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PLASMA MEMBRANE FRAGMENTS IN BOVINE AND CAPRINE SKIM MILKS

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SUMMARY

By either differential or linear gradient ultracentrifugation of bovine or caprine skim milks it was possible to obtain fractions which contained 45–75 % of the lipid phosphorus and unesterified cholesterol of the skim milk. Electron microscopy of these fractions revealed the presence of numerous membrane-bound vesicles, microvilli and membrane fragments. Assay of the fractions for certain membrane-bound enzymes; *i.e.* 5′-nucleotidase, nucleotide pyrophosphatase, alkaline phosphatase and ATPases, established the presence of all but the latter in the membrane-rich fractions. The distributions of the enzymes in the various fractions were correlated with their lipid phosphorus and cholesterol contents.

Compositions of the phospholipids from skim milk membranes, milk fat globule membranes and the plasma membrane of the lactating mammary cell were observed to be similar and unique for having a relatively high level (20–25 %) of sphingomyelin. By virtue of secretory processes, all of these membranes appear to be interrelated with each other and with Golgi vesicle membranes. It is concluded that the membrane material in the skim milk originates primarily from plasma membrane of the lactating cell. The possibility that Golgi vesicle membranes form a substantial part of this material is not precluded by the results of this study.

Separation of bovine skim milk on a Sepharose 4B gel column demonstrated that virtually all of the 5′-nucleotidase and lipid phosphorus are recovered together in the void volume of the column. Considering the particle size discriminating characteristics of this gel, the skim milk membrane material appears to be constituted of relatively large structures rather than of discrete subunits.

INTRODUCTION

Recent findings confirm that approximately 40 % of the phospholipids of milk (bovine and caprine) resides in the skim milk phase, the remainder being associated with the milk fat globules. It was observed further that the kinds and proportions of individual phospholipids in these two locations are very similar[1]. Preliminary investigations established that most (40–70 %) of the phospholipid of bovine skim milk is obtained ultracentrifugally in a so-called "fluff" fraction which is rich in membrane fragments, microvilli and membrane-bound vesicles[2].

This report presents more detailed information on the lipid composition and enzymatic activities of the membrane-rich fraction from bovine and caprine skim milks.

MATERIALS AND METHODS

Isolation of skim milk membrane material

Complete milkings were obtained from individual Jersey cows and goats from the University herds and 200-ml aliquots were immediately separated at ambient temperature into layers of compacted fat globules and skim milk with an International Model K centrifuge operated at 3500 rev./min for 40 min. Skim milk was obtained from under the cream layer by use of a 50-ml syringe fitted with a 6 inch standard gauge needle. This fluid is essentially free of milk fat (total lipid <0.1 %) It was utilized as the starting material in the following two ultracentrifugal techniques.

Differential centrifugal separation of 90-ml aliquots (30 ml/tube) of skim milk was performed in a Spinco Model L2-65 preparative ultracentrifuge at 17 °C utilizing a type 30 rotor operated at 25000 rev./min for 2.5 h ($1.83 \cdot 10^5 \times g \cdot h$ at R_{max}). Each of four fractions (Fig. 1) was pooled with similar fractions from the other centrifuge tubes. The top fraction (D1), consisting of residual fat globules, was removed in as minimum volume as possible (7–9 ml). The clear infranatant (D2) was withdrawn with a pipet until the meniscus coincided with the uppermost portion of the casein pellet (12–16 ml). Fluff fraction (D3) was then obtained with a pipet. This fraction included residual fluff which was removed by gentle washing of the casein pellet (D4) surface with a minimum volume of distilled water. The casein pellet was then collected with a pipet after dispersing it in distilled water.

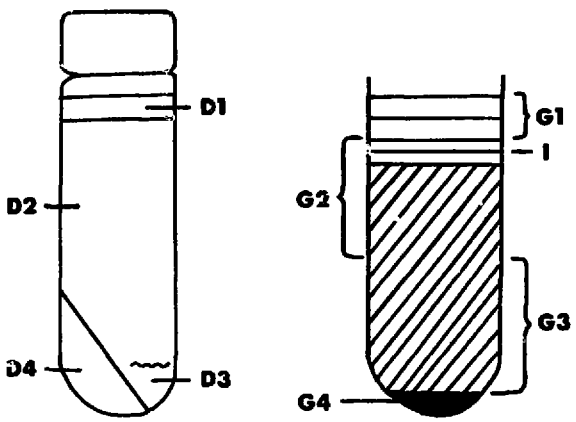

Fig. 1. Left. Diagram of the fractions which resulted when bovine and carpine skim milks were subjected to ultracentrifugation at $1.83 \cdot 10^5 \times g \cdot h$ at 17 °C. Fractions D1, D2 *plus* D4 constitute Fraction R for enzyme assays. Fraction D3 is the skim milk membrane concentrate fraction. Right. Diagram of the fractions which resulted when dialyzed evaporated bovine and caprine skim milks were subjected to sucrose linear gradient ultracentrifugation at $4.18 \cdot 10^6 \times g \cdot h$ at 17 °C. Interface of gradient and sample is designated I.

For sucrose linear gradient ultracentrifugation 300 ml of skim milk were dialyzed continuously for 18 h at 4 °C against distilled water to remove lactose, a component that increased the density of the sample and impaired layering over the gradient. To concentrate the skim milk membranes in the fluid, flash evaporation at 25 °C was employed to remove approximately 90 % of the water. 15 ml of this concentrate were layered over preformed sucrose linear gradients prepared in cellulose

nitrate tubes (60 ml capacity) on a Beckman Linear Gradient Former at room temperature. After many trials the linear gradient selected was prepared from solutions having d_4^{20} of 1.06 g/cm³ and 1.25 g/cm³ as determined by glass pycnometers. 40-ml gradients had a range of 1.10 at the top of the tube to 1.22 g/cm³ at the bottom as determined from a nomograph. Centrifugation conditions were identical to those of the differential technique except for the use of an SW 25.2 rotor ($4.18 \cdot 10^6 \times g \cdot h$ at P_{max}). As shown in Fig. 1, four fractions were secured. While these fractions should not be cross referenced with those obtained by differential centrifugation, we did observe that fraction D3 when placed on the gradient disposed itself in the position of fraction G2.

Extraction and analysis of lipids

Lipid extraction of each fraction was performed immediately by a modified Folch procedure[3]. Fifteen sample volumes of chloroform–methanol (2:1, v/v) were added to each fraction, to which was combined 3 sample volumes of 0.4% aqueous $CaCl_2$. The organic solvent layer from each extracted fraction was removed and brought to near dryness with a rotating evaporator operated at 40 °C and evacuated by a water aspirator. The concentrated extract was quantitatively transferred through a funnel stoppered with glass wool into a vial and stored. The final volumes including rinsings were 2–3 ml.

The lipids in these extracts were separated by thin-layer chromatography on plates coated with 0.5-mm layers of silica gel HR (Merck). A solvent system of petroleum ether–ethyl ether–glacial acetic acid (160:40:2, v/v/v) was used. The areas of the plates corresponding to the total phospholipids (origin) were analyzed for lipid phosphorus by the method of Rouser *et al.*[4]. Unesterified cholesterol was located on the plates by use of a reference standard and brief exposure to iodine vapor. The appropriate areas of silica gel were scraped into funnels stoppered with glass wool and the cholesterol, after elution with diethyl ether, was determined gas chromatographically essentially as described by Luukkainen[5]. Recovery of cholesterol was found to exceed 90% by this method. Cholesterol samples and the reagent for trimethylsilation (250 µl of tetrahydrofuran, 150 µl of hexamethyldisilazane and 50 µl of trichlorosilane) were incubated for 30 min at 60 °C. Gas chromatographic analysis was performed isothermally (220 °C) on a Hewlett Packard Series 5750 instrument fitted with a column 3.175 mm × 63.5 cm packed with 3% OV 17 coated on a stationary phase of Gas Chrom Q 100/120 mesh (Applied Science Laboratories, State College, Pa.). Nitrogen carrier gas was used at a flow rate of 45 ml/min and quantitation was performed by triangulation. For calculation of molar quantities of phospholipid and cholesterol the molecular weight values of 750 and 387 g/mole were used, respectively.

For phospholipid compositional analyses aliquots of total lipids were spotted on 0.25 mm precoated thin layer chromatographic plates (EM Laboratories, Elmsford, N.Y.) and developed two-dimensionally according to the procedure of Parsons and Patton[6]. Phospholipid spots and appropriate blanks were scraped from plates into test tubes for lipid phosphorus analysis by the method of Rouser *et al.*[4].

Enzymatic activity

Since differential ultracentrifugation required less time and manipulation

than the gradient procedure to obtain the membrane material, it was used to prepare samples for enzyme assays. Enzymatic activities were determined for the principal membrane fraction (D 3) and a pooled fraction R composed of residual fat globules (D 1), infranatant (D 2), casein pellet (D 4) and tube washings. The two fractions were dialyzed against distilled water at 4 °C for 18 h prior to analysis.

Alkaline phosphatase was assayed at pH 9.6 according to the colorimetric procedure of Amador and Wacker[7] using *p*-nitrophenyl phosphate as substrate. Assay of nucleotide pyrophosphatase was performed according to the procedure of Skidmore and Trams[8] with NADH as substrate in a total volume of 3 ml at pH 9.5 with 100 mM 2-amino-2-methyl-1,3-propanediol HCl. 5'-Nucleotidase activity with AMP as substrate was determined by the method of Touster *et al.*[9] at pH 9.1 in a total volume of 1.0 ml and was stopped by addition of 2.5 ml of 16 % trichloroacetic acid. Subsequent assay for inorganic phosphate was performed according to the method of Ames[10] using a total volume of 3.0 ml and obtaining absorbance readings at 820 nm in a Bausch and Lomb Spectronic 20 colorimeter. Mg^{2+}-activated ATPase and ouabain sensitive Na^{+}–K^{+}–Mg^{2+}-activated ATPase were assayed according to the method of Post and Sen[11] in a total volume of 3.0 ml using ATP as substrate. The released P_i was quantitated spectrophotometrically following butanol extraction by the procedure of Parvin and Smith[12]. Protein was determined according to the modified Lowry procedure of Miller[13] using bovine serum albumin as standard.

Gel filtration of skim milk

In light of the evidence of membrane material in skim milk it became of interest to explore other methods of isolation which might be applied directly to the skim milk to facilitate purification of the membrane material and to indicate evidence of its size distribution and properties. In this connection gel filtration according to the method of Sachs and Painter[14] was usefully applied to skim milk. Siliconized glass beads were poured into a glass column (2.5 cm × 100 cm) to a level of approximately one-half (41 cm) the height. Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey) in 0.5 % NaCl was then slurried into the column to yield a total column height of 82 cm. After application of the sample (2 ml) to the column, elution was effected in 0.5 % NaCl at 4 °C with a head pressure of 45 cm of the NaCl solution. This provided a flow rate of 15 ml/h. Elution patterns were determined by absorbance readings at 280 nm and fractions from the column were assayed for lipid phosphorus and 5'-nucleotidase according to methods previously specified.

Electron microscopy

All fractions of both centrifugal techniques were viewed in the electron microscope according to the following preparations which are more fully described by Stewart *et al.*[2]. Negative staining was performed by mixing equal volumes of sample and 2.0 % phosphotungstic acid. One drop of this mixture was transferred to a 200 or 300 mesh carbon-coated grid. After standing for 30 s the grid was blotted with filter paper to remove excess stain. Samples for thin sectioning were fixed in 3 % glutaraldehyde and stained in saturated uranyl acetate for 1 h. After embedding the material in the medium of Spurr[15], and hardening, thin sections were prepared

on a Sorvall-Porter and Blum ultramicrotome MT-2 with a glass knife. Subsequently, the sections were stained with lead citrate and examined in an Hitachi HU-11E electron microscope at 75 000 v.

RESULTS

Data concerning the distribution of cholesterol and lipid phosphorus in bovine and caprine skim milks are presented in Tables I and II. Sedimentationof chloresterol and lipid phosphorus was closely correlated in all the various fractions. In confirmation of the findings of Stewart *et al.*[2] most of both lipid components was found in the so-called fluff or membrane-rich fractions (D3 and G2). Free cholesterol to phospholipid ratios of fractions obtained from differential and linear gradient ultracentrifugations also are presented in Tables I and II. Although the values for the bovine and caprine

TABLE I

DISTRIBUTION OF LIPID PHOSPHORUS AND CHOLESTEROL AND CHOLESTEROL–PHOSPHOLIPID RATIOS FOR FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF BOVINE AND CAPRINE SKIM MILK

Values represent average of three samples determined in duplicate. Lipid phosphorus ranges from 0.4 to 0.8 mg/100 ml of skim milk. Unesterified cholesterol accounts for approx. 85% of the cholesterol in bovine milk[16]. Abbreviations: P, lipid phosphorus; C, unesterified cholesterol; C/P, cholesterol–phospholipid molar ratio; D1, residual cream (milk fat globules); D2 clear infranatant; D3 fluff or membrane-rich fraction; D4, casein pellet.

Sample from tube	% *distribution* (*bovine*)			% *distribution* (*caprine*)		
	P	*C*	*C/P*	*P*	*C*	*C/P*
D 1	14	14	0.36	16	24	0.52
D 2	8	—*	—*	13	—*	—*
D 3	62	65	0.31	47	49	0.35
D 4	14	14	0.34	24	30	0.40

* Data not included. Subsequent analyses showed these fractions from other samples to have C/P ratios in the range of 0.30 to 0.40.

TABLE II

DISTRIBUTION OF LIPID PHOSPHORUS AND CHOLESTEROL AND CHOLESTEROL–PHOSPHOLIPID RATIOS FOR FRACTIONS OBTAINED BY LINEAR GRADIENT ULTRACENTRIFUGATION OF BOVINE AND CAPRINE SKIM MILK.

Values represent average of three samples determined in duplicate. Abbreviations: P, lipid phosphorus; C, unesterified cholesterol; C/P, cholesterol–phospholipid molar ratio; G1, residual cream (milk fat globules) *plus* upper portion of gradient; G2, membrane-rich fraction; G3, lower portion of gradient; G4, casein pellet.

Sample from tube	% *distribution* (*bovine*)			% *distribution* (*caprine*)		
	P	*C*	*C/P*	*P*	*C*	*C/P*
G1	16	19	0.45	23	38	1.04
G2	54	49	0.30	54	50	0.60
G3	21	26	0.43	14	8	0.34
G4	9	6	0.25	8	4	0.26

skim milk membrane fractions (0·30–0.60) are lower than those reported for rat liver plasma membrane, for example 0.79–0.83[9], they are similar to the values of 0.24–0.50 (molar basis) reported by Thompson *et al.*[17] for milk fat globule membrane and the 0.31 and 0.30 for bovine mammary Golgi and plasma membranes by Keenan and Huang[18].

A comparison of cholesterol to phospholipid ratios between Tables I and II reveal that a number of the fractions from the gradient separation (Table II) had higher values than those obtained by differential centrifugation (Table I). This almost certainly means that the membrane material in skim milks separated on the gradient contained more cholesterol than those submitted to differential centrifugation. The reason for this difference between these milks is not known. On the other hand, comparing ratios for the two principal membrane fractions in the bovine skim milks, D3 and G2, very nearly identical values were found (0.31 and 0.30 respectively). Regarding the caprine data, it is evident that elevated ratios were found in the surface layers G1 and D1 and in the next lower layer of the gradient separation G2. While the reason for these variations are uncertain we suspect that the smaller fat globules of goat's milk, in comparison to cow's milk, may be removed less efficiently from the skim milk and these, having a higher cholesterol to phospholipid ratio elevate the values for Fractions G1 and D1. The ratio for G2 may have been altered as a result of mixing when G1 was removed.

Two-dimensional thin-layer chromatography of skim milk membrane polar lipids demonstrated the presence of characteristic milk phospholipid classes. As shown by representative results given in Table III skim milk membrane material displays phospholipid composition similar to that of milk fat globule membrane and mammary cell plasma membrane[19]. The relatively high level of sphingomyelin is a distinguishing feature of these membranes. Cardiolipin, which one might expect to find if mitochondrial membranes were present, was not detected. The distribution of enzyme activities in the various skim milk fractions, Table IV, was calculated on the basis of units of activity per ml skim milk. These data demonstrate that enzyme distributions in the tube following differential ultracentrifugation were generally similar to those for phospholipid and cholesterol although the activity for nucleotide pyrophosphatase tended to concentrate in the membrane fraction to a somewhat

TABLE III

TYPICAL PHOSPHOLIPID COMPOSITION OF BOVINE SKIM MILK MEMBRANE CONCENTRATE FRACTION (D 3) COMPARED WITH THE PHOSPHOLIPID COMPOSITION OF BOVINE MILK FAT GLOBULE MEMBRANE AND BOVINE MAMMARY CELL PLASMA MEMBRANE

Individual phospholipids as percentages of total lipid phosphorus. Abbreviations: MFGM, bovine milk fat globule membrane; PM, bovine mammary cell plasma membrane

	D3	*MFGM**	*PM**
Sphingomyelin	22.4	21.9	24.5
Phosphatidylinositol	9.4	10.5	12.7
Phosphatidylserine	12.3	11.6	8.5
Phosphatidylcholine	35.9	28.7	29.0
Phosphatidylethanolamine	20.0	27.5	25.0

* Data from Keenan *et al.*[19].

TABLE IV

DISTRIBUTION OF UNIT ACTIVITY AND SPECIFIC ACTIVITY OF CERTAIN ENZYMES PRESENT IN BOVINE SKIM MILK MEMBRANE CONCENTRATE FRACTION (D3) AND R-FRACTION FOLLOWING DIFFERENTIAL ULTRACENTRIFUGATION OF SKIM MILK

R is the designation given to skim milk *minus* the membrane concentrate fraction (D3) recovered by differential ultracentrifugation. Values are average of four samples analyzed in triplicate.

	% *Distribution**	*Specific activity*
*Alkaline phosphatase***		
D 3	70	2.05
R	30	0.3
*5'-Nucleotidase***		
D 3	6.	132.
R	36	11.4
*Nucleotide pyrophosphatase****		
D 3	82	167.
R	18	4.6

* Units of activity in fraction/total activity · 100.
** Specific activity as nmoles P_i released per min per mg protein.
*** Specific activity as a change of $2.5 \cdot 10^{-5}$ absorbance units/min per mg protein.

greater degree than for the other two enzymes. Specific activities for the enzymes are also presented in Table IV. The values for 5'-nucleotidase and nucleotide pyrophosphatase are similar to those reported by Patton and Trams[20] for milk fat globule membrane. Specific activities of alkaline phosphatase for lipoprotein particles prepared from fat globules and separated milk were reported to be 8.7–57.2 (ref. 21) and 48 (ref. 22). In contrast, skim milk membranes demonstrated much less activity with an average of 2.1 for four determinations which is similar to the value reported by Morton[21] for separated (skim) milk. The skim milk membrane material displayed neither (Na^+–K^+–Mg^{2+})-ATPase activities which verifies preliminary data of E. G. Trams (personal communication). The apparent absence of ATPase was confirmed by three different assay techniques, one of which involved [γ-^{32}P]ATP as substrate. In this latter variation, released $^{32}PO_4^{3-}$ was obtained by butanol extraction[12], and its radioactivity assayed by scintillation counting.

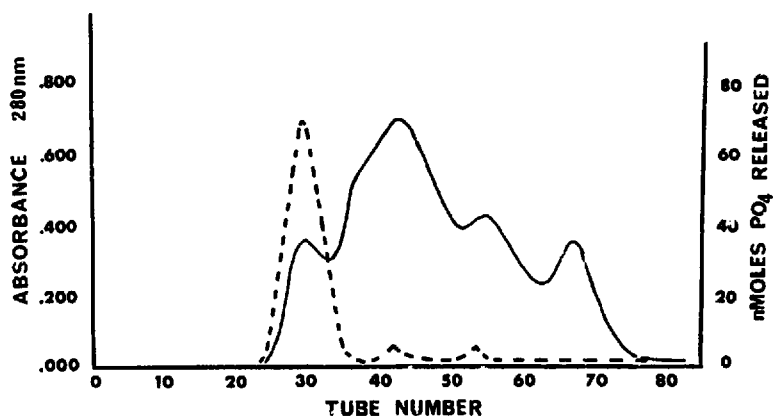

Fig. 2. Separation of bovine skim milk (2 ml) on Sepharose 4B gel: —, material absorbing at 280 nm; ——, pattern of elution for 5'-nucleotidase activity. The void volume, first 95 ml contained >90% of both the 5'-nucleotidase and lipid phosphorus in the sample.

From experiments on direct gel filtration of skim milk it was observed that greater than 90% of both the lipid phosphorus and the 5'-nucleotidase activity were eluted in the void volume, the first 95 ml of eluate. The relationship of 5'-nucleotidase activity to the total elution pattern in a typical gel-filtration run, one of four such separations, is shown in Fig. 2. Following the void volume not more than 2–3% of the total lipid phosphorus or 5'-nucleotidase activity was recovered in the eluate which contained most of the material absorbing at 280 nm.

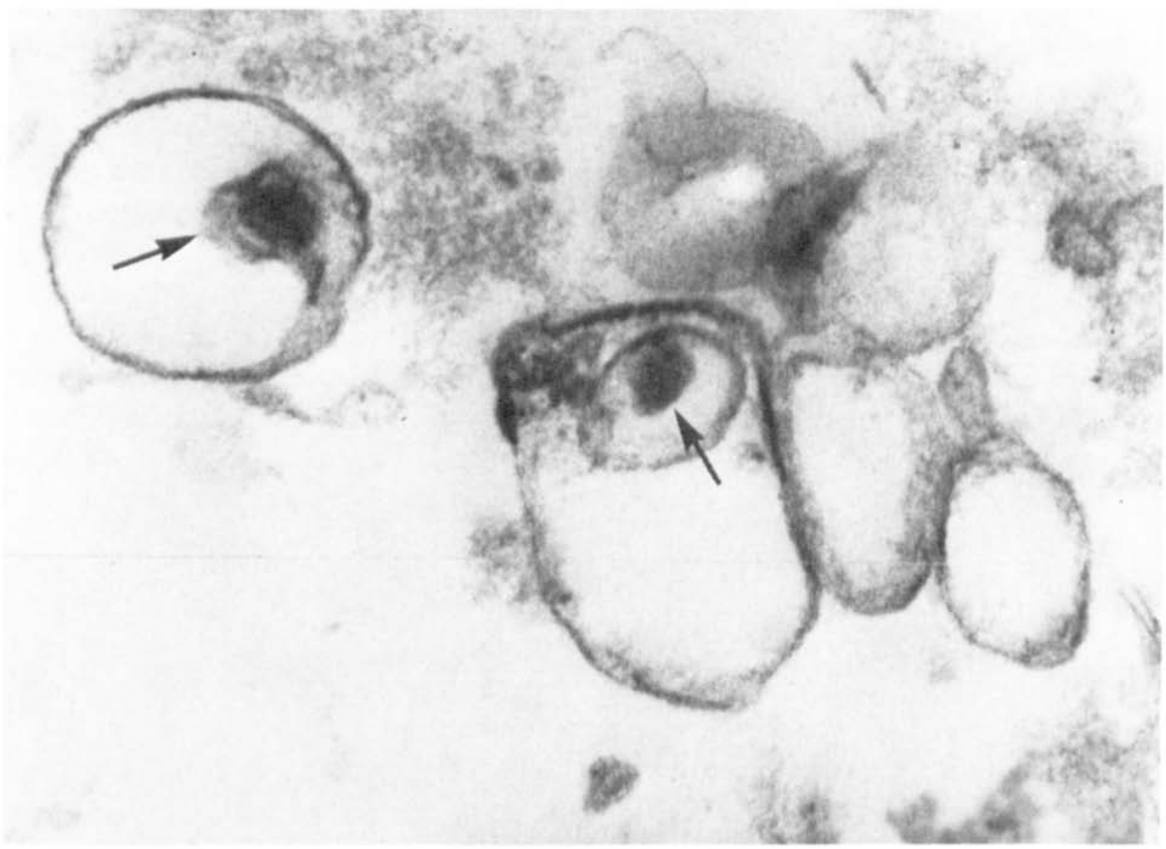

Fig. 3. Electron micrograph of fixed and embedded bovine skim milk membrane material isolated by ultracentrifugation in a sucrose gradient. Membrane-bound vesicles, some of which contain dark casein granules (arrows), are shown. Overall length of arrow represents 0.25 μm.

Electron microscopy generally confirmed the observations of Stewart *et al.*[2] regarding the presence of membrane-bound vesicles and microvilli in the fluff fraction (D 3) from skim milks. Moreover the same kinds of structures were observed (Fig. 3) in the phospholipid cholesterol-rich fraction (G 2) obtained by ultracentrifugation of dialyzed skim milk in a linear gradient.

DISCUSSION

Currently available evidence suggests that the plasma membrane in the apical or secreting end of the lactating cell is in a state of flux. It undergoes loss as it envelopes milk fat globules in their process of secretion and it gains when Golgi vesicles fuse with plasma membrane in emptying vesicle contents out of the cell. This implies an interrelatedness, in fact a precursor–product relationship, between Golgi, plasma and milk fat globule membranes.

The results of our present study support and expand upon earlier evidence[1,2]

that bovine and caprine skim milks also contain cell membrane material. The disposition of free cholesterol in correspondence to that of lipid phosphorus in fractions by the two centrifugal isolation techniques and the further correlation of activity for certain plasma membrane marker enzymes (Table IV and Fig. 2) with the cholesterol–phospholipid distribution strongly supports the membrane nature of the material.

The data indicate that the membrane fragments in question are derived from the plasma membrane or possibly from Golgi vesicles the membranes of which by virtue of the secretion process merge with plasma membrane. The relatively high sphingomyelin (20–25% of lipid phosphorus), and free cholesterol contents and the presence of enzymes with relatively high specific activity characteristic of plasma membrane, such as 5′-nucleotidase, support the identity as plasma membrane. Microvilli, detected in the skim milk membrane fraction in this and an earlier study[2] would necessarily orginate from the plasma membrane. The complete lack of ATPases in the skim milk membranes is somewhat puzzling although it appears to be questionable whether (Na^+–K^+–Mg^{2+}):ATPase is present in plasma membrane in the apical region of the lactating mammary cell (for further discussion see ref. 23). The cholesterol to phospholipid molar ratios for the skim milk membranes (Tables I and II) are relatively low in comparison to values reported for plasma membrane of rat liver. This low ratio could be a natural consequence of rapid turnover of plasma membrane as a result of the secretory mechanisms. Moreover, membrane fusion required in the secretory processes may be facilitated by lower levels of cholesterol in the Golgi and plasma membranes. From the gradient centrifugation experiments skim milk membranes were estimated to have densities of 1.10–1.15 which values are within those reported by Keenan *et al.*[24] for both plasma membrane of the lactating cell and milk fat globule membrane.

The exploratory experiments on use of Sepharose 4B indicated that this material will be useful for separating skim milk membranes from the classical milk proteins. This gel separates proteins in the molecular weight range $1 \cdot 10^5$–$20 \cdot 10^6$. It appeared to hold effectively the milk proteins including the casein micelles of milk which range from 0.03 to 0.3 μm in diameter and exhibit apparent molecular weights of several million. However, the membrane material (Fig. 2) almost exclusively passed through the column in the void volume. We have reasoned that skim milk would be a logical place to search for membrane subunits, if the condensation of subunits represents a mechanism by which membranes might form. Our gel-filtration data suggest that small membrane subunits of a size such as represented by the cross-sectional diameter of a typical plasma membrane (about 0.01 μm) do not exist in milk. From the distribution of the cholesterol and phospholipids as well as from the ultrastructural appearances of the various skim milk fractions we reason that virtually all of the lipid phosphorus and unesterified cholesterol is contained in membrane fragments and vesicles. On ultracentrifugation these fragments either concentrate in the so-called fluff fraction or they are swept to the top or the bottom of the tube in association with residual small milk fat globules or casein micelles respectively.

One of our principal interests in the phenomenon of constant membrane export into milk by the lactating cell is to develop experimental approaches to the turnover of membrane components. The milk system appears to be unique in not

requiring that cells be disrupted (with concomitant generation of artifacts) in order to obtain membrane material.

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