Origin of the Milk Fat Globule[1]

STUART PATTON, Department of Dairy Science,
Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT

Milk fat globules originate as fat droplets within the lactating mammary cell. These droplets are composed largely (>98%) of glycerides. Their constituent fatty acids are derived by lipolysis of very low density lipoproteins and chylomicrons of the blood and by de novo synthesis within the cell. Evidence of two principal routes for the synthesis of milk fat triglycerides has been presented: the so-called glycerolphosphate and monoglyceride pathways. Recent findings on these pathways are discussed. The nature of the milk triglycerides with their unique compliment and distribution of short chain fatty acids appears to depend upon a closely regulated relation between the soluble multienzyme complex that synthesizes the fatty acids and the glyceride synthetase that is bound to the endoplasmic reticulum. The resulting triglycerides appear to self-assemble into droplets from the surface of the endoplasmic reticulum. No special ultrastructures (transport particles, vesicles, etc.) have been detected in relation to this process. Milk fat droplets at the time of secretion average several microns in diameter, there being species variations. The basic secretion mechanism involves envelopment of the droplet in plasma membrane and expulsion of it from the cell. As a consequence there are at least two pools of polar lipids (cholesterol and phospholipids) associated with secreted milk fat globules, i.e., one from the plasma membrane and one entrained earlier from the endoplasmic reticulum at the time of triglyceride synthesis and accumulation. In all, the polar lipids do not make up more than 1-2% of the total lipids in milk and a substantial fraction of them has been identified recently with plasma membrane fragments occurring in the skim milk phase. Radiotracer and ultrastructural studies show that this membrane material does not result simply by shedding of surface from milk fat globules. This dispersed material and the lining around milk fat globules constitute valuable sources for the study of cell membranes.

INTRODUCTION

Interest in the milk fat globule increases as our knowledge of its origin and structure expand. As a cellular product it involves many intriguing biological processes. These include the transport of lipids from blood into the cell and ultimately into milk, the synthesis of triglycerides, their accumulation into fat droplets and the secretion of these fat droplets from the cell. In the process some of the blood lipids are transformed, such as the triglycerides, phospholipids and fatty acids; others such as cholesterol and carotene pass into the milk unchanged. The various metabolic events impart to the milk fat globule qualities that determine its nutritional value and, in the case of milk as an article of commerce, many of its processing properties. Thus studies of the origin and structure of milk fat globules have a number of useful aspects: (a) They may contribute to knowledge of basic cell biology; (b) they may add to understanding of milk biosynthesis; (c) they may yield information enabling the production of improved milk and milk products; and (d) they may indirectly aid in solution of certain biomedical problems involving lipid accumulations (atherosclerosis, obsesity and lipidoses of various kinds).

The present paper is an attempted status report regarding knowledge of the origin and structure of the milk fat globule. This will involve a review of selected literature together with discussion of some recent research findings from our laboratory. Recent reviews in the areas of milk lipid composition (1), ruminant metabolism in relation to the synthesis and secretion of milk fat (2), ultrastructure of milk fat globules (3-5) and secretion of milk (6) are available. The other papers of this symposium will give fresh interpretive treatments of a number of these areas. In addition a comprehensive treatise (7) which will deal with the biochemistry and physiology of lactation, including the milk lipids, is in preparation. Most of what is known regarding the milk fat globule derives from studies of bovine milk, and thus knowledge of the globule of this species is the essential basis of this paper. To a lesser yet important extent, the lactating goat, rat, mouse and guinea pig also have been employed in the research. While these species probably provide an adequate basis for generalizations, we will not know how significant species variations are until more research is conducted on the full spectrum of lactating mammals. At the present writing it is well established that there are wide species variations in the levels of fat and in the fatty acid compositions of milk (8,9). We are led to believe, however, that the mechanism of secretion of milk fat globules is generally the same for all mammals.

GENERAL NATURE AND ORIGIN OF MILK LIPIDS

The bulk (>99%) of bovine milk lipids exists in the form of fat globules which average 2-3 μ in diameter (Figs. 1 and 2). The remainder occurs in membrane-fragments in the skim milk phase (10,11). The lipids of milk fat globules are largely (98-99%) glycerides. These lipids also include ca. 1% phospholipid, 0.06% glycolipid, 0.30% cholesterol, which is 85-90% in the nonesterified form, and traces of many other lipid classes (free fatty acids, hydrocarbons including carotene, sterols and fat soluble vitamins). The composition of bovine milk lipids has been very extensively investigated and the knowledge is fairly complete (1). Two unique recent contributions to the knowledge of milk lipids concern the identification of ceramide (12) and gangliosides (13) in the medium. The fatty acid composition of the ceramide, obtained at a level of 2.2 mg/liter of milk, was observed to be identical to that of the sphingomyelin and cerebrosides of milk, which suggests that ceramide is a precursor of the latter two classes of lipids.

Milk lipids derive in part from lipids circulating in the blood, the remainder being synthesized in the mammary gland. The transport of serum lipids into the lactating cell, their integration into the synthesis of milk lipids within the cell and the secretion of the completed products from the cell constitute a principal research frontier of milk biosynthesis.

It is estimated that ca. 40-60% of the fatty acids of milk fat originate from triglycerides associated with serum lipoproteins (14,15). Raphael (16) has recently established that the bovine serum lipoprotein detected by Glascock et al. (17), which serves as the principal serum source of fatty acids for milk triglyceride synthesis, is a classical very low

[1]One of eight papers presented in the symposium "Milk Lipids," AOCS Meeting, Ottawa, September 1972.

density lipoprotein (VLDL). This class of serum lipoproteins sediments at a density <1.006 and exhibits electrophoretic mobility of a pre-β serum lipoprotein. The triglycerides of VLDL in the lactating bovine are degraded by lipoprotein lipase in the endothelium lining capillaries of mammary tissue (18,19). In nonruminant species chylomicron triglycerides also are substrates in this process (18,19). The resulting fatty acids and glycerol are utilized in the synthesis of milk lipids along with fatty acids and glycerol derived de novo in the mammary tissue. Metabolic pathways involved are discussed subsequently.

In addition to serum triglycerides, carotene, cholesterol and phospholipids are taken up from the blood. Carotene and cholesterol are transferred as such to milk. Serum phospholipids transferred to the mammary gland appear to be completely broken down in the tissue. The evidence is that most, if not all, of the milk phospholipids are synthesized de novo in the mammary epithelium (20).

SYNTHESIS OF MILK TRIGLYCERIDES

Monoglyceride Pathway

Research on the synthesis of milk fat triglycerides has centered on two principal metabolic routes: the monoglyceride pathway and the α-glycerolphosphate pathway. It has been demonstrated that triglyceride synthesis in intestinal mucosa involves monoglyceride as substrate (21). Monoglycerides and fatty acids released by lipase in the lumen of the intestine are transported into the mucosal cells and are there resynthesized into triglycerides. This synthesis requires prior activation of the fatty acids by adenosine triphosphate and coenzyme A. McBride and Korn (22) have demonstrated such a monoglyceride pathway in guinea pig mammary tissue in vitro. On the basis of the positioning of palmitic acid in high and low molecular weight milk triglycerides, Dimick et al. (23) have noted that the 2 position of the high molecular weight fraction appears to be occupied by a palmitate precursor from the blood. Yet when ^{14}C-palmitic acid is given intravenously (goat) it preferentially labels the 1 or 3 positions. This suggests that something other than serum fatty acid in free form, presumably a 2-monoglyceride, accounts for the observations of Dimick et al. Recently Coccodrilli (24) has shown that milk triglyceride glycerol derived from glucose within the mammary gland (goat) is diluted in the high molecular weight fraction by glycerol from another source (Fig. 3). It appears beyond doubt that at least part of this other source is derived from blood triglycerides through action of lipoprotein lipase. It is known that lactating mammary tissue contains glycerol kinase (25), and more recently Kinsella (26) has demonstrated this enzyme in freshly secreted bovine milk. The question seems to be whether the serum triglycerides utilized in the synthesis of milk fat are only partially degraded to an extent that considerable monoglyceride remains or whether there is total breakdown to free fatty acids and glycerol. The most recent findings (27) from continuing investigations of this problem at the laboratories of Annison and Linzell has led them to conclude that the plasma triglycerides are more or less completely hydrolyzed (see also 19). In terms of the findings of Dimick et al. (23), this would seem to establish a fatty acid transport mechanism from serum triglycerides into the lactating cell which specifically positions palmitate in the 2 position of high molecular weight milk triglycerides. In any event, unequivocal evidence that a 2-monoglyceride pathway either does or does not exist in vivo for the synthesis of milk fat triglycerides is yet forthcoming.

α-Glycerolphosphate Pathway

The most ubiquitous known biochemical route in the synthesis of triglycerides is that involving α-glycerolphosphate. According to the Kennedy scheme (28,29) this compound, generated either by glycolysis or the action of glycerol kinase, is acylated with 2 mol fatty acid to yield the 1,2 diacyl phosphatidic acid, which in turn is converted by phosphatidic acid phosphohydrolase to a diglyceride. This latter compound then is acylated with a third mole of fatty acid to yield the triglyceride. Since the experiments of Luick (30), it has been known that glucose is converted to milk triglyceride glycerol in the bovine mammary gland. However the demonstration of phosphatidic acid, a key intermediate in the Kennedy scheme, in either milk or mammary tissue has proven difficult. A number of complicating factors have contributed to this problem. Under normal conditions there appears to be essentially no mass of this compound in either milk or mammary tissue. A number of phosphatidic acid preparations obtained from supply houses appeared to be methyl phosphatidates, as a result of extracting preparative reactions (phosphatidylcholine plus phospholipase D) with solvents containing methanol (see 31) rather than authentic phosphatidic acid. This has created confusion regarding the behavior of the compound in thin layer chromatographic (TLC) systems. Few authors have given adequate detail as to their methods of isolating phosphatidic acid. In addition, phosphatidic acid is always a potential artifact by decomposition of any of the other glycerolphospholipids.

As with the 2-monoglyceride route, the α-glycerolphosphate pathway has been demonstrated in mammary tissue in vitro (32,33). We have sought to provide evidence of the latter path in the intact animal, and a representative experimental result is presented in Figure 4. It shows that a very high specific activity component that coincides precisely in two dimensional TLC systems with phosphatidic acid is detectable in mammary tissue within a few minutes, following iv injection of $H_3{}^{32}PO_4$ into the rat. Details of these experiments will be published subsequently.

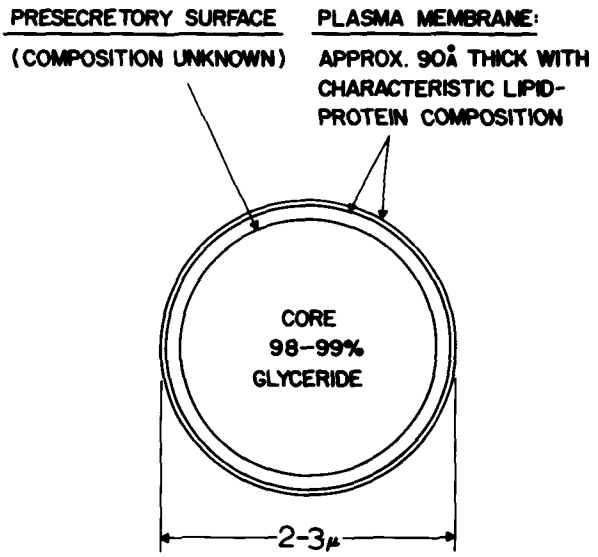

FIG. 1. Scheme for typical milk fat globule.

FORMATION OF MILK FAT GLOBULE

While it is well established that the lactating mammary cell synthesizes triglycerides and readily evident from ultrastructure studies that the cell contains lipid droplets (fat globules) that are secreted, the actual mechanism by which the glyceride molecules join to make a fat droplet is not known. From the electron microscopy autoradiography studies of Stein and Stein (34), it is evident that triglycerides are synthesized in the endoplasmic reticulum of the cell. Kinsella has recently confirmed that milk triglycerides are recovered with the microsomal (mainly endoplasmic

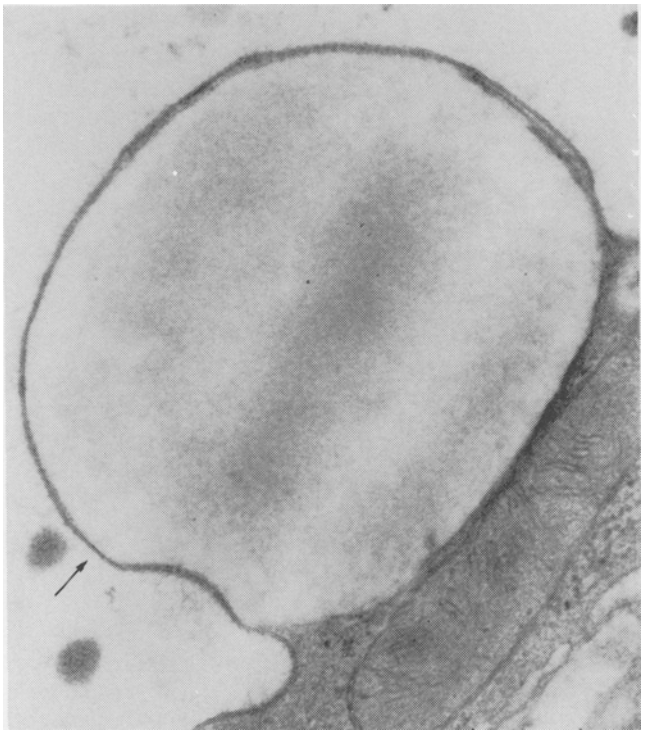

FIG. 2. Milk fat globule (rat) in process of being secreted from cell. Large mitochondrion lies under globule inside cell. Two casein micelles are evident in alveolar lumen (lower left by arrow). Note variable thickness of globule membrane. At one point (arrow) well resolved unit membrane flush against globule core is evident. Globule is 1.60 x 1.27 μ in long and short diameters.

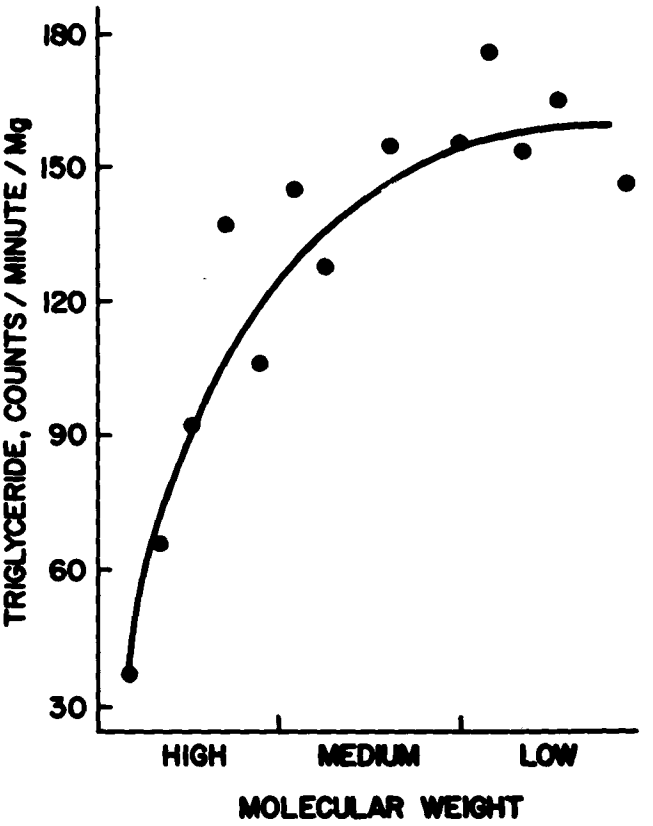

FIG. 3. Specific activity in glycerol of fractionated triglycerides of goat milk following intramammary infusion with U-^{14}C-glucose.

reticulum) fraction of the lactating cell (35) and that this fraction is the site of milk triglyceride synthesis (36). Thus the question remains: How do glycerides synthesized by intracellular membranes gather into droplets?

An interesting consideration in milk fat droplet formation is the melting properties of the constituent triglycerides. Since new molecules of triglyceride must enter the growing milk fat droplet at its outer surface, a liquid state in the droplet would facilitate the uptake of additional triglyceride molecules whereas solidification of the droplet would prevent entering glyceride molecules from disappearing into the interior of the droplet. The ruminant mammary cell has two known devices for ensuring that milk triglycerides are maintained in a relatively liquid state. One is through synthesis of triglycerides which contain short chain fatty acids and thus relatively low melting points. The other is through conversion of stearic acid to the lower melting oleic acid and the use of this latter acid in triglyceride synthesis. It has been shown (37,38) that the ca. 10 mol% of butyric acid in bovine milk fat occurs 1 mol per mole of glyceride, so that ca. 30% of the milk triglyceride molecules contain butyric acid. Of course the other short chain fatty acids (C_6-C_{12}) as components of triglyceries would also tend to maintain liquid conditions in milk fat droplets at body temperature. Kinsella (36) has demonstrated a close association in the stearyl desaturase and glyceride acyl transferase enzymes of lactating mammary tissue. He suggests that these two membrane-bound enzymes are physically proximate to each other on the membrane surface and that stearyl desaturase functions as a regulator of triglyceride synthesis. We are proposing that the actual cytophysical basis of this regulation may be through removal of end product (triglyceride), i.e., if the supply of oleic acid is abundant, the triglycerides will tend to be low melting and as liquid molecules can be moved easily from the site of synthesis into the fat droplet. Another consequence of this idea is that fat droplet growth may be halted by the accumulation of high melting triglycerides at its surface.

A further interesting consideration in the matter of triglyceride synthesis and milk fat droplet formation is the character and functioning of fatty acid synthetase. It appears that the unique fatty acid pattern of ruminant milk fat triglycerides is preserved so long as the mammary cells are maintained intact (39). However when fatty acid synthetase, a multienzyme complex occurring in the cytosol of the mammary cell, is isolated and purified the exclusive product of its synthesis is palmitate (40). This evidence suggests that within the intact cell the pattern of triglyceride fatty acids obtained may be dependent upon a structured relationship between the fatty acid synthetase and the sites of triglyceride synthesis on the endoplasmic reticulum, as appears to be the case with stearyl desaturase. In earlier reports (41) we have suggested several possible mechanisms for milk fat droplet formation within the mammary cell. These are: (a) growth of droplets by synthesis of triglycerides at their cytoplasmic interphases (Fig. 5, upper left); (b) merging of smaller droplets to form larger droplets (Fig. 5, upper right); (c) conveyance of triglycerides by small messenger particles which are the site of triglyceride synthesis or which shuttle from that site to the forming fat droplet (Fig. 5, middle); (d) cytoplasmic flow encouraging triglyceride molecules which have accumulated by synthesis on intracellular membranes to gather into droplets in order to minimize free surface energy; formed droplet then grows in size as glyceride-laden membranes flow against it (Fig. 5, lower left). An addition to this list is: (e) conveyance of triglycerides synthesized on the endoplasmic reticulum by way of the internal phase of lipid bilayer membranes to points of glyceride accumulation (Fig. 5, lower right).

Unfortunately we do not yet have entirely adequate ways of mapping the location of lipids at the molecular level in tissues. However some evidence in literature and our

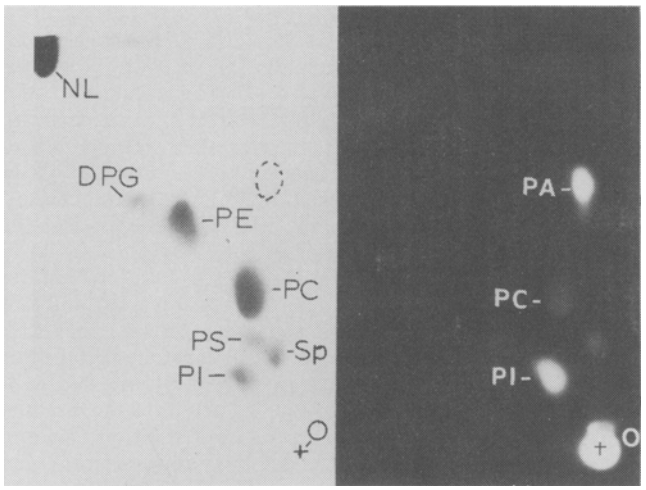

FIG. 4. Left: thin layer chromatographic separation of polar lipids extracted from lactating rat mammary gland 10 min following iv injection of 0.2 mC_i of $H_3{}^{32}PO_4$. Right: autoradiogram of the same thin layer separation showing location of radioactivity (^{32}P) in various lipids. Abbreviations: NL, neutral lipids; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid (position of PA mass where indicated by broken line); PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; Sp, sphingomyelin; O, origin.

own observations with the aid of electron microscopy during the past 5 years suggest that certain of these alternatives are less likely than others. The autoradiographic studies of Stein and Stein (34) do not indicate that the surface of forming milk fat droplets is a preferential site of glyceride synthesis but that cell membranes (endoplasmic reticulum) are. Further, freshly secreted milk fat globules show little capability of incorporating fatty acid into triglycerides, but the skim milk phase does (42). So it would appear that alternative a is unlikely. There is no evidence as yet to support mechanisms b and c. We have seldom, if ever, seen cells with many small fat droplets and we have seen very few instances of fat droplets merging, such as seems commonplace with Golgi vesicles. Rather, there are ordinarily a few fat droplets per cell. If a messenger particle of the type described in mechanism c exists, it has not yet been adequately demonstrated either by microscopy or biochemistry. One would expect an accumulation of such particles to be commonly observable around fat globules within the cell. Mechanisms d and e offer the alternatives that triglycerides synthesized on the membrane accumulate either on the outside or the inside of the membrane. Again we know of no consistent ultrastructural evidence to support either of these possibilities. However, if a lipid bilayer construction of the membrane at the site of milk triglyceride synthesis is assumed, one might conceive of completed triglyceride molecules moving about readily within the bilayer of the membrane rather than accumulating on its more polar surface. Maintenance of a liquid condition in the hydrocarbon region of the membrane would then enable the flow of triglyceride molecules to points of accumulation (droplet formation). Skillful use of electron microscopy should eventually enable one to discern the true mechanism of milk fat droplet formation.

We have observed, as have others (4,43), that lipid droplets in the basal area of the lactating cell are relatively small and that their mean size increases progressively toward the apical (secreting) end of the cell. Since the basal area of the cell must be relatively enriched in fatty acids of serum origin, there probably is a second gradient along the cell in addition to size of the droplet, and that concerns the sources of fatty acid for triglyceride synthesis.

MILK FAT GLOBULE MEMBRANE

It is not possible to understand the nature and origin of the milk fat globule without considering the mechanism of its secretion, for in this process the globule derives unique constituents and properties. It is now well established that the milk fat droplet in the process of its secretion is enveloped in plasma membrane of the lactating cell. The progressive envelopment effectively separates the droplet from the cell (Fig. 2) and the process is completed by a pinching off of the connecting membrane. Extensive evidence supporting this mechanism, which was first documented by ulttastructural studies of Bargmann and Knoop (43), has been presented elsewhere (44-46). Research on the milk fat globule membrane, reviewed by Brunner (47), was strongly oriented in a physicochemical direction for many years. The approach has received much benefit by clarification of the cytological origin of the membrane. Nonetheless, problems involved in the research appear more challenging than ever. It can be stated with some certainty that three principal surfaces are involved in the secreted milk fat globule. There is the surface that it has as a fat droplet before secretion from the cell, and there are the inner and outer surfaces of the plasma membrane which are superimposed on the native surface (Figs. 1 and 2). While it is well known that the core of milk fat globules is composed almost exclusively (98-99%) of glycerides, it is not possible to assume that the surface material is entirely plasma membrane. The surface components on the fat droplet as it exists within the cell have not been characterized. The inner surface may well represent unique pools of cholesterol, phospholipids, proteins and other surface active molecules. Marker enzymes for endoplasmic reticulum, such as DPNH cytochrome C reductase and glucose-6-phosphatase, have been detected in the bovine (48) and human (49) milk fat globule membrane. In any event, the isolation of these droplets from the cell so that they can be analyzed regarding surface and other constituents is fraught with problems of artifact generation (Hood and Patton, unpublished data). The problem is to recover the droplets without

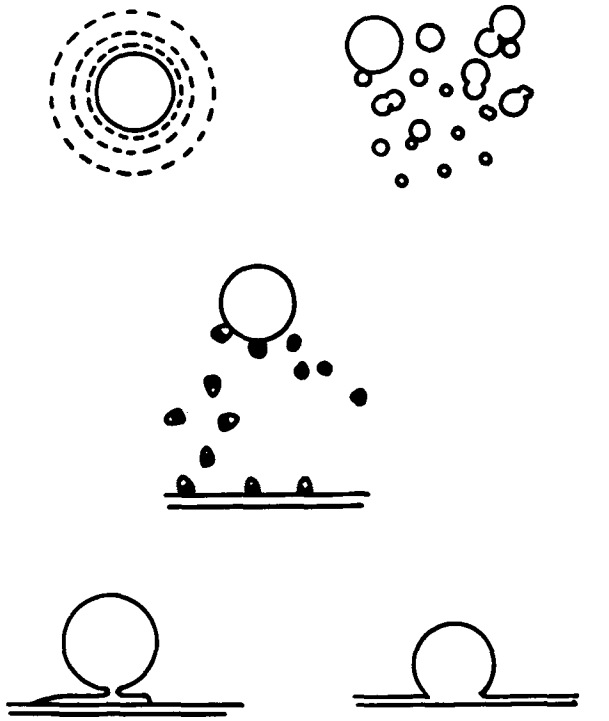

FIG. 5. Some possible mechanisms of milk fat droplet growth within cell. Upper left: droplet grows by synthesis of glycerides at its surface; upper right: droplet grows by small droplets merging to form larger droplets; middle: growth of droplet results from addition of lipid to its surface by messenger particles laden with triglyceride; lower left: droplet grows by adhesion of glycerides on surface of lipid-synthesizing membrane; lower right: droplet grows by triglycerides moving within bilayer membrane from their points of synthesis to points of accumulation (nonpolar collecting regions).

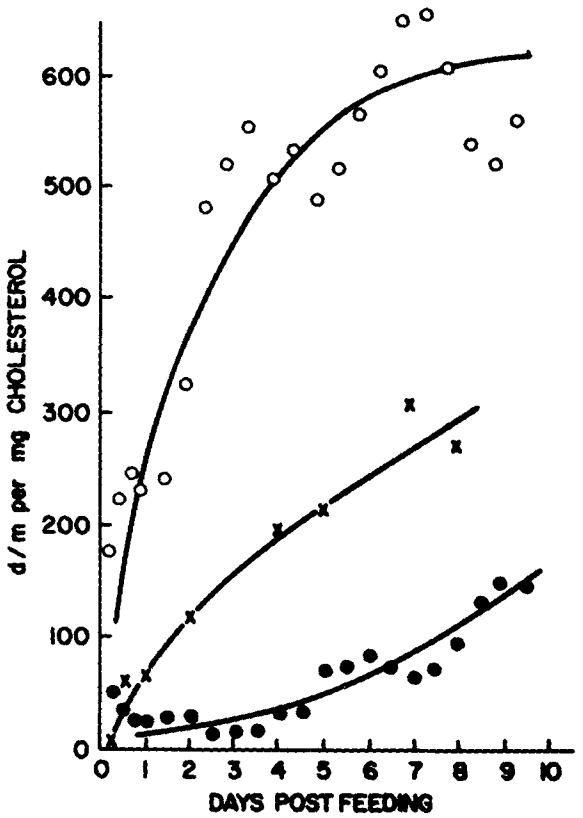

FIG. 6. Incorporation of dietary cholesterol into milk by lactating goat mammary gland. X—X, Blood serum cholesterol; •——•, cream (milk fat globule) cholesterol; ○——○, skim milk cholesterol. 4-^{14}C-Cholesterol (50 μ Ci) in 20 ml corn oil was administered by injection directly into abomasum.

entraining, extracting or absorbing other ceullular components.

There are also difficulties of interpreting the ultrastructure of the milk fat globule membrane from electron photomicrographs. It must be borne in mind that at best the electron microscope enables us to see reproducible artifacts. It is difficult, if not impossible, to fix, embed and section milk fat globules in a completely satisfactory way. The saturated nature of milk fat does not facilitate reaction with osmium or glutaraldehyde. Consequently the lipids frequently extract, slip or contract, leaving holes, tears, ridges or smears in the sectioned material. In spite of this, researchers are able to make some consistent observations about the ultrastructural appearance of milk fat globules. It is generally agreed that the milk fat droplet within the cell is not surrounded by the classical dark-light-dark unit membrane, but that a finer single osmiophilic line at the surface of the droplet is sometimes observed. It is further observable that a classical membrane (the plasma membrane) surrounds the fat globule at the time of its secretion. Crescents of cytoplasm are occasionally caught between the lipid surface and the surrounding membrane at the time of fat globule secretion; this cytoplasmic material containing varying amounts and kinds of cell organelles may be a source of some of the enzymes detected in association with milk fat globules. Wooding et al. (50) estimate that 1-5% of secreted bovine milk fat globules in their study had crescents associated with them.

It is also generally held, since the observations of Bargmann and Knoop (43) to the present time (5,51), that there appears to be a rearrangement of the milk fat globule membrane as the globule undergoes postsecretion aging in the milk serum. There is no question that the appearance of the membrane seems to change, given any one method of attempting fixation; however the meaning of this observation is unclear. In our experience many variations in the ultrastructural appearance of fresh or aged fat globule membranes are observed. Even with a single globule, differences are frequently seen. In the case of the micrograph (Fig. 2), it is evident that the appearance of the membrane around the protruding (secreted) portion of the globule varies. There is one point (arrow, lower left) where the globule has a thin, well resolved unit membrane. Along the top of the globule, the membrane is much thicker and more diffuse, and at the upper right it again seems clear-cut in appearance but with a layered effect.

We have discussed elsewhere (44) the London-Van der Waals attraction forces between the fat droplet and the plasma membrane at secretion. It seems reasonable that hydrophobic forces of this type would continue to act after secretion, expelling additional water (cytoplasm) and pulling the membrane more and more tightly onto the globule. Bearing in mind the difficulties of lipid fixation, we feel that differences in appearance of membrane around aged globules may result from progressing dehydration of the membrane which makes it more difficult to fix, embed and section. Wherever globules contain cytoplasmic inclusions (moisture) under the membrane, definition of the membrane under the electron microscope is improved (Fig. 10, Reference 5). In addition, membranes removed from milk fat globules appear to show reasonably good unit membrane appearance (52).

Another type of postsecretion change in milk fat globules about which there is continuing speculation concerns loss of globule membrane material to the skim milk phase. In electron microscopic studies of the milk fat globule membrane, Wooding (51) has observed that membrane is lost into the milk plasma by a process of vesiculation, leaving a structureless secondary membrane on the globules. On the other hand, milks (cow and goat) normally collected from the gland show ca. 60% of their phospholipids (53) and ca. 85% of their cholesterol (54) associated with the fat globules. One is inclined to wonder how the membrane is lost to the skim milk when most of the membrane lipids are retained by the globule. Moreover some of the lipid-bearing membrane material in the skim milk phase is microvilli (10), no doubt derived by sloughing of the cell surface. Further, tracer studies (53) indicate that the time courses and intensities in labeling phospholipids of skim milk and fat globules are quite different. An interesting recent observation at our laboratory is that milk fat globules exhibit substantial Mg^{+2}/ATPase activity (46), whereas skim milk shows none of this enzyme (11). While none of the foregoing observations precludes the possibility that some fat globule membrane material is shed into the skim milk, the quantitative significance of such a transfer needs to be evaluated. Incubations of radiotracer-labeled, freshly secreted milk fat globules in unlabeled skim milk and vice versa might help clarify the extent to which material leaves the globule surface.

The question of milk fat globule membrane stability is important. This membrane and the membrane material in the skim milk phase (10,11) afford a valuable research opportunity to study membrane structure and function, in that plasma membrane of the cell is made available in a physiologically satisfactory condition. Normally cells must be disintegrated in order to obtain plasma membrane, which poses problems of artifacts and impurities. However, if the globule membrane is undergoing profound structural changes following secretion, it will make reliable information on structure and function of the membrane more difficult to obtain. Research on the molecular architecture of the milk fat globule membrane is just beginning. We have suggested a conceptual approach to the sidedness of the membrane regarding certain plasma membrane marker enzymes (46). For example, all the 5'-nucleotidase appears to be on the outer surface of the plasma (milk fat globule) membrane.

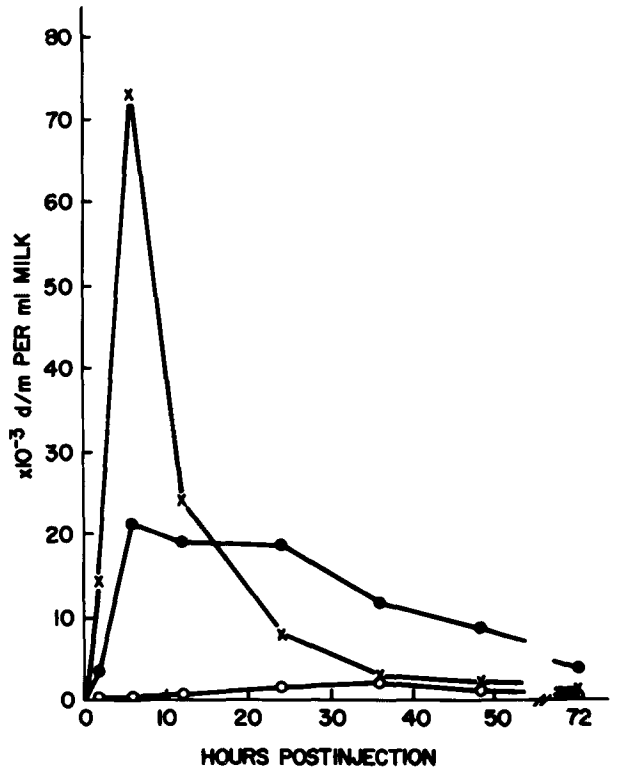

FIG. 7. Incorporation of 2-^{14}C-mevalonate into milk lipids by lactating rat mammary gland. Cream and skim milk activities expressed as equivalent to 1 ml whole milk. X—X, Triglyceride radioactivity in cream fraction; •—•, cholesterol radioactivity in cream (milk fat globule) fraction; ○—○, cholesterol radioactivity in skim milk fraction. Dose of mevalonate was 20 μ Ci in 0.2 ml saline injected intravenously.

This question can be raised: From whence comes the membrane to replace plasma membrane lost from the cell in the secretion of milk fat globules? We have suggested previously (44) that membranes around Golgi vesicles may replenish plasma membrane at the time these vesicles merge with the plasma membrane in emptying their contents out of the cell; Wooding (4) has presented evidence that Golgi vesicle membranes may at times partially bound the fat droplet surface before and during its secretion. What keeps the amount of plasma membrane in balance is an interesting question, because there would appear to be far more membrane around mature Golgi vesicles than is required to replenish that lost around milk fat globules.

ORIGIN OF SOME MINOR GLOBULE CONSTITUENTS

Cholesterol, phospholipids and β-carotene are among the more important minor components of the milk fat globule. The ultimate origin of these compounds leads to considerations of membrane synthesis and transformation beyond the scope of this paper. However we would like to give our understanding of the research frontier with regard to the origin of these globule constituents.

Phospholipids

There may be two pools of phospholipids associated with the milk fat globule, one relating to the droplet surface before secretion and one involved in the plasma membrane that encompasses the globule at secretion. The studies of Easter et al. (55) indicate that most, if not all, the phospholipids of milk are synthesized de novo in mammary tissue. This results from evidence that intravenously administered $^{32}P_i$ labels (goat) milk phospholipids more promptly and intensely than it labels blood serum phospholipids and that autoradiograms from such experiments reveal all the milk phospholipids to be labeled

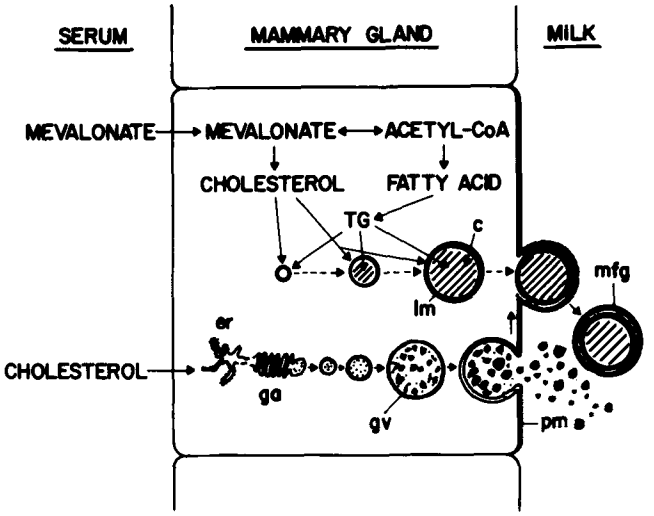

FIG. 8. Proposed scheme (by D.J. Easter) for incorporation of exogenous and endogenous cholesterol into milk fat globule. Abbreviations: TG, triglyceride; lm, limiting membrane (of developing globule); er, endoplasmic reticulum; ga, golgi apparatus; gv, golgi vesicles; pm, plasma membrane; mfg, milk fat globule.

approximately in proportion to their masses. Further, in $^{32}P_i$ experiments with both the rat and the goat, the ratios between masses for total milk phosphorus and milk lipid phosphorus and ratios of their total radioactivities were essentially identical, which suggests that inorganic phosphorus and lipid phosphorus of milk were being derived from a single pool of phosphate within the gland. An additional observation from the study was that no dietary phospholipid (U-^{14}C-phosphatidylcholine) was transferred intact into rat milk. The capacity of isolated mammary cells to synthesize the glycerophospholipids of milk has been demonstrated using radioactive glycerol (56). However additional experiments with such cells regarding their capacity to incorporate $^{32}P_i$ into phospholipids of the cell and into secretory lipids would be desirable.

Cholesterol

Despite the importance of cholesterol in the diet, its origin and distribution in milk and milk products is not very well established. This results from there having been few comprehensive studies and from the somewhat diverse forms and locations of cholesterol in milk. Milk cholesterol occurs in both free and esterified form and in skim milk as well as in association with fat globules. In addition, fat globules may contain the two forms in the surface (membrane) layer as well as in the globule core. The two fairly comprehensive analyses of cholesterol and cholesterol ester distribution in milk (54,57) have yielded conflicting results. From literature values and our own unpublished findings we conclude that ca. 20% of total cholesterol occurs in the skim milk phase, and the remainder is associated with the cream (milk fat globules) phase; that of the total milk cholesterol (ca. 10%) occurs in esterified form. Data (54,57) regarding the amount and form of cholesterol associated with the core of fat globules pose difficulties, because methods for partitioning the surface and cores of globules may easily lead to artifactual cholesterol distributions. Additional comprehensive research on cholesterol distribution in milk is clearly needed.

Milk cholesterol may be of dietary origin, synthesized endogenously and carried to the mammary gland via the circulation or synthesized in the mammary gland (58,59). While there appears to be species differences in the relative contributions of these three sources, the data from which to draw firm conclusions regarding any species are relatively meager.

The fact that serum cholesterol is transported into milk raises some highly intriguing questions regarding which

serum lipoprotein(s) is the vehicle and how the cholesterol is taken up and transported through the lactating cell into milk. Data from our laboratory by Easter (59 and unpublished findings) give interesting insight on these problems. In the lactating rat he observed that the turnover time for dietary cholesterol from blood serum into milk was 17-20 hr, suggesting that this relatively long time was required for the cholesterol to evolve through the membrane system of the lactating cell into the milk (59). Results from subsequent studies (unpublished) with the lactating goat and rat indicated that ^{14}C-serum cholesterol tends to preferentially label the skim milk membrane material (Fig. 6), while cholesterol synthesized from ^{14}C-mevalonate in the mammary tissue labels fat globule cholesterol (Fig. 7). These findings led Easter to propose a two pool scheme for cholesterol metabolism by the lactating cell, as shown in Figure 8. This scheme is provocative in that it can be readily tested by additional experiments.

One of the more interesting implications of the data from Figure 6 is that the serum precursor of milk cholesterol must be a relatively minor serum lipoprotein from the standpoint of cholesterol content, because the specific activity of the skim milk cholesterol greatly exceeded that of the blood serum.

Another interesting point regarding cholesterol metabolism by the mammary gland concerns the rapid turnover of fatty acid through the cholesterol ester fraction of milk (60,61). It is now of much interest to establish which of the several sites (fat core, globule membrane or skim milk membrane) contains this metabolically active fraction and what the acceptor is for the acyl moieties that it is transferring.

Carotene

The yellow color of bovine milk fat globules is due to carotenoids (presumably β-carotene). So far as is known this milk fat globule pigment and provitamin is exclusively of dietary origin. Essentially little or no carotene reaches the goat milk fat globule or milk fat globules of most other species because of its destruction or conversion to vitamin A within the animal body. The transport of carotene into bovine milk would seem deserving of careful study, since it is an interesting marker of lipid metabolism and may represent a model to be compared, for example, with transport of cholesterol and its esters, organic pesticide compounds and other nonpolar molecules. Analysis of bovine serum lipoproteins (D.L. Puppione and S. Patton, unpublished data) reveal them to be rich in carotenoid. Interestingly enough, the lipoprotein class known to be taken up by the mammary gland, the VLDL, appears to have very low levels of carotenoid in comparison to the other serum lipoprotein classes. Further studies will be required to establish which of these classes carry the carotenoid destined for the milk fat globule. How carotene is transported through the mammary cell and establishment of its precise location in the milk fat globule are additional research challenges. Earlier work has indicated that carotene is distributed in the bovine milk fat globule in accord with surface area (62) and that it is concentrated in the globule membrane (47).

We have not touched on the surface of the milk fat globule (plasma membrane) as a purveyor of species specific immunologic information, as a possible site to investigate cell recognition phenomenon or as a structure that may reveal aging effects in the membranes of a differentiated cell. But it is clear that the milk fat globule presents an expanding array of interesting research possibilities.

ACKNOWLEDGMENT

This research was supported in part by Grant HL 03632 from the U.S. Public Health Service. P.S. Stewart prepared and discussed electron photomicrographs, and C. Thoele provided technical assistance.

REFERENCES

1. Morrison, W.R., in "Topics in Lipid Chemistry," Vol. I, Edited by F.D. Gunstone, John Wiley and Sons, Inc., New York, 1970, p. 51.
2. Storry, J.E., J. Dairy Res. 37:139 (1970).
3. Knoop, E., Milchwissenschaft 27:364 (1972).
4. Wooding, F.B.P., J. Cell Sci. 9:805 (1971).
5. Henson, A.F., G. Holdworth and R.C. Chandan, J. Dairy Sci. 54:1752 (1972).
6. Linzell, J.L., and M. Peaker, Physiol. Rev. 51:564 (1971).
7. B.L. Larson and V.R. Smith, "Lactation: a Comprehensive Treatise," Edited by Academic Press, In press.
8. Jenness, R., and R.E. Sloan, Dairy Sci. 1970:599 (Abstr. 32).
9. Glass, R.L., H.A. Troolin and R. Jenness, Comp. Biochem. Physiol. 22:415 (1967).
10. Stewart, P.S., D.L. Puppione and S. Patton, Z. Zellforsch. 123:161 (1972).
11. Plantz, P.E., and S. Patton, Biochim. Biophys. Acta 291:51 (1973).
12. Fujino, Y., and T. Fujishima, J. Dairy Res. 39:11 (1972).
13. Keenan, T.W., C.M. Huang and D.J. Morré, Biochem. Biophys. Res. Comm. 47:1277 (1972).
14. Anninson, E.F., J.L. Linzell, S. Fazakerley and B.W. Nichols, Biochem. J. 102:637 (1967).
15. Bishop, C., T. Davies, R.F. Glascock and V.A. Welch, Ibid. 113:629 (1969).
16. Raphael, B.H., Ph.D. thesis, Pennsylvania State University, 1972.
17. Glascock, R.F., V.A. Welch, C. Bishop, T. Davies, E.W. Wright and R.C. Noble, Biochem. J. 98:149 (1966).
18. Schoefl, G.I., and J.E. French, Proc. Roy. Soc. B. 169:153 (1968).
19. Scow, R.O., M. Hamosh, E.J. Blanchette-Mackie and A.J. Evans, Lipids 7:497 (1972).
20. Easter, D.J., S. Patton and R.D. McCarthy, Ibid. 6:844 (1971).
21. Senior, J.R., J. Lipid Res. 5:495 (1964).
22. McBride, O.W., and E.D. Korn, Ibid. 5:448 (1964).
23. Dimick, P.S., R.D. McCarthy and S. Patton, Biochim. Biophys. Acta 116:159 (1966).
24. Coccodrilli, G.D., Ph.D. thesis, Pennsylvania State University, 1971.
25. McBride, O.W., and E.D. Korn, J. Lipid Res. 5:442 (1964).
26. Kinsella, J.E., Int. J. Biochem. 3:89 (1972).
27. West, C.E., R. Bickerstaffe, E.F. Anninson and J.L. Linzell, Biochem. J. 126:477 (1972).
28. Kennedy, E.P., Ann. Rev. Biochem. 26:119 (1957).
29. Weiss, S.B., E.P. Kennedy and J.Y. Kiyasu, J. Biol. Chem. 235:40 (1960).
30. Luick, J.R., J. Dairy Sci. 44:652 (1961).
31. Yang, S.F., S. Freer and A.A. Benson, J. Biol. Chem. 242:477 (1967).
32. Pynadeth, T.I., and S. Kumar, Biochim. Biophys. Acta 84:251 (1964).
33. Kuhn, N.J., Biochem. J. 105:213 (1967).
34. Stein, O., and Y. Stein, J. Cell Biol. 34:251 (1967).
35. Kinsella, J.E., Lipids 7:165 (1972).
36. Kinsella, J.E., Ibid. 7:349 (1972).
37. Patton, S., and R.D. McCarthy, J. Dairy Sci. 46:916 (1963).
38. Dimick, P.S., and S. Patton. Ibid. 48:444 (1965).
39. Kinsella, J.E., and R.d. McCarthy, Biochim. Biophys. Acta 164:518 (1968).
40. Smith, S., and S. Abraham, J. Biol. Chem. 245:3209 (1970).
41. Dimick, P.S., R.D. McCarthy and S. Patton, in "Physiology of Digestion and Metabolism in the Ruminant," Edited by A.T. Phillip and Son, Oriel Press Ltd., Newcastle-upon-Tyne, England, 1970, p. 529.
42. McCarthy, R.D., and S. Patton, Nature 202:347 (1964).
43. Bargmann, W., and A. Knoop, Z. Zellforsch. 49:344 (1959).
44. Patton, S., and F.M. Fowkes, J. Theoret. Biol. 15:274 (1967).
45. Keenan, T.W., D.J. Morré, D.E. Olson, W.N. Yunghans and S. Patton, J. Cell Biol. 44:80 (1970).
46. Patton, S., and E.G. Trams, FEBS Letters 14:230 (1971).
47. Brunner, J.R., in "Fundamentals of Dairty Chemistry," Edited by B.H. Webb and A.H. Johnson, AVI Publishing Co., Inc., Westport, Conn., 1965, p. 403.
48. Dowben, R.M., J.R. Brunner and D.E. Philpott, Biochim. Biophys. Acta 135:1 (1967).
49. Martel-Pradal, M.B., and R. Got, FEBS Letters 21:220 (1972).
50. Wooding, F.B.P., M. Peaker and J.L. Linzell, Nature 226:763 (1970)
51. Wooding, F.B.P., J. Ultrastructure Res. 37:388 (1971).
52. Keenan, T.W., D.E. Olson and H.H. Mollenhauer, J. Dairy Sci. 54:295 (1971).
53. Patton, S., and T.W. Keenan, Lipids 6:58 (1971).
54. Mulder, H., and T.A. Zuidhof, Neth. Milk Dairy J. 12:173 (1958).

55. Easter, D.J., S. Patton and R.D. McCarthy, Lipids 6:844 (1971).
56. Kinsella, J.E., Biochim. Biophys. Acta 164:540 (1968).
57. Huang, T.C., and A. Kuksis, Lipids 2:453 (1967).
58. Connor, W.E., and D.S. Lin, Amer. J. Physiol. 213:1353 (1967).
59. Easter, D.J., Lipids 6:645 (1971).
60. Patton, S., and R.D. McCarthy, J. Dairy Sci. 46:396 (1963).
61. Keenan, T.W., and S. Patton, Lipids 5:42 (1970).
62. White, R.F., H.D. Eaton and S. Patton, J. Dairy Sci. 37:147 (1954).

[Received October 26, 1972]