 2 min followed by centrifuging at $50000 \times g$ for 1 h and 2–4°C will isolate membrane in a quick and convenient manner. Reaction times and effects of using various washing media have not yet been studied although casual observations suggest that yields of membrane are comparable whether distilled water, saline, 0.25 M sucrose or phosphate buffered (pH 7.2) salt solution are used as washes. Partly or completely avoiding the globule washing step is a compelling objective. A useful product with greater integrity may result simply by treating very fresh milk, or an unwashed, compacted layer of globules therefrom, with detergent and deriving a membrane-rich fraction by centrifugation.

Applicability of the detergent method to milk fat globules of species other than the goat will require further research. There is an anomalous response with bovine globules and our satisfactory application to human globules should be tested on additional samples. We note that with some bovine milks if fat globules are isolated at room temperature or lower, little or no membrane is obtained on treating with 0.2% Triton X-100. However, there appears to be no difficulty if the globules are first isolated at 37°C. It is suspected that a globulin which binds to bovine globules on cooling may interfere with action of the detergent. This globulin, which promotes globule clustering and rising (creaming) in cow's milk is absent from goat's milk [22].

Triton X-100 should be useful as a probe to reveal the molecular arrangement of the milk fat globule exterior. By comparing what is released progressively by the detergent to what is retained by the globule one may be able to assign components to one or another of the several surfaces involved. In this connection, Freudenstein et al. [18] using membrane prepared by churning, from milk fat globules of several species, have shown that Triton X-100 releases a characteristic coat protein (approx. mol. wt. 70000) located on the inner surface of the membrane, Application of this technique to the intact fat globule may be complicated somewhat by post-secretion rearrangements of the globule membrane reported by electron microscopists (see Refs. 3 and 23 for discussion). However, such changes have never been determined quantitatively. Use of detergent may help to resolve the problem in that rearranged membrane (no longer undercoated with cytoplasm) may not be released by detergent.

Acknowledgements

I thank Dr. John S. O'Brien for use of his laboratory and facilities at the University of California, San Diego, Bridget Stemberger for preparation of electron photomicrographs and staff at the Bundesanstalt für Milchforschung, Kiel, F.R.G. for technical assistance in the preparation of this manuscript. This research was supported by National Science Foundation grant PCM 79-27252 and funds of the Pennsylvania Agricultural Experiment Station.

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