MICRO LIPID DROPLET PRECURSORS OF MILK LIPID GLOBULES

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(ABSTRACT)

The lipid in milk (milk fat) is found in the form of droplets known as milk lipid globules (MLG). These milk lipid globules are encompassed by a unit membrane known as the milk lipid globule membrane (MLGM) which is derived from the apical plasma membrane of the mammary epithelial cell during secretion. In lactating mammary epithelial cells, immediate precursors of milk lipid globules appear to be cytoplasmic lipid droplets (CLD). These cytoplasmic lipid droplets have diameters >1 μm and are characterized by an electron dense, granular surface coat. A previously unrecognized group of structures with diameters <.5 μm, which resemble cytoplasmic lipid droplets in matrix and surface coat appearance, has been observed. The surface coat of these triacylglycerol containing structures, termed micro lipid droplets (μLD), was similar to that of cytoplasmic lipid droplets in enzyme and polypeptide composition. Morphological evidence suggested that these small structures may originate from rough endoplasmic reticulum (RER) and fuse with cytoplasmic lipid droplets. Immunochemical studies showed homology of certain proteins among the rough endoplasmic reticulum, micro lipid droplets and cytoplasmic lipid droplets, which supported the possibility of an

endoplasmic reticulum origin of these droplets. The rate of incorporation of [1-^{14}C]-palmitate and [1,2,3-^{3}H]-glycerol into lipid of RER, μLD, CLD and MLG fractions suggested a possible translocation pathway of triacylglycerols from the rough endoplasmic reticulum to cytoplasmic lipid droplets. The micro lipid droplets seem to provide triacylglycerols to support growth of cytoplasmic lipid droplets. In addition, morphological evidence suggested that these micro lipid droplets can be secreted directly in a manner similar to cytoplasmic lipid droplets, providing for the small lipid globules in milk. Little is known concerning the biochemical processes of milk lipid secretion but it is thought that butyrophilin, a glycoprotein found in milk lipid globule membrane, may play a role. After treatment of mammary epithelial cells with tunicamycin, butyrophilin content of this membrane is reduced. Thus a method for the study of the physiological role of this glycoprotein is proposed.

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INTRODUCTION

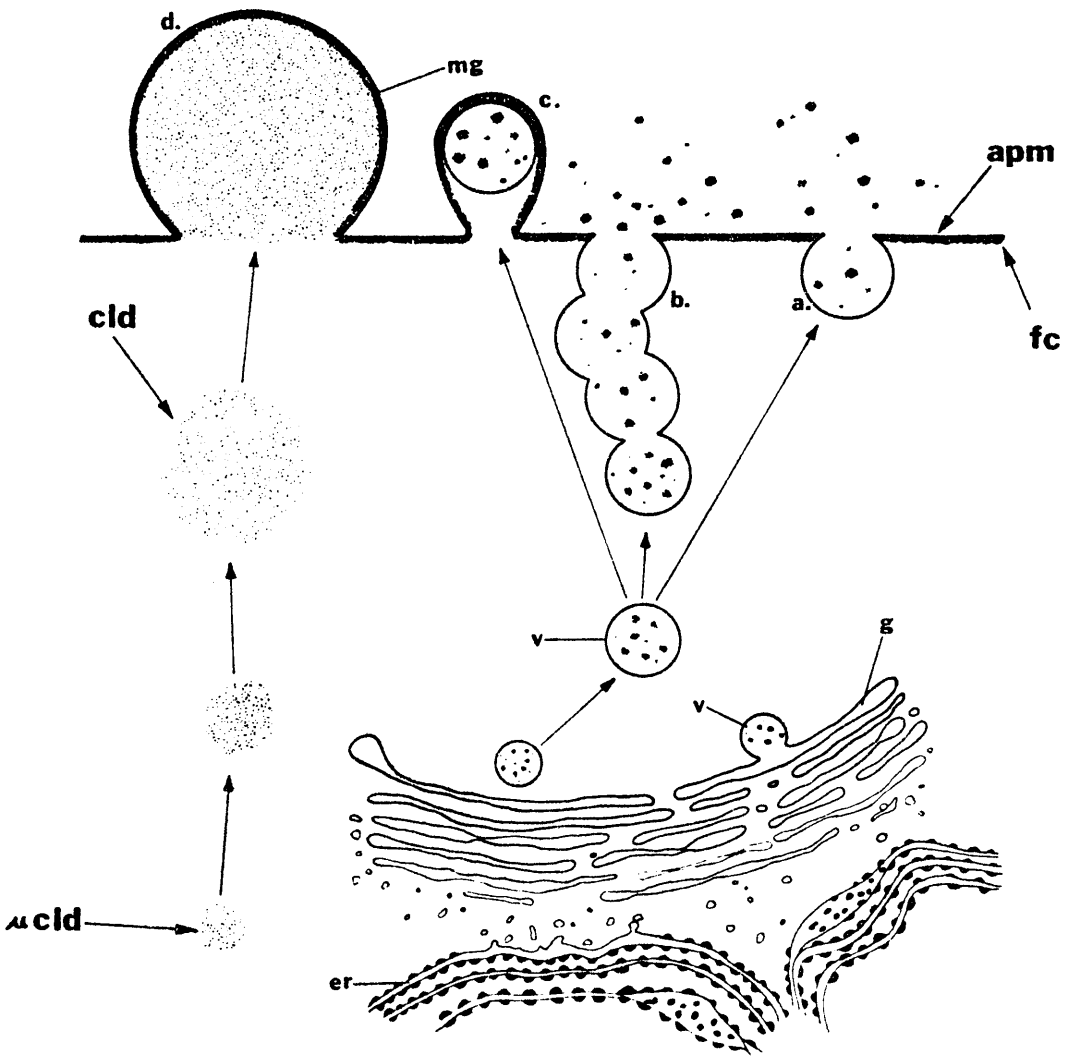
Milk and the processes of milk synthesis and secretion have been studied intensively during the past 100 years. Innovations in lipid and protein chemistry along with development of electron microscopic techniques have enabled scientists to see more clearly the complexity of milk. To date, milk fat has been determined to consist of over 400 different fatty acids (1) while a large number of different proteins have been determined to exist in milk from various species (2).

Milk synthesis can be divided into three major categories. These are :the synthesis of proteins, of lipids, and of carbohydrates. Once synthesized these components are secreted from the cell through two major pathways (Fig. 1). Proteins and carbohydrates are packaged in the Golgi apparatus and are secreted exocytotically via Golgi secretory vesicles (3). Lipids are secreted by a different route in which the lipid droplet buds from and becomes enveloped in the apical plasma membrane of the cell, much like the budding of a virus from its host cell (4).

The following discussion is concerned with the synthesis of milk lipids. In particular, the development of the cytoplasmic lipid droplet (CLD), which is the intracellular precursor of the milk lipid globule, will be examined. It is proposed that growth of the cytoplasmic lipid droplet as it moves apically through the mammary epithelial cell is a direct result of fusion processes with smaller lipid droplets originating from the endoplasmic reticulum. These small intracellular lipid droplets, known as micro lipid droplets (μLD), have recently

been isolated and characterized (5). Evidence supporting the proposed role of micro lipid droplets in growth of cytoplasmic lipid droplets is presented (6). In addition, some aspects of secretion of cytoplasmic lipid droplets will be discussed.

Figure 1. Secretion of milk constituents from mammary epithelial cells. Abbreviations: er, endoplasmic reticulum; g, Golgi apparatus; v, secretory vesicle; apm, apical plasma membrane; fc, fuzzy coat material on cytoplasmic face of apical plasma membrane; μCLD, micro cytoplasmic lipid droplet; CLD, cytoplasmic lipid droplet; mg, forming milk lipid globule. a and b) Observed pathways of carbohydrate and protein secretion involving exocytosis of secretory vesicles. c) Secretion of a secretory vesicle as if it were a lipid droplet. d) The cytoplasmic lipid droplet grows in size as it moves apically through the cell to be secreted. During secretion the droplet is encompassed by apical plasma membrane forming a milk lipid globule membrane.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LITERATURE REVIEW

Triacylglycerol synthesis in the mammary gland

Early experiments designed to elucidate the origin of cellular milk lipid precursors measured the difference in lipid composition of blood plasma before and after it had circulated through the mammary gland. It was determined, in lactating cows, that the portion of plasma lipid taken up by mammary epithelial cells was the neutral lipids, consisting largely of the blood triacylglycerols (7). However, this could not be the only source of lipids utilized by the mammary gland since there existed short and medium chain length fatty acids in milk that were not present in the blood plasma (8). In addition, it was observed that the lactating mammary gland possessed a high respiratory quotient, suggesting that some part of the milk fat was synthesized in the gland (9). The enzyme responsible for fatty acid synthesis in animals is the cytosolic fatty acid synthetase complex (10). Fatty acids of varying chain length are synthesized by successive addition of two carbon units derived from acetyl-CoA. Chain termination responsible for the synthesis of short and medium chain length fatty acids has been shown to be a result of transacylation reactions. This is in contrast to the acylthioester hydrolase responsible for release of longer chain fatty acids (11).

To determine the contribution of de novo synthesis to the fatty acid pool within the cell, a survey of the fatty acids in milk from lactating goats after an IV injection of [^{14}C]-acetate was undertaken (12). It was determined that fatty acids less than 16 carbons long

were synthesized by the goat mammary gland while those greater than 16 carbons were absorbed from the blood plasma. Palmitate was determined to come from both sources. The fatty acid composition of milks from different species varies, reflecting differences in the contributions of de novo synthesis and transport of these fatty acids (Table I) (13).

Lipids derived from the circulation enter the mammary epithelial cell through its basal membrane. These lipids are made available to the cell by the hydrolysis of chylomicrons and very low density lipoproteins (VLDL) of blood plasma (14) by lipiprotein lipase (15). It has been proposed that the mammary gland synthesizes lipoprotein lipase as a proenzyme which can then be exported to the capillary endothelial cells (16). The mode of transport of fatty acids into the cell remains to be determined. In conclusion, the lipids utilized by both ruminant and nonruminant mammary epithelial cells to synthesize milk triacylglycerols are obtained through two separate pathways. These are the uptake of plasma lipids from the circulation and the de novo synthesis of fatty acids of varying chain length within the cell.

Before these fatty acids can be incorporated into triacylglycerols or other complex lipids they must first be acetylated. Acyl-CoA synthetases are responsible for this activation. The activated product is the Acyl-CoA ester which can be utilized in triacylglycerol synthesis (8).

Various routes have been proposed to account for triacylglycerol synthesis in the mammary gland (17). The glycerol-3-phosphate pathway is the route of primary importance (8). This pathway requires the

Table I. Fatty acid composition of milk fats of various species

Species	Approx. mol% of fatty acids												
	4:0	6:0	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	>18:3
Rabbit	-	1	45	23	1	1	9	1	3	8	9	1	-
Rat	-	1	4	14	12	15	30	2	2	13	18	-	-
Sheep	13	6	4	12	6	11	21	3	9	15	2	1	-
Cow	12	4	2	4	4	10	24	2	12	24	2	1	-
Goat	9	6	5	11	4	10	21	2	12	21	2	-	-

initial phosphorylation of glycerol via a glycerol kinase followed by the addition of two molecules of fatty acids forming phosphatidic acid. Phosphatidic acid is converted to a diacylglycerol by phosphatidic acid phosphorylase and is subsequently acetylated at position 3 to form the triacylglycerol. This sequence of reactions was proposed by Kennedy for triacylglycerol synthesis in liver (18). Pynadath and Kumar (19) determined this pathway also exists in the lactating goat mammary gland by incorporating [1-^{14}C]-acyl-CoA into glycerides with DL-α-glycerophosphate or α,β-diglyceride as the acyl acceptors in vitro. Additional evidence for the existence of this pathway in the mammary gland was provided when Patton showed the existence of phosphatidic acid in rat mammary gland after an IV injection of ^{32}P labeled phosphoric acid (20).

Other pathways proposed to play a role in the synthesis of triacylglycerols require 2-monoglyceride or dihydroxyacetone-3-phosphate as acceptors. These pathways seem to make a smaller contribution to the overall triacylglycerol synthesis than does the glycerol-3-phosphate pathway but the relative contribution of these additional pathways must still be determined (13).

Since the lipid droplet is greater than 95% triacylglycerol (17,21) it has been assumed that lipid droplet synthesis takes place at the site of triacylglycerol synthesis. The enzymatic activities involved in triacylglycerol synthesis have been localized in the microsomal fraction of lactating rat (22,23) and goat (24) mammary gland. These results support the earlier observations of Stein and Stein, who demonstrated the incorporation of radiolabeled palmitate into lipid at the endoplasmic reticulum of lactating mouse mammary

gland within 1 minute after an IV injection of the labeled fatty acid (25). Together these observations suggest that the rough endoplasmic reticulum is the site of triacylglycerol synthesis in the mammary epithelial cell.

Formation and growth of the cytoplasmic lipid droplet

Although the mechanism of triacylglycerol synthesis is well understood, relatively little is known about the process by which the cytoplasmic lipid droplet is formed or how this droplet grows in size as it moves apically through the epithelial cell to be secreted. Although various theories have been postulated for these processes, there is no overwhelming support for any one particular theory.

In considering the synthesis and growth of these cytoplasmic lipid droplets it is important to discuss some general characteristics of these events. Both the synthesis and subsequent growth of these lipid droplets require the accumulation of triacylglycerols; thus the physical state of these triacylglycerols becomes an important consideration. If a lipid droplet is composed mainly of triacylglycerols possessing low melting points, the lipid droplet will be in a liquid state at the animals' body temperature. In a liquid state, new triacylglycerols added to the surface of the forming droplet can be incorporated into the core of the droplet much easier than if the droplet were solidified in whole or in part (17). The fluidity of the triacylglycerols synthesized in the cell is dependent on the melting points of its constituent fatty acids. Fatty acids of shorter chain lengths have lower melting points than do longer chain length fatty

acids. It can be seen from Table I that 35 to 50% of the total fatty acids associated with triacylglycerols of milk from rabbit, rat, sheep, cow, and goat consist of chain lengths of less than 16 carbons. Incorporation of these shorter fatty acids primarily into the sn-3-position of the triacylglycerol can help to insure the necessary fluidity of the intracellular lipid droplet (26). This has been proposed to be a possible regulatory control on lipid droplet formation. A second mechanism proposed to increase the fluidity of triacylglycerols in the lipid droplet is the conversion of stearic acid (18:0) to the lower melting oleic acid (18:1) (27). The ratio of oleic to stearic acids in ruminant milk lipid is about 2 to 1 (Table 1).

Synthesis of lipid droplets at the endoplasmic reticulum has been proposed to occur in different ways. One proposal is that, due to the hydrophobic nature of triacylglycerols, the lipid droplet would likely form within the bilayers of the endoplasmic reticulum (4). This proposal raises the question of how the bilayer of the endoplasmic reticulum containing transmembrane proteins could open, allowing the lipid droplet room for growth within the membrane. An answer to this question has been proposed by Long and Patton (28), who determined that there existed a direct relationship between triacylglycerol synthesis and the synthesis of phosphatidylcholine. These investigators suggested that the addition of newly synthesized phosphatidylcholine to the endoplasmic reticulum membrane may allow for the increased fluidity of the membrane, providing for clearance of transmembrane proteins from the particular site of lipid synthesis. This could

facilitate separation of the bilayer, supporting growth of the lipid droplet within the membrane. A similar model has been proposed for synthesis of lipid droplets within the bilayers of the endoplasmic reticulum of various tissues (mammary gland, adipose tissue, skeletal muscle, etc.) by Scow et al. (14).

A second possibility is that the lipid droplet could be synthesized within the lumen of the endoplasmic reticulum cisternae (8). As the lipid droplet is released by the endoplasmic reticulum these models propose that it is encompased by membrane material derived from the endoplasmic reticulum. In the case where the droplet forms within the bilayer of the membrane, the lipid droplet is covered by one of the leaflets of the bilayer, while it would seemingly be enveloped in an intact membrane if the droplet were synthesized within the lumen of the endoplasmic reticulum. Biochemical evidence for such a coating, assumed to be derived from the endoplasmic reticulum encompassing the cytoplasmic lipid droplet, has been obtained (4,17,29).

In contrast, Peixoto De Menezes and Pinto da Silva (30) have proposed a different process of lipid droplet formation based on observations from freeze fracture electron microscopy. These investigators have proposed a 3 step process for the formation of cytoplasmic lipid droplets which still utilizes the endoplasmic reticulum as a nucleation site for droplet synthesis but in a manner different than previously proposed. The first event in this proposed scheme is the clearing of proteins from the area of endoplasmic reticulum responsible for droplet synthesis, forming a particle-free membrane area as viewed

by freeze fracture. This step is not unlike what Long and Patton propose to occur as a result of increased levels of phosphatidylcholine within the membranes of the endoplasmic reticulum. The second and third steps are the apposition of these particle-free bilayers of endoplasmic reticulum membrane to the periphery of the growing lipid droplet, followed by the conversion of the membrane lipids to triacylglycerols which are incorporated into the lipid droplet. Thus the scheme suggests that the growing lipid droplet develops in the cytosol attached to and at the expense of the membranes of the endoplasmic reticulum. This proposed mechanism does not account for the proteins incorporated at the periphery of the droplet as part of the monolayer or coat material seemingly derived from the endoplasmic reticulum.

There is little definitely known about the formation of the lipid droplet in the mammary epithelial cell and there is even less known about the mechanism(s) of growth of these droplets as they move apically through the cell to be secreted. The fact that these lipid droplets do increase in size is based on the relative size distribution of these droplets within the cell. An attempt to quantify such growth was made by Stemberger and Patton (19). They observed two populations of lipid droplets within the epithelial cell. These were small droplets (<1.5 μm) found throughout the cell and larger droplets (>1.5 μm) found primarily in the apical regions of the cell. Different models have been proposed to account for this growth of the cytoplasmic lipid droplet as it moves to be secreted (17).

The results of Stein and Stein (25) show the incorporation of [^{3}H]-palmitate into cytoplasmic lipid droplets found in the apical

region of the cell along with milk lipid globules found in the lumen within 10 minutes after injection of isotope into mice. They suggested that this could be due to the addition of newly synthesized lipid to preexisting droplets as well as the synthesis of new lipid droplets. The way in which newly synthesized lipid could combine with preexisting lipid droplets has been a subject of much speculation.

One explanation for the growth of cytoplasmic lipid droplets is the possibility of synthesis of triacylglycerols at the droplet surface. These new triacylglycerols are then incorporated into the growing droplet (17). This is an unlikely possibility, since most evidence indicates that the acyl transferases responsible for triacylglycerol synthesis are localized in the rough endoplasmic reticulum of the mammary epithelial cell (22,24), with little activity associated with the surface coat of the lipid droplet (32). Alternatively, growth could be a result of diffusion of triacylglycerols in the form of micelles from their site of synthesis (RER) to the growing lipid droplet (33). It could also be proposed that triacylglycerols are transported to the growing lipid droplet via some lipid exchange protein similar to the transport of phospholipids between cellular membranes as shown by Wirtz (34). Carrier proteins involved in triacylglycerol transport to the cytoplasmic lipid droplet have not been identified.

The proposal which may seem to be the most obvious is the fusion of small lipid droplets with the larger cytoplasmic lipid droplets in order to provide triacylglycerols necessary for their growth (5,35). Although this mechanism seems reasonable, until recently no direct evidence has been presented which would support this process. In fact,

negative evidence, such as the existence of lipid droplets observed in apposition to one another without any signs of fusing, would suggest that this is not the case (31,36). In addition, many of the small lipid droplets found within the epithelial cells (31) are eventually secreted without undergoing much growth. This is supported by the observations of Walstra (37), who showed that small lipid globules in cow's milk account for about 80% of the total number of globules. Although the number of these lipid globules is large, their contribution to the total milk lipid volume is not great (<5%) due to their small size.

It has recently been observed that small lipid droplets are capable of fusing with larger lipid droplets within the epithelial cell, suggesting that these structures can supply triacylglycerols to the growing lipid droplet (5). In addition, these small lipid droplets have been determined to be biochemically and morphologically similar to the larger cytoplasmic lipid droplets.

Secretion of the cytoplasmic lipid droplet

During secretion of milk lipid from mammary epithelial cells the lipid droplet is enveloped in the apical plasma membrane of the cell; this membrane forms the milk lipid globule membrane (4,38,39). Although morphological evidence has existed for many years, nothing is really known of the factors which are responsible for the movement of lipid droplets from the endoplasmic reticulum to the apical region of the cell. Similarly nothing factual is known of the interactions between the cytoplasmic lipid droplet and the apical plasma membrane

during secretion.

Patton and Fowkes (40) proposed that, upon close approach of the lipid droplet to the apical plasma membrane, London-Van der Waal's forces could play a role in attracting the plasma membrane to the surface of the cytoplasmic lipid droplet, allowing for envelopment of this droplet with the membrane. This was refuted by Wooding (36), whose observations indicated that the lipid droplet never approached close enough to the apical plasma membrane to generate such forces due to the existence of a fuzzy coat material associated with the cytoplasmic face of the membrane.

More recently comparisons of protein found in milk lipid globule membrane and the apical plasma membrane of intact mammary epithelial cells have led some investigators to suggest that there may exist some proteins in the apical plasma membrane that are responsible for binding of the lipid droplet prior to secretion (41). Butyrophilin (Mr 67,000) is a glycoprotein localized in both milk lipid globule membrane and the apical plasma membrane of the mammary epithelial cell (41). Carbohydrate analysis of butyrophilin eluted from SDS polyacrylamide gels indicates that the glycoprotein possesses one carbohydrate chain of the high mannose variety, suggesting that butyrophilin is an N-linked glycoprotein (42). It has been proposed that butyrophilin may possess a hydrophobic domain exposed at the cytoplasmic face of the apical plasma membrane which specifically binds to the intracellular lipid droplet, facilitating the secretion process (41). A second possible role proposed for butyrophilin is the

attachment of the fuzzy coat material to the cytoplasmic face of the apical plasma membrane and thus to the milk lipid globule membrane (43). This fuzzy coat material has been isolated from milk lipid globule membrane and has been determined to be enriched in both butyrophilin and xanthine oxidase (43). Xanthine oxidase, in addition to being found in the fuzzy coat, is also located in apical plasma membrane and milk lipid globule membrane (44). Its role in lipid secretion, if any, has not been elucidated.

The extent to which the milk lipid globule membrane resembles the apical plasma membrane of the cell has not been established. Comparisons of lipid, protein,and enzyme composition of the milk lipid globule membrane to that of plasma membrane of the mammary epithelial cell have been made. In polar lipid composition the two membranes are very similar as determined by thin layer chromatography followed by determination of lipid phosphorus (45). The distribution of fatty acids in the major phospholipid classes is also similar (45). Milk lipid globule membrane is enriched in proteins such as xanthine oxidase and butyrophilin while apparently containing only small quantities of proteins found in the plasma membrane fraction. Many of the dissimilarities between these membranes appear to be due to quantitative rather than qualitative differences (39). 5'-nucleotidase has been shown to be a marker enzyme for plasma membrane isolated from mammary epithelial cells,with a 10 - 15 fold increase in the specific activity relative to the homogenate (46). This enzyme has also been determined to be a constituent of the milk lipid globule membrane,with

a 25 fold increase in specific activity relative to the homogenate (47). Thus,a plasma membrane origin of the milk lipid globule membrane is suggested by various biochemical observations. Such comparisons must be made on the basis of total plasma membrane instead of the more desirable apical plasma membrane since the latter has not been isolated to this point. There is some evidence to suggest that the apical plasma membrane undergoes structural changes allowing for secretion of the lipid droplet. Freeze fracture eletron microscopy suggests a clearing of particles from the portion of the membrane encompassing the lipid droplet as it is secreted (48). This would suggest that the lipid droplet is secreted through a membrane which yields little resistance, since many of the membrane proteins other than butyrophilin and xanthine oxidase seem to be redistributed along areas of apical plasma membrane seemingly not involved in the lipid secretion process.

It can be seen with electron microscopy that during and after secretion of the lipid droplet from the cell there is a close association of the milk lipid globule membrane, the inner fuzzy coat material of this membrane, and the cytoplasmic lipid droplet coat material (4,29). Determining the role of butyrophilin and xanthine oxidase within these membranes may answer some of the questions concerning the process of lipid secretion.

MATERIALS AND METHODS

Materials

The following materials were used: ascorbic acid, $AgNO_3$, AMP, ATP, Bovine Serum Albumin, citrate, ethanolic hydroxylamine, cytochrome c, dithiothreitol, Folin phenol reagent, coomassie blue, α-lactalbumin, NAD, oxytocin, 2-(P-indophenol)-3-(P-nitrophenyl)-5-phenyl tetrazolium (INT), Na_2-P-nitrophenyl phosphate, Triton x-100, tunicamycin, sodium carbonate, and sucrose from Sigma; dextrose, ethyl acetate, 1-amino-2-napthol 4-sulfonic acid, KH_2PO_4, sodium azide, trichloroacetic acid, $MnCl_2$, urea, KSCN, sodium potassium tartrate, $MgCl_2$, NaOH, sulfuric acid, acetic anhydride, Na_2HPO_4, perchloric acid, KCL, methanol, diethyl ether, rexane, acetic acid and silica gel G TLC plates from Fisher; succinate, KCN, cupric sulfate, NaCl, chloroform and acetone from J.T. Baker; acrylamide, bis acrylamide, Tris, SDS and glycine from BRL; Biorad AG1-X2 resin; nitrocellulose from Schleischer and Schuell; hypoxanthine from Kodak; ferric perchlorate from Alfa products; ammonium molybdate from Mallinckrodt; Ready Solv from Bechman; [1-^{14}C]-palmitic acid, 56 mCi/mmol, ^{125}I-Protein A, 30 mCi/mg protein from Amersham; [1,2,3-^{3}H]-glycerol, 38.2 mCi/mmol, UDP-galactose, 302 mCi/mmol from New England Nuclear; goat anti-rabbit IgG-colloidal gold complex from Janssen Pharmaceutica, Beerse, Belgium; Lowicryl K4M low temperature embedding medium, Epon 812, araldite, glutaraldehyde, cacodylate, osmium tetroxide, uranyl acetate, lead acetate, and formvar coated grids from Polysciences. Antibodies against butyrophilin, CLD coat material, μLG membrane,and the material remaining on the droplet after removal of this

membrane were raised in rabbits by T.W. Keenan at VPI & SU and Purdue University.

Methods

Animal sources

Samples for morphological observations were collected from primiparous Sprague-Dawley rats between the 7^{th} and 12^{th} days of lactation and Holstein cows from 2 to 7 years old between the 2^{nd} and 12^{th} months of lactation. For isolation of subcellular fractions mammary tissue was collected from lactating Sprague-Dawley rats or from lactating Holstein or Jersey cows at commercial meat packing plants and transported to the laboratory on ice. The alpine goat used in the tunicamycin experiment was in the 2^{nd} month of its 3^{rd} lactation.

Transmission electron microscopy

Pieces of bovine and murine mammary tissue approximately 1 to 2 mm^3 were quickly removed from sacrificed animals and fixed by immersion in a freshly prepared 1"1 mixture of 4% glutaraldehyde in .1 M cacodylate (pH 7.0) and 2% osmium tetroxide for 1 hour. Samples were then washed briefly with .1 M cacodylate (pH 7.0) and post fixed with buffered 2% osmium tetroxide for 2 hours (49). Following fixation the samples were rinsed in cold buffer, dehydrated in ethanol and acetone and embedded in Epon 812 (50) or a mixture of Epon and Araldite (51). Ultrathin sections of material were cut on a microtome and collected on Formvar coated 100 mesh copper grids. The sections were then stained in aqueous uranyl acetate (52) and lead citrate (53). Tissue sections were examined with a Phillips EM300 or a Zeiss EM10 electron microscope operated at 60 kV.

Freeze-fracture electron microscopy

Samples of rat mammary tissue were fixed by immersion in 2.5% glutaraldehyde in .1 M cacodylate buffer (pH 7.2) for 2 hours at 4°C. These samples were then rinsed with buffer and transferred to a 20% glycerol solution in .1 M cacodylate buffer pH 7.2. Samples were then frozen in liquid Freon-22 cooled by liquid nitrogen. The frozen tissue samples were freeze fractured at -103°C and shadowed at 40°C with platinum-carbon in a Balzers BAR 300 freeze-etch unit (Balzers AG, Liechtenstein). The platinum-carbon replicas were cleaned in chromerge for 2 hours and rinsed in distilled H_2O. The replicas were then washed in a 2:1 chloroform-methanol mixture and mounted on 200 mesh nickel grids. The replicas were examined in a Zeiss EM10 electron microscope operated at 80 kV.

Immunocytochemistry

Samples of bovine and murine mammary tissue were fixed by immersion in 2.5% glutaraldehyde in .1 M cacodylate buffer pH 7.2 for 1 hour at 0°C and then rinsed in cold buffer. The samples were then dehydrated in methanol at -35°C and embedded in Lowicryl K4M for 12 hours at this temperature (54). The Lowicryl resin was polymerized with UV light for 3 days at -35°C. Thin sections were cut and mounted on Formvar coated 200 mesh nickel grids.

Grids were then floated face down on a drop of 1% ovalbumin in phosphate buffered saline, pH 7.4, (PBS = 10 mM Na_2HPO_4, 150 mM NaCl), for 5 minutes at room temperature. These grids were then transferred onto drops of serially diluted affinity purified rabbit anti-CLD protein for

2 hours in a moist chamber. The grids were then washed in PBS and placed on drops of a 20-fold dilution of affinity purified goat anti-rabbit antibody bound to particles of colloidal gold (5 mm) (55). The grids were washed with PBS and stained with 5% aqueous uranyl acetate and lead acetate (56). Two control experiments were performed. Thin sections were incubated with anti-CLD adsorbed with CLD protein for 24 hours followed by the goat anti-rabbit IgG - gold complex and sections were incubated with the anti-rabbit IgG-gold complex alone.

Isolation of subcellular fractions

All operations were done at 0 to 4°C unless otherwise stated. Isolation of cytoplasmic lipid droplets, micro lipid droplets and rough endoplasmic reticulum was achieved utilizing sucrose density gradients (5). Tissue was minced finely and washed to remove entrained milk by suspension in 5 to 10 volumes of .25 M sucrose in 250 ml bottles which were centrifuged at 3000 g for 10 minutes in a Beckman model J-6B centrifuge. This process was repeated until the suspending medium remained clear. The tissue was then homogenized in 1 M sucrose (2 ml/gram tissue) with a Polytron PT-20 for approximately 1 minute at a setting of 5. The homogenate was then filtered through increasing layers of cheesecloth until rapid flow through 2 overlaying double layers of cheesecloth was achieved.

The volume of the homogenate was measured and an equal volume of 2.5 M sucrose was added. The resulting solution was mixed thoroughly and transferred to clear SW27 Beckman centrifuge tubes (15 ml/tube). The homogenate was then carefully overlayed with 10 ml of .8 M sucrose.

H_2O was carefully layered over the .8 M sucrose layer until the volume was within a centimeter from the top of the tube. The gradients were then centrifuged using a SW27 rotor at 100,000 g for 1 hour in a Beckman model L5-50B ultracentrifuge. The cytoplasmic lipid droplet fraction floated to the top of the gradient and was removed with a spatula. This fraction was dispersed into H_2O by brief homogenization with the Polytron at low speed and refloated by centrifuging for 30 minutes at 100,000 g.

The micro lipid droplet fraction was found at the interface between the H_2O and .8 M sucrose layers. This fraction was collected with a Pasteur pipette and mixed with an equal volume of 2.5 M sucrose. The mixture was then placed in centrifuge tubes (15 ml/tube) and overlayed first with .8 M sucrose and then with H_2O as before. The micro lipid droplet fraction was obtained as before and was diluted with several volumes of H_2O. The diluted micro lipid droplets were then centrifuged at 100,000 g for 30 minutes and harvested as a pellet.

Alternately, micro lipid droplets were harvested as two separate subpopulations, termed heavy and light micro lipid droplets. Heavy and light micro lipid droplets were separated on sucrose density gradients consisting of homogenate prepared as previously described and overlayed with successive 8 ml layers of 1 M sucrose, .5 M sucrose,and H_2O. Heavy micro lipid droplets were collected from the 1 M-.5 M sucrose interface, while light micro lipid droplets were found at the .5 M sucrose-H_2O interface. The micro lipid droplet fractions obtained in this way were further purified by repeating this centrifugation. Heavy and light micro lipid droplets were subsequently pelleted as previously described.

For isolation of rough endoplasmic reticulum the supernatant and pellicle lying below the .8 M sucrose layer in the first gradient were pooled, gently homogenized,and diluted with cold distilled H_2O (1 ml H_2O/ 2 ml suspension) (57). This diluted suspension was then centrifuged in a Sorvall RC-2B centrifuge using a SS34 rotor for 15 minutes at 21,000 g. After centrifugation the supernatant was decanted carefully without disturbing any of the pellicle. This supernatant was then layered over a gradient consisting of 10 ml of 1.5 M sucrose layered over 5 ml of 2 M sucrose in TKM buffer (TKM = 50 mM Tris, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$). This gradient was then centrifuged for 16 hours in a SW27 rotor at 100,000 g. The band resting on the 2 M sucrose was collected along with the 2 M sucrose layer. The pooled suspension was then diluted to 2 to 3 times its volume with .25 M sucrose. This solution was centrifuged at 100,000 g for 30 minutes in a SW27 rotor The rough endoplasmic reticulum was harvested as a pellet at the end of this centrifugation.

For preparation of milk lipid globule membrane (35) and the material remaining on the lipid globule after removal of this membrane, cream was separated from milk by centrifugation at 5000 g for 15 minutes. The cream was then washed 2 to 3 times with .9% NaCl by centrifugation under similar conditions. The cream was then resuspended in 2 to 3 volumes of saline wash buffer, chilled and churned in a Waring blender until a hard butter was formed. The aqueous phase was squeezed from the butter and centrifuged at 100,000 g for 3 hours. Milk lipid globule membrane was harvested as a brown pellet. In the cases where the cream fraction was very small due to the limits placed on milk

collection, (i.e. rat), the churning method was not used. In these cases the washed cream was frozen in 15 ml of buffer in a centrifuge tube. The cream was then thawed in a 37°C water bath and centrifuged as were larger volumes.

The butter resulting from the preparation of milk lipid globule membrane was extracted with chloroform:methanol (2:1, v/v) according to Folch (58) and the proteins which partitioned into the aqueous phase and which remained at the interphase of the two phases were precipitated with acetone. These proteins constituted the material remaining with the lipid droplets after the milk lipid globule membranes were removed.

Enzyme determination

The following enzyme activities were determined: acid phosphatase (EC 3.1.3.2) (59), alkaline phosphatase (EC 3.1.3.1) (60), 5'-nucleotidase (EC 3.1.3.5) (61) Mg-ATPase (EC 3.6.1.15) (62), succinate dehydrogenase (EC 1.3.99.1) as succinate-tetrazolium reductase (63) and as succinate cytochrome c reductase (62), xanthine oxidase (EC 1.2.3.2) (64) and lactose synthetase (EC 2.4.1.22) (65). Turbidity of lipids in cytoplasmic lipid droplet fractions interfered with certain spectrophotometric assays. This interference was minimized by extraction of lipids with diethyl ether after termination of the reaction.

Lipid analysis

Lipids were extracted with chloroform:methanol (2:1, v/v) and extracts were washed to remove nonlipid contaminants (58). Total lipids were determined gravimetrically on aliquots of extracts dried to constant weight. Total lipids were separated into phospholipids,

cholesterol,and triacylglycerols on silica gel thin layer chromatography plates developed in hexane - diethyl ether - acetic acid (85:15:1) (66). The plates were dried and then separated lipids were scraped from areas of the plates corresponding to rf's of appropriate standards. Triacylglycerols and cholesterol were eluted from the silica gel with chloroform and the chloroform fraction was clarified by centrifugation. Triacylglycerols were dried under nitrogen to remove the chloroform. Triacylglycerol concentration was determined according to Snyder and Stephens (67). The cholesterol sample was diluted to 5 ml with chloroform and the concentration of cholesterol was determined according to Stadtman (68). Phospholipid concentration was determined according to Rouser et al (69) without prior elution from the silica gel.

Incorporation of radiolabeled precursors into lipids

Primiparous rats nursing 6 to 10 pups received a single intraperitoneal injection of 100 μCi of [1-^{14}C]- potassium palmitate, or 200 μCi of [1,2,3-^{3}H]-glycerol between 7 and 12 days of lactation. At intervals from 3 minutes to 6 hours after injection of radioisotope, rats were sacrificed and inguinal mammary glands were rapidly removed. Endoplasmic reticulum, micro lipid droplets and cytoplasmic lipid droplets were prepared from this tissue as described. Milk was collected from rats prior to sacrifice starting at 10 minutes after label injection. 50 IU of oxytocin was administered intraperitoneally 2 minutes before milking. Rats were milked for approximately 1 minute before sacrifice. Lipids were extracted from milk and subcellular

fractions by the method of Folch (58) and were separated into classes on thin layer plates as described under lipid analysis. Triacylglycerols and phospholipids were also determined as previously described. Radioactivity was determined in a Beckman model LS-3150T liquid scintillation counter.

Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (8%) was done by the method of Laemmli (70). Gels were stained with coomassie blue or by the silver staining method (71). Some protein samples such as the surface coat material of cytoplasmic lipid droplets were prepared by extraction. Lipid was extracted with diethyl ether at 37°C and proteins were precipitated with diethyl ether:acetone (1:1, v/v). The precipitated protein was then washed successively with 100%, 90% and 80% acetone. The protein was then pelleted by centrifugation and dissolved in sample fix solution (10 mM phosphate buffer, pH 6.8; 10% β-mercaptoethanol; 10% glycerol; 5% SDS). This protein solution was then boiled for 5 minutes. Before applying samples to gels, protein was determined by the method of Lowry et al. (72).

Affinity purification of antibodies

Affinity purification of rabbit anti-CLD coat antibody was achieved by passing the antibody fraction through a CNBr-activated Sepharose 6B column on which the antigen had been immobilized (73). Nonspecific antibodies were washed through the column with 5 column volumes of washes A through D listed under immunoblots. The specific antibody was eluted with 3M KSCN and collected in PBS. The antibody was concentrated

in an Amicon filtration cell with a XM100A membrane.

Immunoblots

After electrophoresis, the slab gel was soaked in a pretransfer buffer for 1 to 2 hours to remove SDS (74,75). The gel was then soaked in transfer buffer for 1/2 hour to prevent it from shrinking or expanding during the transfer (75,76). The blotting apparatus was based on the design of Towbin (77) and consisted of the following successive layers: stainless steel plate (cathode), plastic grid, dacron sponge, filter paper, SDS gel, nitrocellulose, filter paper, dacron sponge, plastic grid, stainless steel plate (anode). This sandwich was then placed in a chromatography tank which was filled with transfer buffer to which was added .1% SDS in order to facilitate transfer of larger molecular weight proteins (76). Proteins were transferred at 300 mAmps for 5 hours. After transfer the protein blot was soaked in 5% bovine serum albumin (BSA) in PBS overnight to saturate any free binding sites still remaining on the nitrocellulose filter (77).

Detection of specific proteins was achieved by incubating the blot with affinity purified polyclonal antibodies in 1% BSA•PBS followed by ^{125}I-Protein A in .1% BSA•PBS. The final concentration of antibody during incubation with the blot was 25 to 75 µg of protein/ml. ^{125}I-Protein A (sp.act. >30 mCi/mg protein) was diluted to yield a solution containing .1 to .5 x 10^6 cpm/ml before incubation. Each of these incubations was for 2 hours at room temperature. Excess antibody was removed from the nitrocellulose filter prior to incubation with ^{125}I-Protein A by washing for 20 minutes with each of the following solutions:

A) .5% Triton X-100 in PBS

B) .1% Triton X-100 in PBS

C) .5 M NaCl in PBS

D) PBS

The same series of washes were used to remove excess ^{125}I-Protein A (74). The blot was then dried between filter papers. Autoradiography was done at -70°C. Pretransfer and transfer buffers are defined below.

500 ml Pretransfer Buffer (pH 7.5)		5 l Transfer Buffer (pH 8.8-9.0)	
4 M urea	120 g	25 mM Tris	15 g
50 mM NaCl	1.5 g	12 M glycine	71.5 g
2 nM EDTA	.4 g	±.1% SDS	
10 mM Tris	.6 g		
1 mM dithiothreitol	8 mg		

Tunicamycin infusion

25 mg of tunicamycin was dissolved in 2 ml of ethanol:H_2O (4:1, v/v). This solution was infused into the right half of the mammary gland of a lactating goat. The left side of the gland was not treated in any way and was used as a control. The goat was milked prior to and at 12 hour intervals after infusion. Milk lipid globule membrane was isolated from milk obtained from control and tunicamycin treated glands as previously described. Comparison of the membrane was made on the basis of protein content as determined by SDS gel electrophoresis.

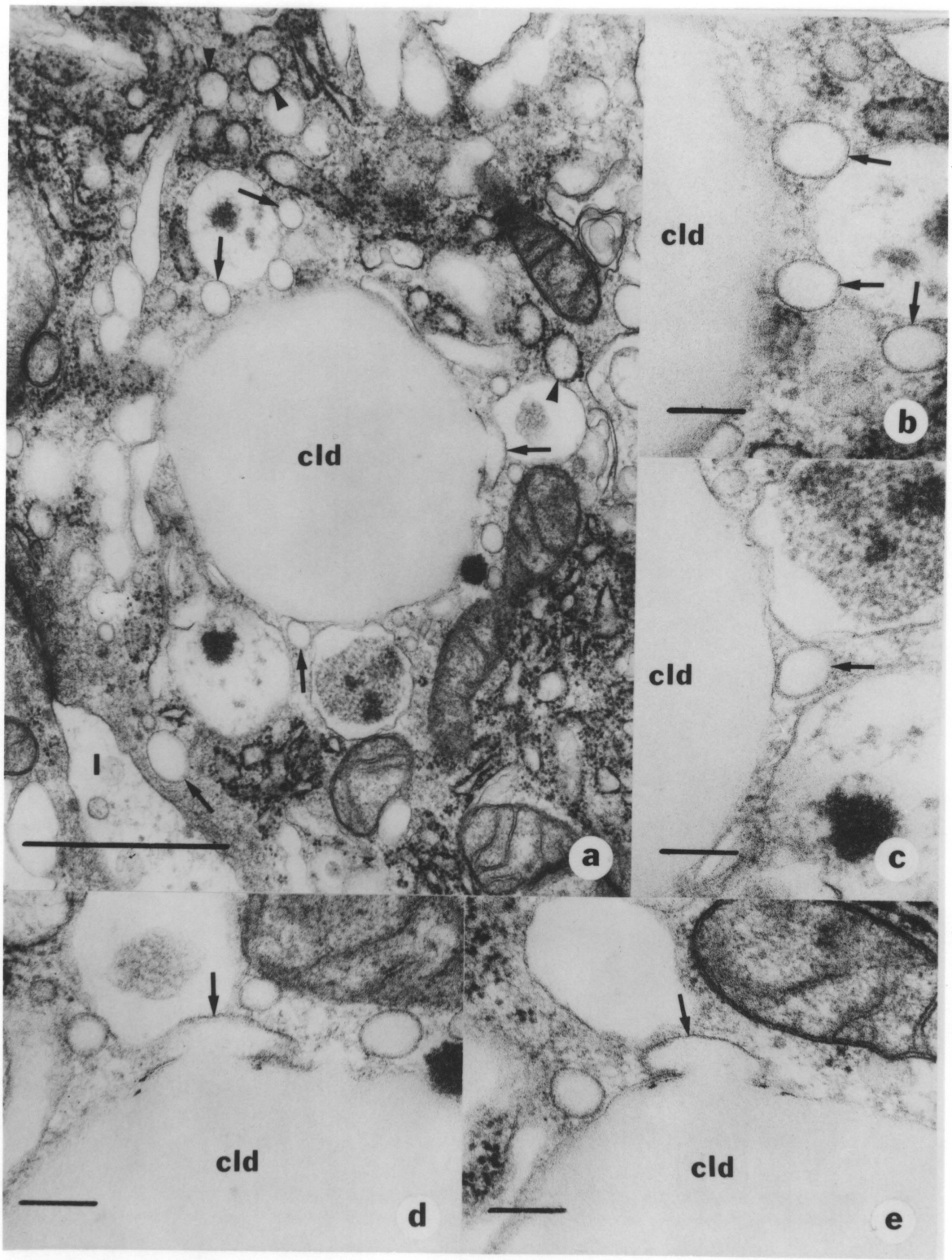
RESULTS AND DISCUSSION

Morphological evidence for the existence of micro lipid droplets

The immediate precursor of the milk lipid globule appears to be the cytoplasmic lipid droplet (5,39). It has been observed that these cytoplasmic lipid droplets seem to grow in size as they move from basal to apical regions of the mammary epithelial cell prior to secretion (31,35). The mechanism(s) by which these cytoplasmic lipid droplets increase in size has not been determined. Although various models have been proposed to describe this process there is no compelling evidence which supports any one particular model (17). Recently in our laboratory a population of small lipid droplets, henceforth known as micro lipid droplets (μLD), (<.5 μm), has been observed. These micro lipid droplets are seemingly capable of fusing to larger cytoplasmic lipid droplets providing triacylglycerols for their growth (5).

In figure 2a, a cytoplasmic lipid droplet (>1.0 μm) can be seen in the midst of a number of micro lipid droplets (<.2 μm). Some of these micro lipid droplets were located close to the larger lipid droplet (2b) while others were actually in contact with the larger lipid droplet (2c). In all cases the micro lipid droplets appeared similar to the larger lipid droplets both in the lack of staining of their constituent triacylglycerols and in the existence of a densely staining coat material surrounding the lipid droplet. This coat material lacked unit membrane structure in both the micro lipid droplet and the larger cytoplasmic droplet (28,29,39). Adjacent sections are shown which

Figure 2. Adsorption of micro lipid droplets onto peripheral surfaces of cytoplasmic lipid droplets in mammary epithelial cells of lactating cow. a) Micrograph of apical region of an epithelial cell showing apparently mature (arrows) and immature (arrowheads) micro lipid droplets in the vicinity of a cytoplasmic lipid droplet (cld) and throughout the cytoplasm. b) Higher magnification of portion of fig. 1a showing three micro lipid droplets (arrows) near the cytoplasmic lipid droplet (cld). c) Higher magnification of portion of 1a showing micro lipid droplet (arrow) in contact with surface coat of cytoplasmic lipid droplet (cld). d and e) Adjacent sections showing apparent fusion of a micro lipid droplet (arrows) onto the peripheral surface of a cytoplasmic lipid droplet (cld). a. Bar 1.0 μm. b to e. Bars 0.2 μm. a. 37,000X. b to e. 70,000X.


cld
l
a
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b
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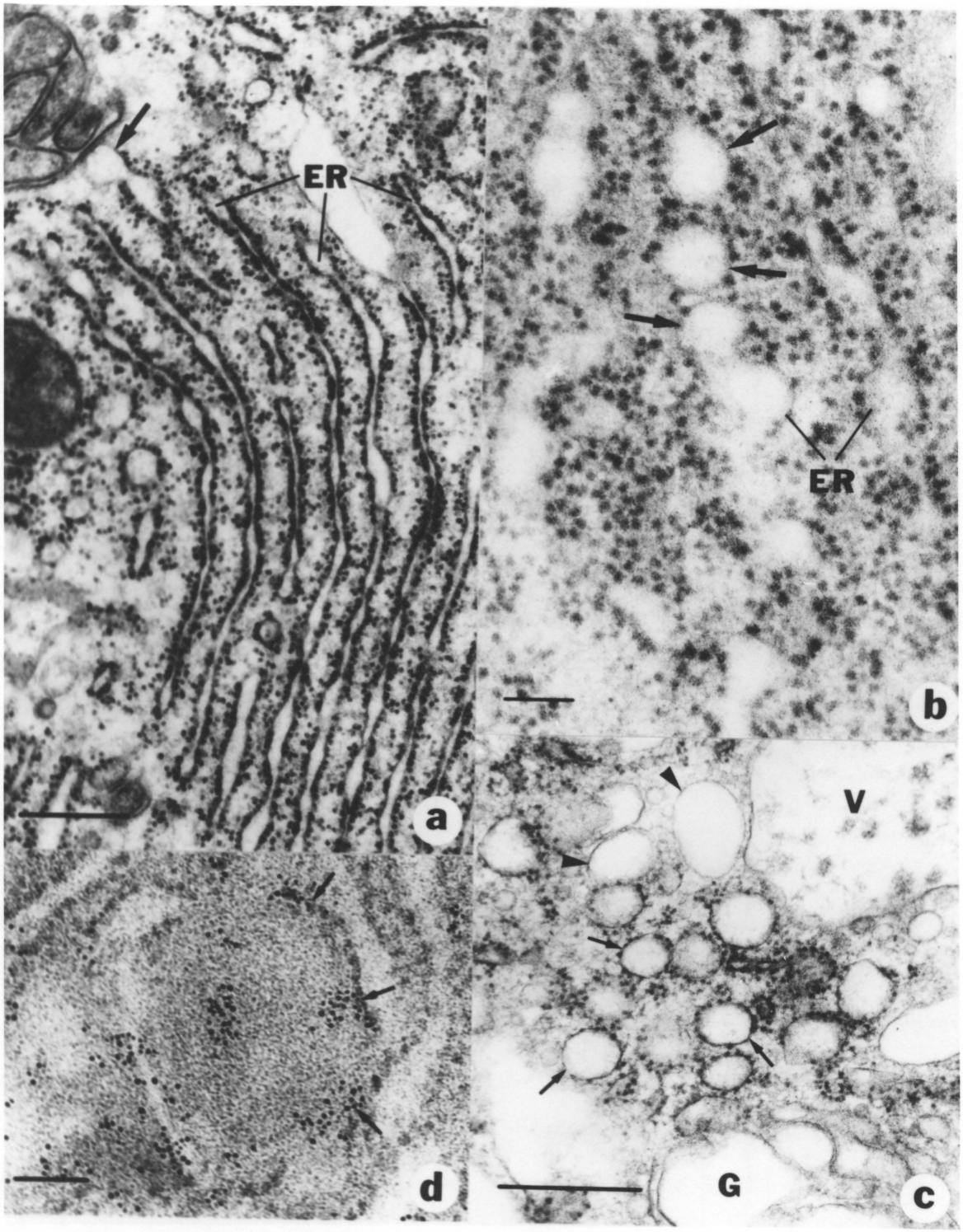
d
cld
e

depicted the apparent fusion of a micro lipid droplet to a larger cytoplasmic lipid droplet (Fig. 2 d and e). The stain-free triacylglycerol regions of the droplets seemed to be continuous and the coat materials of the lipid droplets appeared to be fused. These micrographs represent the first morphological evidence of fusion of lipid droplets in mammary epithelial cells.

Morphological observations of the origin of micro lipid droplets

Since most biochemical (22,23,24) and morphological (25) evidence suggests that the origin of cytoplasmic lipid droplets in the mammary epithelial cell is the rough endoplasmic reticulum, the origin of micro lipid droplets was investigated. In figure 3a, a stack of cisternae of the rough endoplasmic reticulum can be seen. At terminal regions of these endoplasmic reticulum cisternae, dilations, possibly due to lipid synthesis and droplet formation (35), were frequently seen. In addition, structures similar to micro lipid droplets were often seen, apparently being blebbed from these regions of the endoplasmic reticulum (arrow). Dilations along the length of a cisternae of rough endoplasmic reticulum were also observed and appeared similar to micro lipid droplets in size and characteristic lack of matrix staining (Fig. 3b). The presence of these dilated areas could be due to lipid droplet formation at these sites also. If lipid droplets were to bleb from sites on the rough endoplasmic reticulum it would not be unreasonable to propose that at least some micro lipid droplets would have ribosomes attached to their surface. Structures which resembled micro lipid droplets were seen in basal regions of the cell with ribosomes seemingly attached to their

Figure 3. Evidence for possible origin of micro lipid droplets from endoplasmic reticulum. Abbreviations: G, Golgi apparatus; V, secretory vesicle. a) Small structures, seemingly lacking a unit-like membrane, the contents of which were amorphous or finely granular appeared to bleb from terminal regions (arrow) of endoplasmic reticulum cisternae (ER). b) Structures similar to those in 2a are seen to bleb from distensions (arrows) along the length of an endoplasmic reticulum cisternum (ER). c) Many structures which resemble micro lipid droplets in size and matrix appearance had ribosomes associated with their surfaces (arrows), while others apparently lacked surface associated ribosomes (arrowheads). d) Immunogold conjugates were present in patches (arrows) over regions of endoplasmic reticulum and over vesicle-like structures in the size range of micro lipid droplets in sections incubated with antibodies against surface proteins of cytoplasmic lipid droplets (see p. 53). a and c. Bars 0.5 μm. b and d. Bars 100 nm. a. 36,000X. b. 109,000X. c. 46,000X. d. 118,000X.

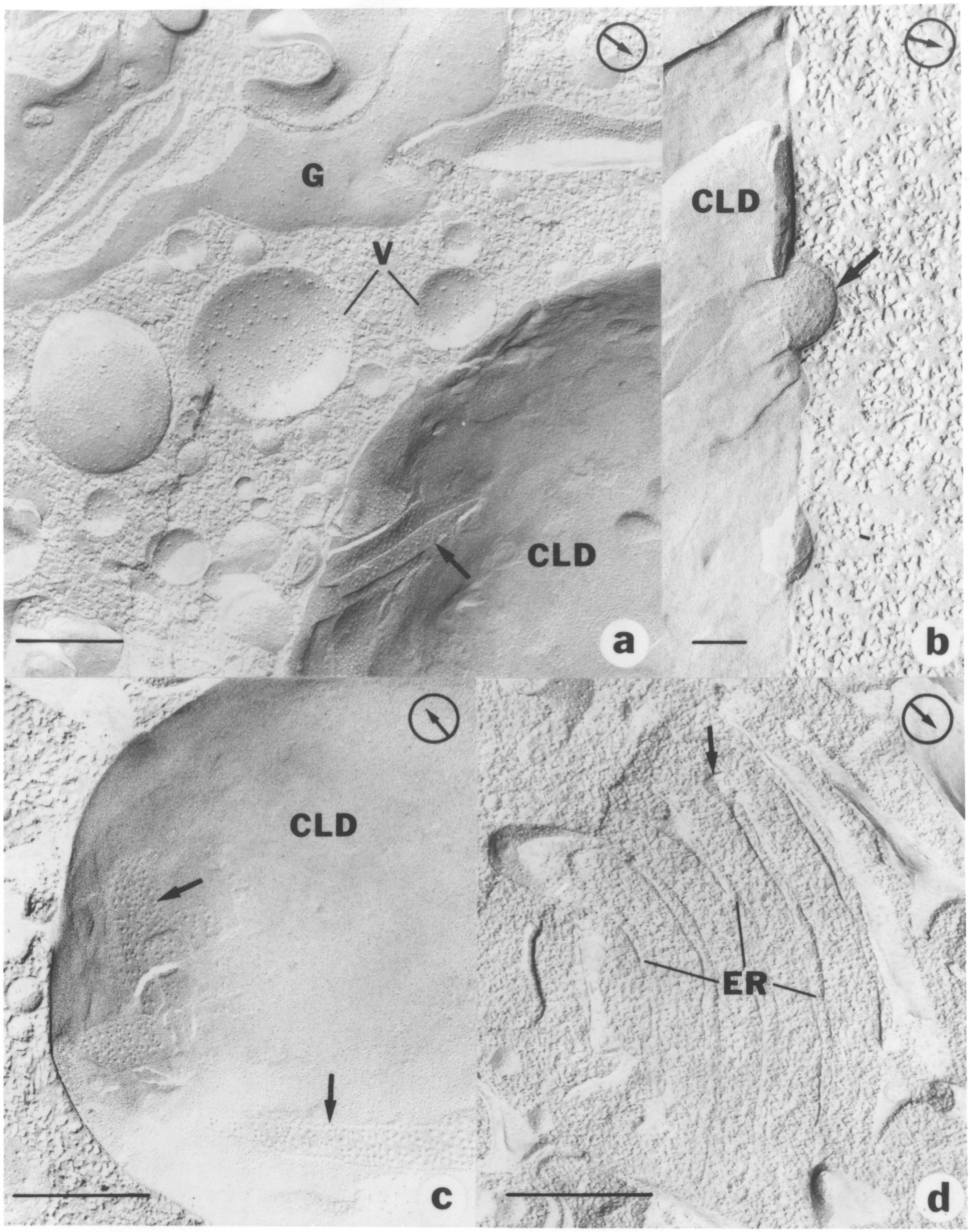

ER
a
ER
b
V
G
c
d

periphery (Fig. 3c, arrows). Observations of micro lipid droplets having ribosomes associated with their surface gives morphological support to the proposed rough endoplasmic reticulum origin of lipid droplets (5).

Freeze-fracture electron microscopic observations

An attempt was made to determine if the same phenomena proposed on the basis of transmission electron microscopy could be seen using freeze fracture electron microscopy. The results obtained are from rat and are presented in figure 4. Figure 4a shows a freeze fracture replica of the Golgi apparatus along with secretory vesicles, in addition to a cross fracture through a cytoplasmic lipid droplet. The crystalline structure of the triacylglycerols throughout the cytoplasmic lipid droplet formed the characteristic pattern obseved by others upon cross fracturing (30,48). The triacylglycerols located at the interior of the lipid droplet exhibited a smooth crystalline surface while those located more exteriorly sometimes exhibited an array of particles (Fig. 4a, arrows) or a similar array of pits as observed on the opposite fracture face of the droplet (Fig. 4c, arrows). It is important to note that the particle size and arrangement (density), resulting from crystallization of triacylglycerols on the surface of the cytoplasmic lipid droplet, was distinct from that observed associated with secretory vesicle or Golgi apparatus membranes. In figure 4b a cross fracture through a cytoplasmic lipid droplet can be seen in which there appeared to be a smaller structure fused to the surface of the cytoplasmic lipid droplet (arrow). This small structure is

Figure 4. Freeze-fracture replicas of mammary epithelial cells from lactating rat. Shadow direction is indicated on each micrograph with a circled arrow. Abbreviations: CLD, cytoplasmic lipid droplet, G, Golgi apparatus, V, secretory vesicle, ER, endoplasmic reticulum. a) Apical region of epithelial cell showing the Golgi apparatus, secretory vesicles and a cytoplasmic lipid droplet (PE face). Based on the size, number,and distribution of particle-like structures, crystalline lipid was morphologically distinct from all endomembranes and structures within the cell. b) Apparent fusion of a micro lipid droplet (arrow) onto the peripheral surface of a cytoplasmic lipid droplet. Note the similarity in crystalline lipid morphology between the micro lipid droplet (arrow 3b) and cytoplasmic lipid droplet (arrow 3a). c) Cross-fracture view of cytoplasmic lipid droplet (P face). Regions of crystalline lipid are shown (arrows). d) Cross fracture view of endoplasmic reticulum. A bleb-like structure is seen to be continuous with a terminal region of an endoplasmic reticulum cisternum (arrow). a and c. Bars 250 nm. b. Bar 100 nm. d. Bar .5 μm. a. 69,000X. b and c. 89,000X. d. 47,000X.


G
V
CLD
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CLD
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ER
d

proposed to be a micro lipid droplet, because the particle arrangement over its surface was more characteristic of that associated with the crystallized surface triacylglycerols of the cytoplasmic lipid droplet than with any intracellular membrane structure examined. Figure 6d is a cross fracture through a stack of rough endoplasmic reticulum cisternae. The dilations of these membranes, proposed to be characteristic of lipid droplet formation from transmission electron microscopic observations, can be observed in this replica (arrow). Thus the possible rough endoplasmic reticulum origin of micro lipid droplets, along with the fusion of these droplets to the larger cytoplasmic lipid droplets, is supported by these freeze fracture electron microscopic observations.

Biochemical comparison of micro and cytoplasmic lipid droplets

A method for isolation of micro lipid droplets was developed (5). Centrifugation through a step sucrose gradient separated cytoplasmic lipid droplets and micro lipid droplets based on their relative densities. The micro lipid droplet fraction was separated into two subpopulations, termed heavy or light micro lipid droplets. Light micro lipid droplets were larger in diameter and contained more triacylglycerols than heavy micro lipid droplets, which allowed this fraction to float more readily. Heavy micro lipid droplets were often observed with ribosomes bound to their surfaces. Light micro lipid droplets were also found with ribosomes bound to their surface but to a lesser extent than the heavier fraction. Since cytoplasmic lipid droplets have no ribosomes associated with their surfaces and light micro lipid droplets have fewer bound ribosomes than heavy micro lipid

droplets, it may be that micro lipid droplets with bound ribosomes have not fully matured. This maturation process would entail the shedding of ribosomes from the droplets surface. The fact that micro lipid droplets with bound ribosomes were not observed to fuse with larger cytoplasmic lipid droplets would seem to support this hypothesis. For the purposes of most of this study heavy and light micro lipid droplets were not separated but were harvested as a composite fraction, as described in methods.

Rough endoplasmic reticulum, micro lipid droplet and cytoplasmic lipid droplet fractions obtained from rat were characterized with regard to various marker enzymes. The values obtained were compared to the total homogenate (Table II). Acid phosphatase is a marker enzyme for lysosomes and is also found in milk lipid globules, while alkaline phosphatase is associated with various fractions, including milk lipid globules (4). 5'-Nucleotidase and Mg-ATPase are plasma membrane markers while succinate-tetrazolium reductase and succinate-cytochrome c reductase are markers for mitochondria (62,63). Xantine oxidase has been shown to be a constituent of milk lipid globule membrane and lactose synthetase is a marker for Golgi apparatus (3,44). Enzyme activities associated with micro lipid droplets and cytoplasmic lipid droplets ranged from <5% of the homogenate for lactose synthetase and <35% of the homogenate for succinate-tetrazolium reductase. Thus the lipid droplet fractions from rat appeared to have no major contamination from other cellular organelles or membranes.

Cytoplasmic lipid droplet and micro lipid droplet fractions were compared on the basis of lipid content (Table III). Micro lipid droplets

Table II. Enzymatic activities of cytoplasmic lipid droplets (CLD) and composite micro lipid droplet (micro LD) preparations from rat mammary gland.

Enzyme	CLD	Micro LD	ER	Homogenate
Acid phosphatase	0.34	0.54	0.36	5.14
Alkaline phosphatase	0.67	0.55	9.20	21.5
5'-Nucleotidase	0.02	0.02	0.11	0.67
Mg-ATPase	0.01	0.01	0.02	0.17
Succinate-tetrazolium reductase	0.18	0.14	0.19	0.49
Succinate-cytochrome c reductase	0.01	0.02	0.01	0.10
Xanthine oxidase	0.01	0.01	0.01	0.05
Lactose synthetase	0.004	0.008	0.03	0.22

Values for endoplasmic reticulum (ER) fractions and total homogenates were included for comparison. Values were averaged for three to five preparations. Specific activities were expressed as μmol/hr/mg protein.

Table III. Lipid composition of fractions from rat mammary gland.

Lipid Fraction	CLD	H-LD	L-LD	ER
Total Lipid, mg/mg protein	82.5	1.05	1.96	0.49
Cholesterol, µg/mg protein	200.0	54.0	14.2	14.6
Phospholipids, mg/mg protein	0.69	0.38	0.11	0.26
Triacylglycerols, mg /mg protein	81.6*	.62	1.84	.22

*CLD were > 85% triacylglycerols.

Abbreviations: CLD, cytoplasmic lipid droplet; H-LD, heavy-micro lipid droplet; L-LD, light-micro lipid droplet; ER, endoplasmic reticulum. Values for CLD were averages for 3 preparations; values for other fractions were for single preparations from tissue combined from 4 animals.

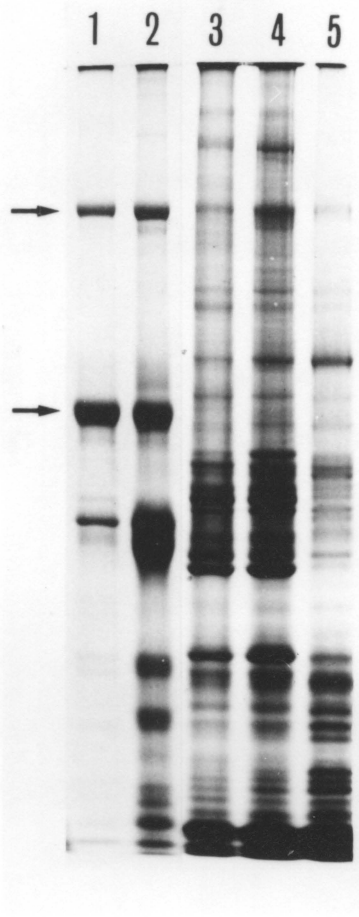
isolated from rat had a lower total phosphlipid and triacylglycerol content compared to cytoplasmic lipid droplets as measured on a protein basis. The fact that these micro lipid droplets contained triacylglycerols suggested that they may be the translocator of triacylglycerols from the rough endoplasmic reticulum to the growing cytoplasmic lipid droplet.

Polypeptide patterns of membranes and lipid droplet coat material

The micro lipid droplet surface coat material derived from the extracted micro lipid droplet fraction was obtained and compared to the surface coat material of the cytoplasmic lipid droplet and with rough endoplasmic reticulum, milk lipid globule membrane, and the material remaining with the milk lipid droplet after the membranes had been removed, using SDS polyacrylamide gel electrophoresis (Fig. 5). The surface coats of cytoplasmic lipid droplets and micro lipid droplets (lanes 3 and 4, respectively) were similar. This suggests that these coat materials have similar origins within the cell. The apparent fusion of micro lipid droplets to growing cytoplasmic lipid droplets is also suggestive of such a relationship in that this fusion seemingly involves the incorporation of micro lipid droplet coat material into the cytoplasmic lipid droplet coat material (Fig. 2 d and e).

The coat material of the micro lipid droplet was similar but not identical in polypeptide composition to the rough endoplasmic reticulum (lanes 4 and 5, respectively). This may be expected if one considers that the coat material may be derived from a single leaflet of the rough endoplasmic reticulum membrane bilayer. The similarities in polypeptide

Figure 5. Electrophoretic separation of polypeptides in fractions from bovine mammary gland. Polypeptide patterns were of milk lipid globule membrane (lane 1), surface material which remained associated with milk lipid globules after removal of membranes (see Fig. 13) (lane 2), cytoplasmic lipid droplets (lane 3), micro lipid droplets (lane 4) and endoplasmic reticulum membrane (lane 5). Arrows denote positions of xanthine oxidase (Mr 155,000) and butyrophilin (Mr 67,000). The gel was 8% polyacrylamide and stained with coomassie brilliant blue. Protein was applied as follows: lane 1, 40 µg protein; lane 2, 70 µg protein; lanes 3,4,5, 100 µg protein.


1
2
3
4
5

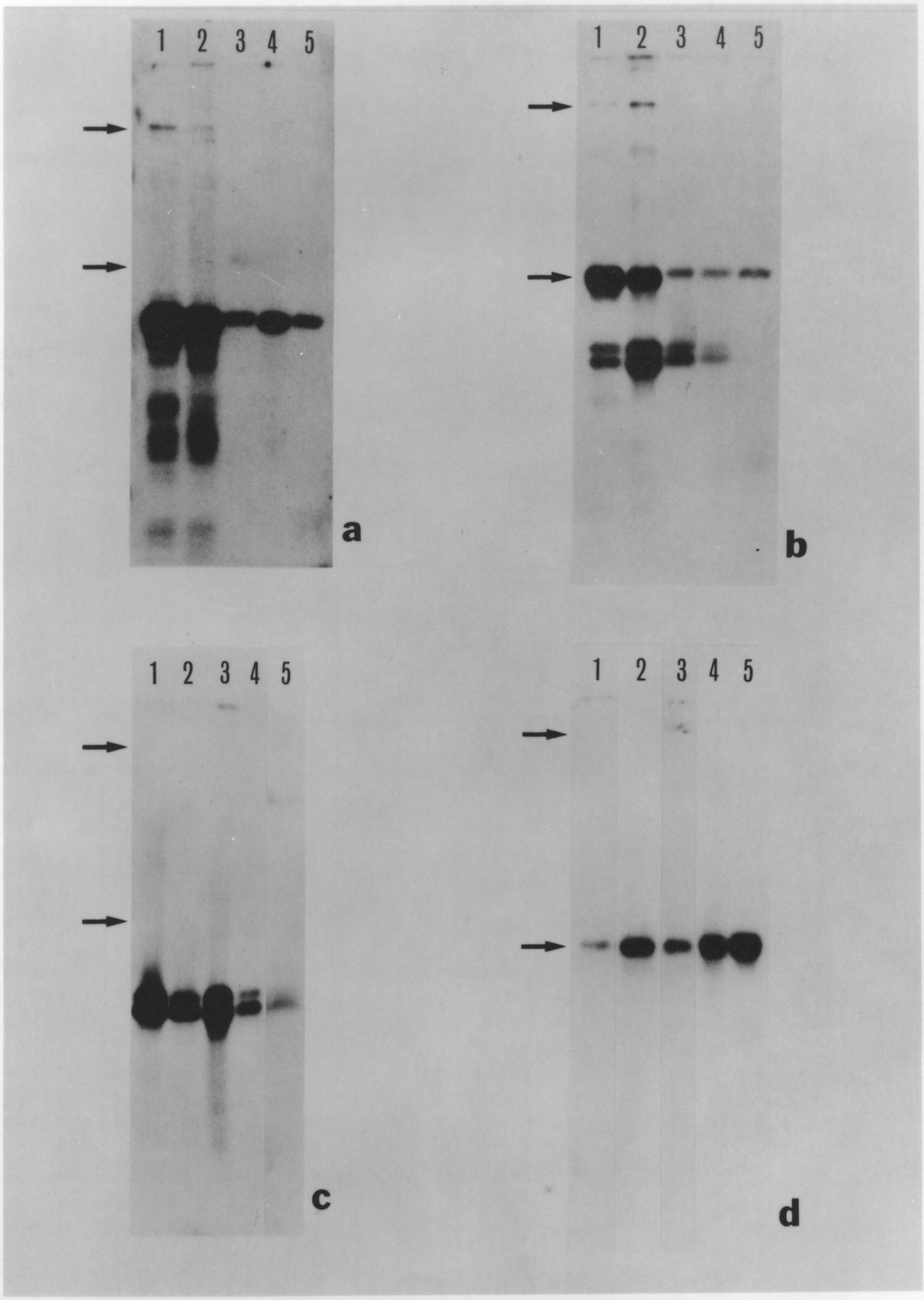
composition between these two fractions do not prove that the coat material is derived from the endoplasmic reticulum, but this observation can be said to be suggestive of such a relationship.

The milk lipid globule membrane (lane 1), enriched in xanthine oxidase (Mr 155,000) and butyrophilin (Mr 67,000), was quite different in polypeptide composition from the intracellular lipid droplet coat fractions and rough endoplasmic reticulum. The material which remained associated with the milk lipid globule after removal of milk lipid globule membrane (lane 2) appeared to be intermediate between milk lipid globule membrane and cytoplasmic lipid droplet coat in polypeptide pattern on SDS gels. This material was enriched in xanthine oxidase and butyrophilin as is milk lipid globule membrane but also contained faster migrating proteins which were characteristic of the intracellular lipid droplet protein fractions and rough endoplasmic reticulum.

Protein homologies based on immunoblotting

In an attempt to show more definitively protein homologies among the fractions being examined, immunoblotting was performed as described. The results are presented in figure 6. Blot a was incubated with antibodies raised against the protein material remaining with the milk lipid droplet after the removal of milk fat globule membrane. In this blot a single band (Mr ∿44,000) was observed to be present in all of the fractions. Milk lipid globule membrane and the material remaining on the milk lipid droplet after the removal of this membrane showed common antigenic reactivity of lower molecular weight proteins. These

Figure 6. Immunological identification of polypeptides in fractions from bovine mammary gland. Polypeptides separated in 8% polyacrylamide gels were electrophoretically transferred to nitrocellulose filters and incubated with affinity purified antibodies to (a) the material remaining on the surface of milk lipid globules after removal of membrane, (b) to milk lipid globule membrane, (c) to the surface proteins of cytoplasmic lipid droplets or (d) to butyrophilin from milk lipid globule membrane. a, b and c) Lane 1, milk lipid globule membrane; lane 2, surface material of milk lipid globules after removal of membranes; lane 3, cytoplasmic lipid droplets; lane 4, micro lipid droplets; and lane 5, endoplasmic reticulum membrane. d) Lane 1, endoplasmic reticulum membrane; lane 2, micro lipid droplets; lane 3, cytoplasmic lipid droplets; lane 4, surface material on milk lipid globules after removal of membrane; and lane 5, milk lipid globule membrane. Antibodies bound to the nitrocellulose filter were detected with ^{125}I-protein A and autoradiography at $-70^{o}C$. Arrows denote positions of xanthine oxidase (Mr 155,000) and butyrophilin (Mr 67,000). Protein concentrations are as in figure 7.


1 2 3 4 5
a
1 2 3 4 5
b
1 2 3 4 5
c
1 2 3 4 5
d

polypeptides appeared to be absent from the other fractions examined (CLD, μLD, RER). Blot b was incubated with anti-milk lipid globule membrane antibody. A protein that comigrated with butyrophilin (Mr 67,000), seen in comparison to the stained blotted gel, acted as an antigen to this antibody (arrow). Butyrophilin is concentrated in milk lipid globule membrane and in the material remaining on the milk lipid droplet after the removal of the membrane, which would explain the greater reactivity of this band in these two fractions as compared to the others. In addition there was a doublet which migrated at a molecular weight of about 44,000, which can be seen to increase in intensity from micro lipid droplets to the cytoplasmic lipid droplet surface coat material. This doublet was also seen in milk lipid globule membrane, the material remaining with the droplet after its removal, and in overexposed films, in rough endoplasmic reticulum. A faint band comigrating with xanthine oxidase (Mr 155,000) (arrow) was seen in both milk lipid globule membrane and the material remaining with the lipid droplet after the removal of this membrane but was not detected in the lipid droplet fractions or in rough endoplasmic reticulum. Blot c was incubated with anti-cytoplasmic lipid droplet protein antibodies. This blot exhibited similar staining in all fractions. The weakest reaction was with RER. The reaction was stronger in the micro lipid droplet fraction and very strong in the cytoplasmic lipid droplet fraction. The doublet in this fraction seemed to comigrate with the doublet seen in Blot b. Finally Blot d was incubated with anti-butyrophilin antibodies. All of the fractions showed reactivity with the antibody, producing a band upon

autoradiography which comigrated with butyrophilin. This strongly suggests that butyrophilin is not totally exclusive to apical plasma membrane and milk lipid globule membrane (41) but that it may also be found, albeit in small quantities, in the lipid droplet fractions and rough endoplasmic reticulum. This was also indicated in Blot b, with anti-milk lipid globule membrane.

These results raise some interesting questions concerning the origin and secretion of lipid droplets. Butyrophilin has been shown to be localized in apical plasma membrane, milk lipid globule membrane, and the coat material attached to the cytoplasmic face of this membrane (42). It has also been observed that xanthine oxidase is enriched in these fractions (41). In attempts to isolate butyrophilin from these fractions it has become evident that butyrophilin and xanthine oxidase have strong affinity for each other. This was suggested by the difficulty in purifying butyrophilin (Mr 67,000) from solubilized milk lipid globule membrane without contamination of xanthine oxidase (Mr 155,000) with molecular sieve chromatography. When considering binding interactions between the cytoplasmic lipid droplet and the apical plasma membrane it may not be unreasonable to suggest that these two proteins may play a role. An interaction between butyrophilin located in the lipid droplet coat with xanthine oxidase located in the apical plasma membrane may promote binding of the lipid droplet to the membrane. In addition there may be lower molecular weight proteins associated with the milk lipid globule membrane partly responsible for such interactions as Blot a in figure 6 may suggest. Future experiments

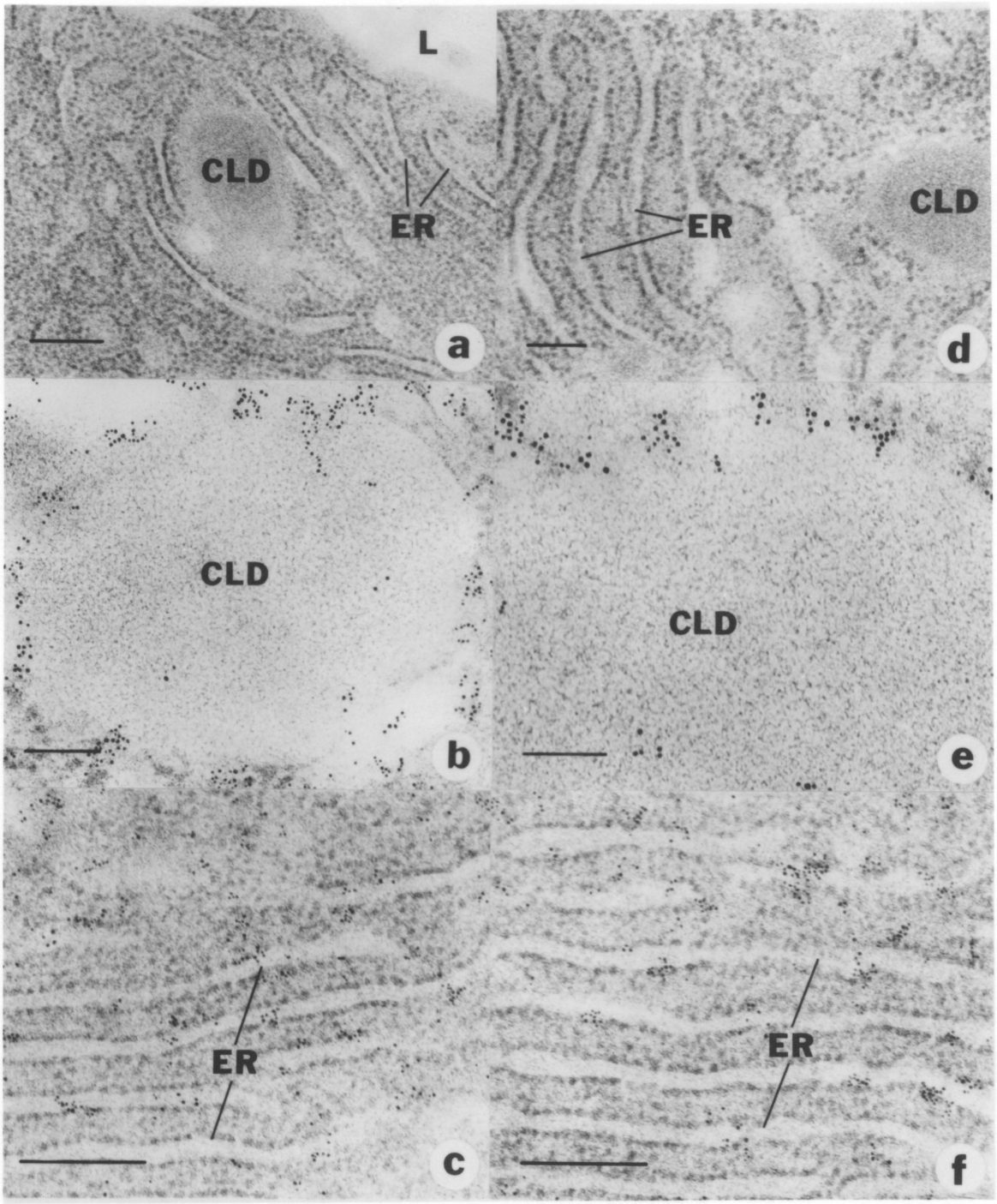
must deal with this possibility and the general binding capacity of the lipid droplet to the membrane.

A second question is raised by the differential reactivity os some proteins among the fractions considered. The doublet which migrated at a molecular weight of about 44,000 seemed to be present in higher concentrations in cytoplasmic lipid droplet coat material relative to the rough endoplasmic reticulum. Since it has been proposed that this coat material is derived from the endoplasmic reticulum (35) this may suggest that micro lipid droplets originate from specific areas along the rough endoplasmic reticulum.

Origin of cytoplasmic lipid droplet coat material

To show more definitively that the rough endoplasmic reticulum is the origin of the coat material surrounding cytoplasmic lipid droplets and to examine the possibility that discrete areas of the rough endoplasmic reticulum may be responsible for lipid droplet formation, an immunocytochemical experiment was performed. Tissues from both cow and rat were fixed and embedded with Lowicryl K4M at low temperatures as described by Roth (54). This embeddment procedure was used to enhance the immunochemical reactivity of the tissue slices. Thin sections of tissue were placed on grids and incubated with affinity purified antibodies to cytoplasmic lipid droplet proteins followed by incubation with a goat anti-rabbit IgG-colloidal gold (5 nm) complex. The major reactivity of this antibody with the fractions considered was a protein doublet (Mr 44,000), as determined from immunoblot c (Fig. 6). The results of this experiment are shown in figure 7.

Figure 7. Immunocytochemical localization of antibodies to proteins from the surface of cytoplasmic lipid droplets in mammary epithelial cells from rat (a,b,c) and cow (d,e,f). Abbreviations: CLD, cytoplasmic lipid droplet; ER, endoplasmic reticulum; L, alveolar lumen. a and d) Control sections incubated with gold-conjugated second antibody but without primary antibody (a) or with primary antibody saturated with excess antigen (d) had no observable gold particles. b and e) Gold particles were distributed in clusters over the entire periphery of the cytoplasmic lipid droplet. The numbers of gold particles present over the triacylglycerol core of the droplets was no greater than background. c and f) Clusters of gold particles were found to be nonuniformly distributed over localized regions of the endoplasmic reticulum cisternae. a,d,e,f. Bars 250 nm. b and c. Bars 100 nm. a. 47,000X. b. 92,000X. c. 135,000X. d. 48,000X. e and f. 80,000X.


L
CLD
ER
a
ER
CLD
d
CLD
b
CLD
e
ER
c
ER
f

Thin sections of tissues incubated with the goat IgG-colloidal gold complex without first being exposed to the primary antibody or with goat IgG-colloidal gold presaturated with primary antibody were used as the controls (Fig. 7 a and d). These sections were devoid of colloidal gold particles, indicating the absence of nonspecific binding of the goat IgG-colloidal gold complex to the tissue sections. When the tissue was incubated with the antibody prior to the addition of the goat IgG-gold complex, the surface coat of the cytoplasmic lipid droplet was labeled, its presence marked by the electron dense colloidal gold particles (Fig. 7 b and e). The colloidal gold particles marked the binding of the antibodies to the surface material of these droplets in a previous incubation. The rough endoplasmic reticulum of both cow and rat after incubations with the antibody and colloidal gold-protein A complex are shown in figure 7, c and f. The electron dense colloidal gold particles were localized in patches along the length of the rough endoplasmic reticulum cisternae of both cow and rat.

Mitochondria, nuclei, Golgi apparatus, and secretory vesicles exhibited no labeling with the colloidal gold. Thus it was concluded that immunological reactions did not occur at any other intracellular organelles. The only structures labeled were the rough endoplasmic reticulum and cytoplasmic lipid droplets. Upon closer scrutiny of the cell, structures in the size range of micro lipid droplets could be found in basal regions of the cell. These structures were also labeled with colloidal gold over their periphery (Fig. 3d). This experiment strongly supports the morphological observations which suggested that the origin of the cytoplasmic and micro lipid droplet coat materials

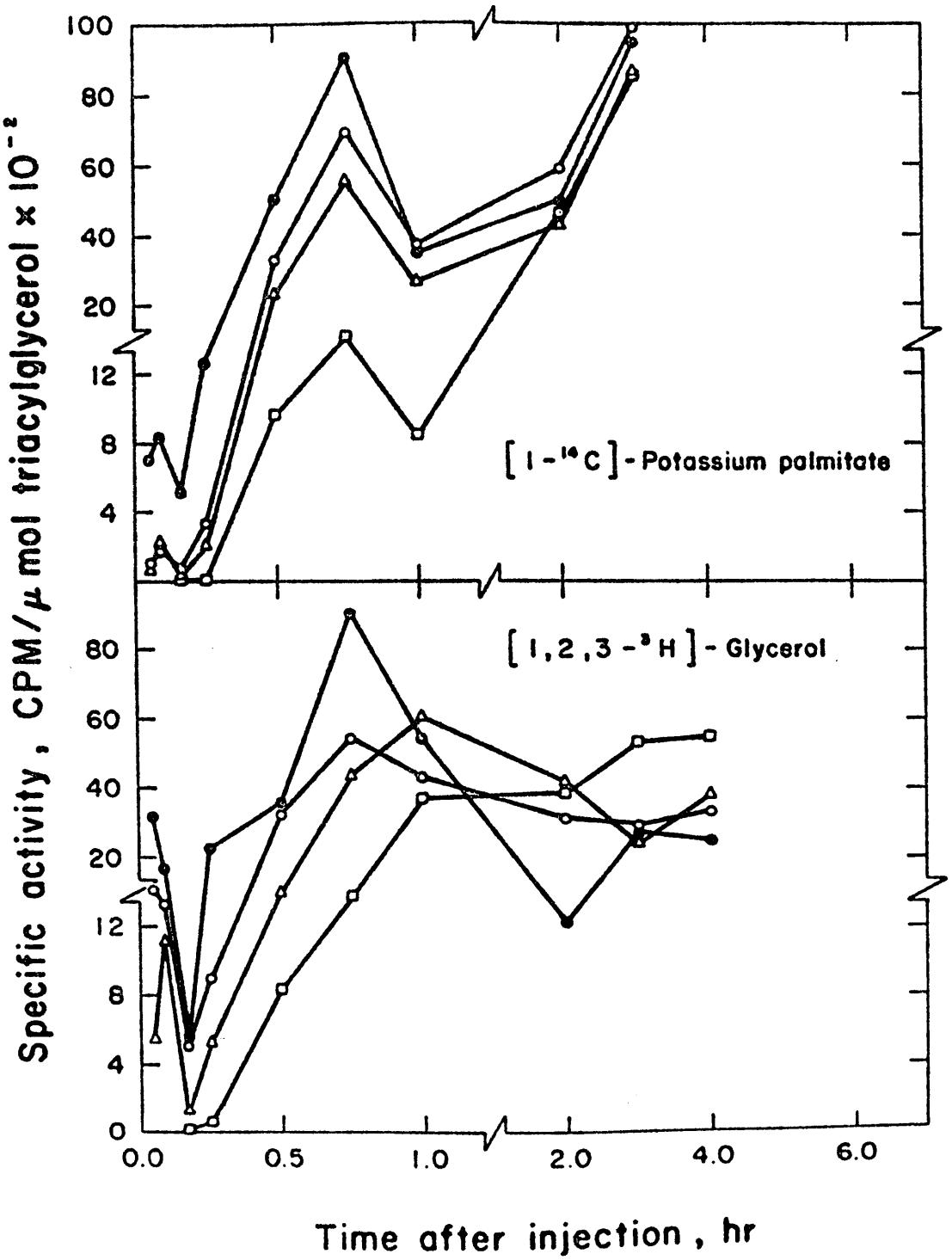
and thus the droplets themselves is the rough endoplasmic reticulum. It also suggested that lipid droplet formation may take place at specific sites along the rough endoplasmic reticulum as the labeling of those membranes (Fig. 7 c and f) was patchy rather than uniform over the length of the rough endoplasmic reticulum.

Incorporation of radiolabeled lipid precursors into triacylglycerols

Radiolabeled lipid precursors were administered to rats intraperitoneally and milk lipid globules, cytoplasmic lipid droplets, micro lipid droplets,and rough endoplasmic reticulum were isolated at various times after injection. The triacylglycerols and phospholipids of these fractions were determined as described in methods; results are given in figures 8 and 9 respectively.

The incorporation of [1-^{14}C]-potassium palmitate into triacylglycerols as measured by the specific activity (cpm/μmole of triacylglycerol) was primarily in rough endoplasmic reticulum at the earliest time points measured. A smaller amount of activity was associated with the cytoplasmic and micro lipid droplets in the first 10 minutes of the experiment. There was no detectable activity in milk at these early time points. In the time period between 10 minutes and 1 hour the activity of all the fractions increased simultaneously, peaking at 45 minutes. At 45 minutes the greatest activity was associated with the rough endoplasmic reticulum fraction followed by micro lipid droplets, cytoplasmic lipid droplets and finally milk lipid globules. The potential of micro lipid droplets to contribute triacylglycerols to cytoplasmic lipid droplets may exist, but if such a process occurs it

Figure 8. Specific activity of triacylglycerols of endoplasmic reticulum (●), micro lipid droplets (○), cytoplasmic lipid droplets (△) and milk (□) at intervals after intraperitoneal injection of radiolabeled lipid precursors into lactating rats. Each time point represents the average of 3 rats. Variance was within ±10% of the average value.

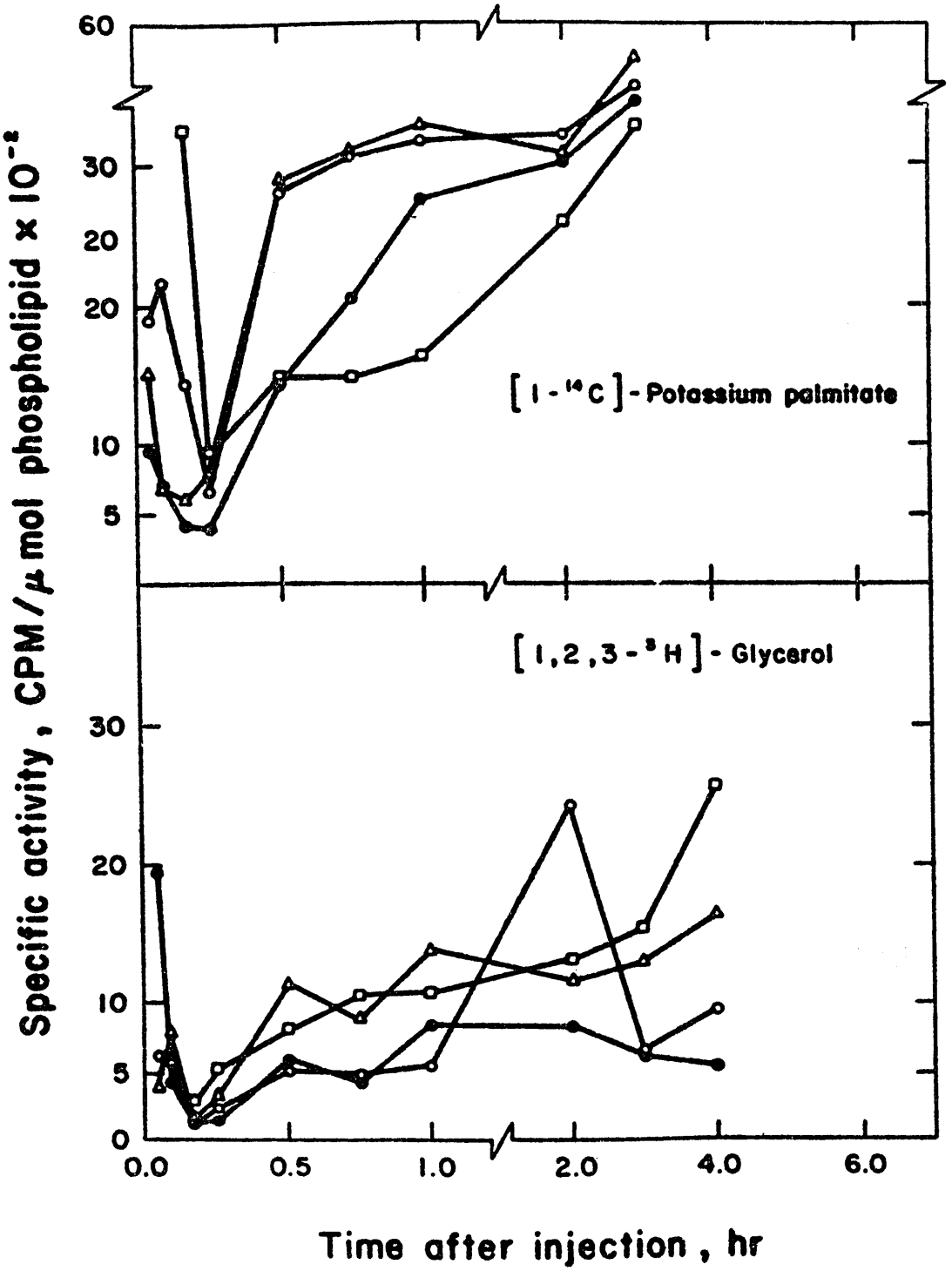

Specific activity, CPM/μ mol triacylglycerol x 10⁻²
100
80
60
40
20
12
8
4
[1-¹⁴C]-Potassium palmitate
80
60
40
20
12
8
4
0
[1,2,3-³H]-Glycerol
0.0
0.5
1.0
2.0
4.0
6.0
Time after injection, hr

seems to be very rapid since there is no detectable lag between the labeling of these two fractions. Thus the flow of triacylglycerols not only into new lipid droplets (micro lipid droplets) but also into preexisting cytoplasmic lipid droplets as proposed by Stein and Stein (25) would seem to be supported by these results. From 1 to 3 hours the triacylglycerol activity of these fractions continued to increase in parallel with one another.

The results obtained from the incorporation of [1,2,3-^{3}H]-glycerol into the triacylglycerols of these fractions suggested a flow of lipids from the rough endoplasmic reticulum into milk lipid globules. Rough endoplasmic reticulum, micro lipid droplets, and cytoplasmic lipid droplets all exhibit small peaks in specific activity at 3 to 5 minutes after injection. From 10 minutes to 4 hours all fractions had peaks in triacylglycerol specific activity. Unlike the palmitate experiment, however, these fractions peaked at different times. Rough endoplasmic reticulum and micro lipid droplets contained their greatest activity at 45 minutes. The specific activity of the cytoplasmic lipid droplets peaked at 1 hour and finally milk lipid globules showed the highest level of incorporation into triacylglycerols after 4 hours post injection. This would seem to suggest that triacylglycerols synthesized at the rough endoplasmic reticulum are first packaged into micro lipid droplets prior to incorporation into cytoplasmic lipid droplets and eventually into milk.

Incorporation of precursors into phospholipids of rough endoplasmic reticulum, micro lipid droplets, cytoplasmic lipid droplets,

Figure 9. Specific activity of total phospholipids of endoplasmic reticulum (●), micro lipid droplets (○), cytoplasmic lipid droplets (△), and milk (□) at intervals after intraperitoneal injection of radiolabeled lipid precursors into lactating rats. Each time point represents the average of 3 rats. Variance was within ±10% of the average value.


60
30
20
20
10
5
[1-14C]-Potassium palmitate
[1,2,3-3H]-Glycerol
30
20
10
5
0
0.0
0.5
1.0
2.0
4.0
6.0
Specific activity, CPM/μ mol phospholipid x 10-2
Time after injection, hr

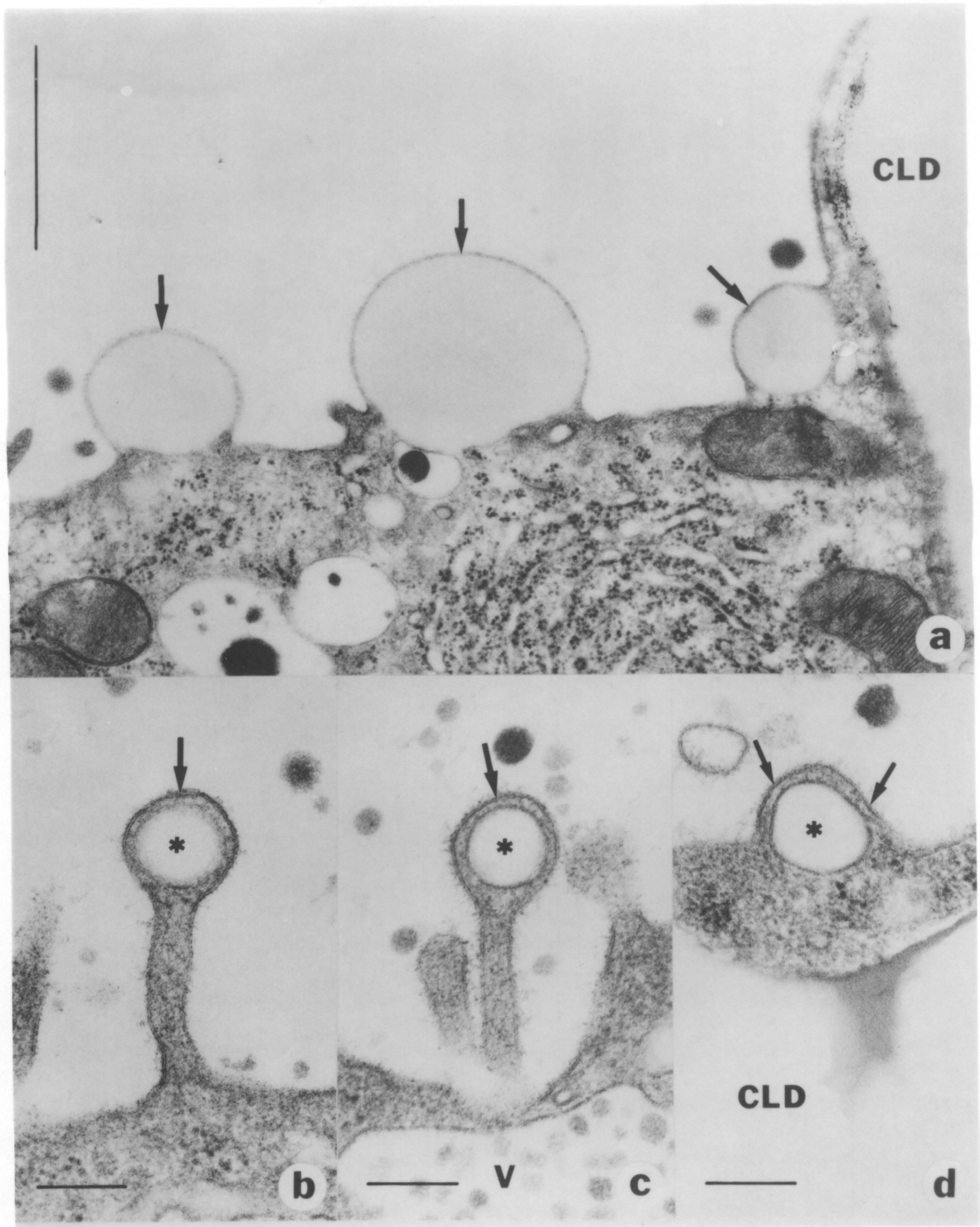
and milk lipid globules was also examined. Results are shown in figure 9. Incorporation of palmitate and glycerol was seen in the phospholipids of rough endoplasmic reticulum, micro and cytoplasmic lipid droplets at short time periods after injection. After these early peaks in phospholipid incorporation all of the fractions showed a steady increase in incorporation of radioactivity into phospholipids.

Secretion of micro cytoplasmic lipid droplets

The activity associated with milk lipid globules within 1 hour after injection of [1-^{14}C]-potassium palmitate (Fig. 8) can possibly be explained by two different phenomena. According to Stemberger and Patton (31) most of the growth of cytoplasmic lipid droplets takes place in the apical region of the cell during the actual process of secretion of the lipid droplet. Thus large lipid droplets protruding into the lumen of the gland may still incorporate newly synthesized lipid into their core by allowing micro lipid droplets to fuse. In this way large lipid droplets containing activity would be secreted into the lumen fairly early compared to other lipid droplets that must undergo considerable growth before secretion.

A second possibility is that micro lipid droplets may be secreted from the epithelial cell after undergoing only a limited number of fusions. Small intracellular lipid droplets (<1.5 μm) are seen throughout the mammary epithelial cell (31) and small milk lipid globules account for 80% of the total number of lipid globules within milk (37). The secretion of small cytoplasmic lipid droplets or micro-cytoplasmic lipid droplets was observed both in rat (Fig. 10 a and b) and in

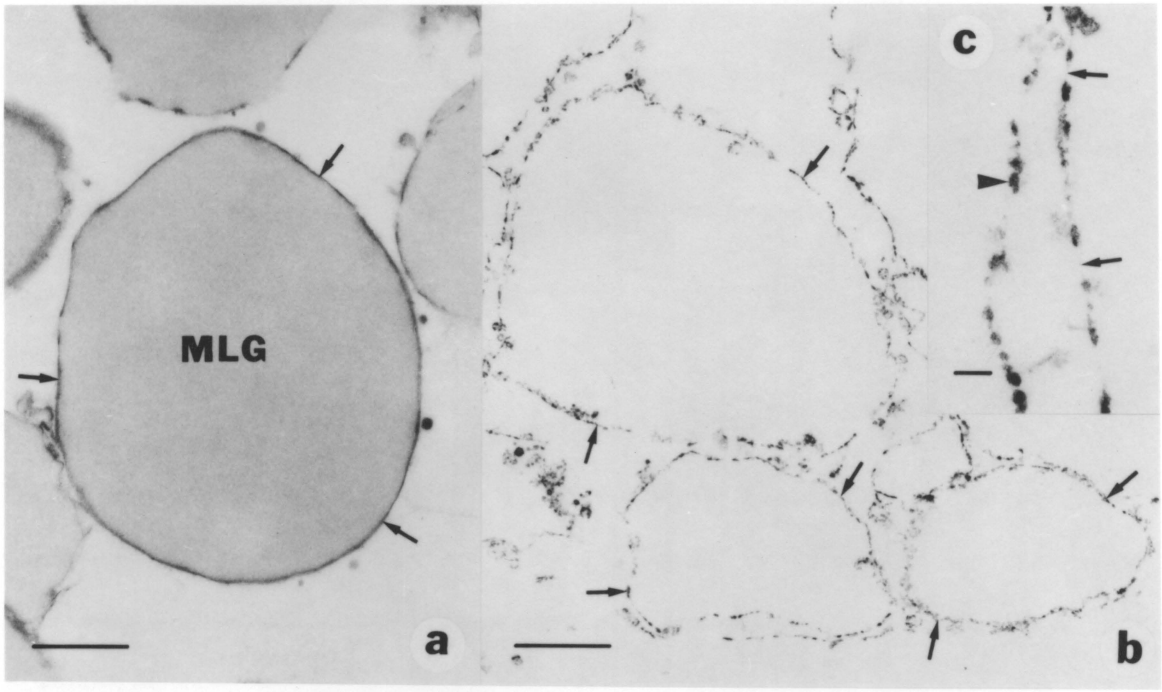
Figure 10. Secretion of lipid droplets from mammary epithelial cells of lactating rat (a and b) and cow (c and d). a) Small lipid droplets (arrows) appear to be partially secreted along with a cytoplasmic lipid droplet (CLD) of more typical size. b and c) Two micro lipid droplets appear to be small enough to be found at the ends of microvilli (arrows). d) A micro lipid droplet is shown associated with apical plasma membrane (arrows). Asterisks mark micro lipid droplets. a. Bar 1.0 μm. b,c,d. Bars 0.25 μm. a. 34,000X. b,c,d. 59,000X.


CLD
a
b
V
c
CLD
d

cow (Fig. 10 c and d). These lipid droplets are so small that they can be observed being secreted from the ends of microvilli (Fig. 10 b and c). A comparison between the size of a cytoplasmic lipid droplet and micro-cytoplasmic lipid droplet can be seen in figure 10a.

The secretion of the micro-cytoplasmic lipid droplets appeared to occur in the same way as that of the larger cytoplasmic lipid droplets. In both cases the lipid droplet is encompassed by apical plasma membrane, forming a milk lipid globule membrane as the droplet is secreted (Fig. 10). During secretion there appeared to be a close association of the milk lipid globule membrane with the coat material of the cytoplasmic lipid droplet. An electron micrograph of a milk lipid globule (Fig. 11a) demonstrates this fact. The electron micrograph in 11b shows milk lipid globules after the milk lipid globule membrane has been removed by the churning method. These lipid globules retained a modified cytoplasmic lipid droplet coat material after the milk lipid globule membrane was removed. This cytoplasmic lipid droplet coat material appeared to have portions of the milk lipid globule membrane fuzzy coat attached, forming localized thickened regions of surface material along the periphery of the droplet (Fig. 11c). This is not totally surprising when considering the close interaction that occurs between these materials. The fuzzy coat of the milk lipid globule membrane has been shown to be enriched in butyrophilin and xanthine oxidase (43). These two proteins are also prominent components of the milk lipid droplet surface material after the removal of membranes (Fig. 5, lane 2). Thus these two proteins are found to be enriched in

Figure 11. Dense staining material remained on the surface of bovine milk lipid globules after removal of the milk lipid globule membrane. a) Milk lipid globules (MLG) were surrounded by a unit-like milk lipid globule membrane (arrows) which had a dense staining coat material associated with its inner surface. b) After removal of the milk lipid globule membrane (see methods), a dense staining material (arrows) which lacked unit membrane appearance remained associated with the surface of the lipid globules. c) Higher magnification of (b) showing localized thickened regions (arrowheads) in this surface material. a and b. Bars 1.0 µm. c. Bar 100 nm. a and b. 14,000X. c. 52,000X.


MLG
a
b
c

the surface material of milk lipid globules after removal of membranes, but are present only in trace amounts in the cytoplasmic lipid droplet coat material itself. This suggests that the densely staining material which remains with the milk lipid droplet after removing the milk lipid globule membrane is composed in part of butyrophilin and xanthine oxidase. The seemingly tight binding of butyrophilin and xanthine oxidase (localized in the apical plasma membrane, milk lipid globule membrane, and the fuzzy coats of these membranes) to the surface of the secreted lipid droplet supports the hypothesis that these proteins may play a binding role in the attachment of the cytoplasmic lipid droplet to the apical plasma membrane prior to or during secretion.

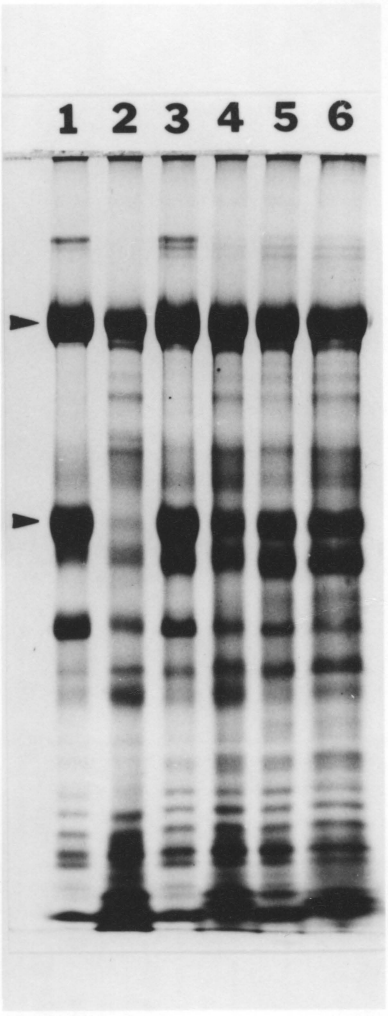
The effect of tunicamycin on milk lipid globule membrane

The design of the mammary gland affords an opportunity to study the possible role of binding proteins in the secretion of cytoplasmic lipid droplets. Butyrophilin, a glycoprotein localized in the apical plasma membrane and thought to play a role in lipid secretion, has been determined to possess one carbohydrate chain of the high mannose variety suggesting that it is an N-linked glycoprotein (42). Tunicamycin, an inhibitor of N-linked glycosylation, has been shown to act effectively in inhibiting the incorporation of some N-linked glycoproteins into plasma membrane both *in vitro* and *in vivo* (78,79). Thus tunicamycin was infused into the lumen of the mammary gland of a goat via the teat canal to determine if it could possibly prevent the glycosylation, and as a result the incorporation of butyrophilin into the apical plasma membrane of the epithelial cell. This method takes advantage of the

accessibility of mammary epithelial cells via the teat canal. Thus in this case the mammary gland of the goat was treated as if it were a cell culture system *in vivo*. The effect of tunicamycin on milk lipid globule membrane and thus on the apical plasma membrane from which it is derived is shown in figure 12. This coomassie stained SDS polyacrylamide gel shows the polypeptide patterns of various milk lipid globule membrane samples obtained from milk of the control and tunicamycin treated glands of a lactating alpine goat. Butyrophilin (Mr 76,000) appeared to be present in the 12 hour control but was almost completely absent from the 12 hour tunicamycin treated gland. Some recovery of the tunicamycin treated gland relative to the control was evident at 24 hours, and at 36 hours the tunicamycin treated and control glands appeared very similar. Thus the incorporation of butyrophilin into milk lipid globule membrane and thus apical plasma membrane can be inhibited almost completely with tunicamycin and this inhibition seems to be reversible as determined by the recovery of the treated membrane by 36 hours. This suggests that butyrophilin is in fact an N-linked glycoprotein and that the carbohydrate moiety of this glycoprotein is a necessary requirement for its insertion into the apical plasma membrane of the mammary epithelial cell. These assumptions remain to be confirmed.

Now that it has been determined that butyrophilin can be selectively excluded from the apical plasma membrane with tunicamycin, its physiological role in lipid secretion can be more closely examined. Thus if butyrophilin plays a role in binding of the lipid droplet

Figure 12. The effect of tunicamycin on the incorporation of butyrophilin in milk lipid globule membrane. Polypeptides were separated on an 8% polyacrylamide gel and stained with coomassie blue. Abbreviations: MLGM, milk lipid globule membrane; TM, tunicamycin treated. Time represents hours after infusion. Samples are as follows: lane 1, MLGM 12 hour control; lane 2, MLGM 12 hour TM; lane 3, MLGM 24 hour control; lane 4, MLGM 24 hour TM; lane 5, MLGM 36 hour control; lane 6, MLGM 36 hour TM. Arrows denote positions of xanthine oxidase (Mr 155,00) and butyrophilin (M4 67,000). 50 µg of protein was applied to each lane.


1 2 3 4 5 6

to the apical plasma membrane during secretion, lipid secretion may be decreased in its absence. This remains to be determined. In addition the possibility that butyrophilin is involved in the binding of the fuzzy coat to the apical plasma membrane must be explored.

CONCLUSIONS

A previously unrecognized group of structures with diameters $< .5\ \mu m$, which resembled cytoplasmic lipid droplets in matrix and surface coat appearance, was observed. These structures were shown to be similar to cytoplasmic lipid droplets both in enzymatic activity and constituent lipid classes. These small structures, termed micro lipid droplets, were proposed to provide triacylglycerols to support the growth of cytoplasmic lipid droplets as they move apically through the cell to be secreted (Fig. 13 pathway II).

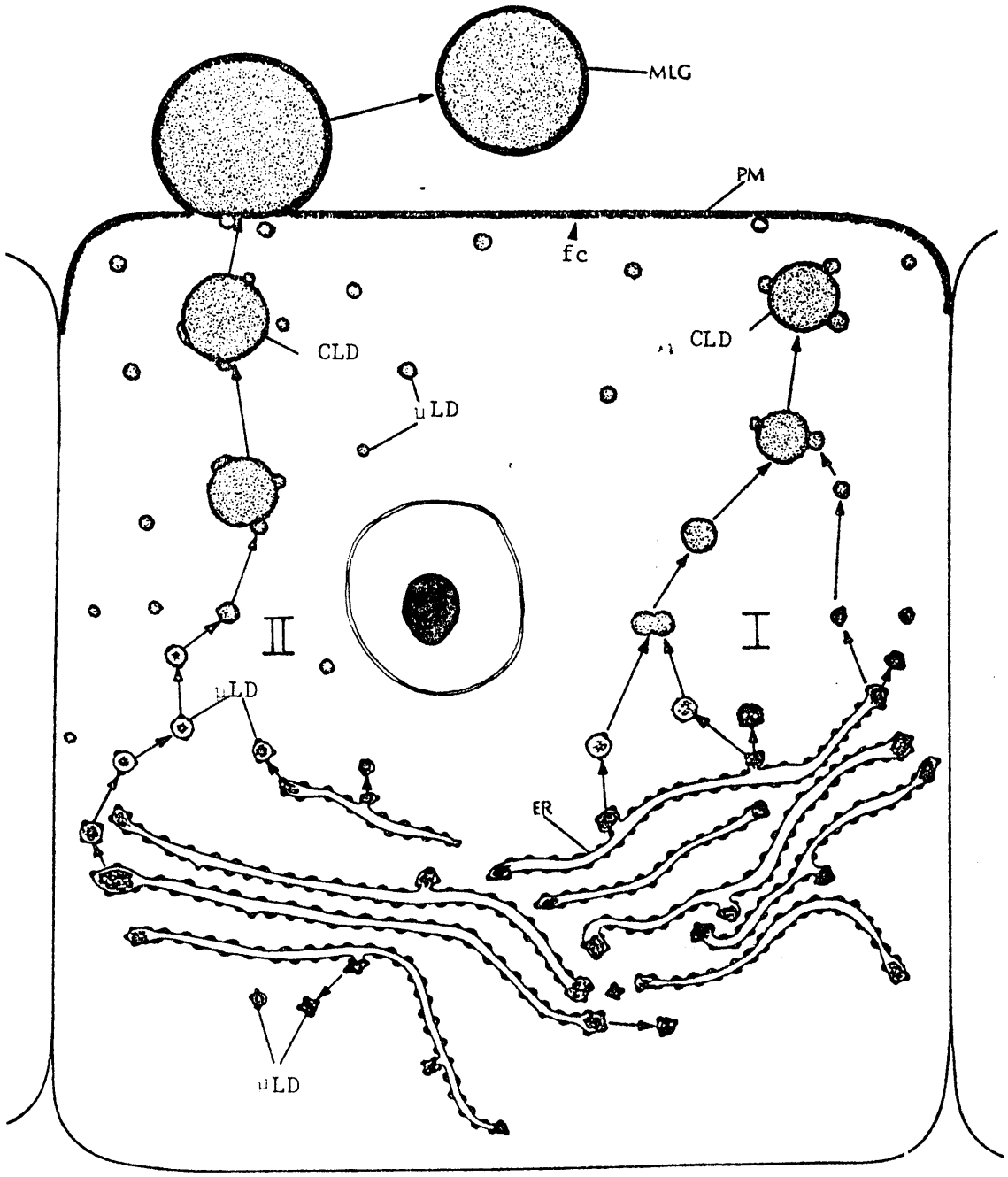
The origin of micro lipid droplets and the apparent fusion of these droplets onto cytoplasmic lipid droplets ($> 1\ \mu m$) has been observed with both transmission and freeze-fracture electron microscopy. These results suggest that the origin of the micro lipid droplet is the rough endoplasmic reticulum. The existence of homologous proteins between these fractions, demonstrated with immunoblotting techniques, was suggestive of a possible endoplasmic reticulum origin of the micro lipid droplets. In addition, immunocytochemical results further suggested that both micro and cytoplasmic lipid droplets originate from the endoplasmic reticulum. These results also suggest that lipid droplet formation may take place at specific sites along the cisternae of the endoplasmic reticulum.

The fact that micro lipid droplets could supply triacylglycerols for the growth of cytoplasmic lipid droplets was demonstrated by the time course of incorporation of radiolabeled lipid precursors into triacylglycerols or phospholipids of milk and various fractions

isolated from lactating mammary gland. These experiments suggested that the synthesis of micro lipid droplets is a very rapid process. Cytoplasmic lipid droplets were labeled almost as fast as the micro lipid droplets, suggesting that the proposed fusion of micro lipid droplets to cytoplasmic lipid droplets is also a very rapid process.

In addition, it was suggested that micro lipid droplets are the direct precursors of the small lipid globules of milk. Morphological evidence was obtained which showed the secretion of micro-cytoplasmic lipid droplets in a manner similar to the secretory mechanism for cytoplasmic lipid droplets. Tight association of the fuzzy coat material of the milk lipid globule membrane with the surface of the milk lipid globule was demonstrated. Such association is suggestive of possible binding interactions which may occur between the cytoplasmic lipid droplet surface coat and the fuzzy coat of the apical plasma membrane of the cell. This interaction may involve butyrophilin and xanthine oxidase and may facilitate the secretion of the lipid droplet. Tunicamycin has been shown to inhibit the incorporation of butyrophilin into milk lipid globule membrane and thus apical plasma membrane. The significance of this fact in relation to lipid secretion has yet to be determined.

Figure 13. Pathways for the proposed incorporation of micro lipid droplets into cytoplasmic lipid droplets. Abbreviations: ER, endoplasmic reticulum; μLD, micro lipid droplet; CLD, cytoplasmic lipid droplet; PM, plasma membrane; fc, fuzzy coat; MLG, milk lipid globule. I) Micro lipid droplets bleb from rough endoplasmic reticulum, fuse with each other to form cytoplasmic lipid droplets and subsequently fuse to the newly formed cytoplasmic lipid droplet to provide triacylglycerols for growth. Fusion of micro lipid droplets to each other forming a cytoplasmic lipid droplet has not been proved or disproved in this study. II) The fusion of micro lipid droplets originating from the rough endoplasmic reticulum to preexisting cytoplasmic lipid droplets to provide triacylglycerols for growth as these droplets move apically to be secreted.


MLG
PM
fc
CLD
CLD
μLD
μLD
μLD
II
I
ER

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