# Negligible release of cardiolipin during milk secretion by the ruminant

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ABSTRACT The presence of cardiolipin (diphosphatidyl glycerol) in lactating mammary tissue (cow and goat) was investigated. The tissue was separated into subcellular fractions by sedimentation; the identities of the fractions were confirmed by electron microscopy. Polar lipids recovered from the fractions, the whole tissues, and milks were analyzed by two-dimensional thin-layer chromatography and the percentages of cardiolipin were determined.

The phospholipids of whole mammary tissue from the cow and goat contain 3–5% cardiolipin which is concentrated largely, if not exclusively, in the mitochondria. Although milk may on occasion have up to 1% cardiolipin in its phospholipids, some normal milks contain less than 0.15%. Since tissue contains 20–30 times the amount (mg/g) of phospholipids in milk, the quantitative ratio of tissue to milk cardiolipin is several hundred to one.

We interpret this to mean that the mechanism of milk secretion is highly selective and insures retention of mitochondria within the cell even though they are decidedly smaller than milk fat globules which are continuously secreted. Our findings substantiate the conception that there is very little disintegration of the cell or disruption of the plasma membrane during milk secretion.

The fatty acids of cardiolipin from lactating mammary tissue of cow, goat, and pig are highly unsaturated; they contain 50% or more octadecadienoic acid.

SUPPLEMENTARY KEY WORDS mammary tissue · mitochondria · diphosphatidyl glycerol · thin-layer chromatography · fatty acid composition

Abbreviations: cardiolipin, di(3-*sn*-phosphatidyl) 1′,3′-*sn*-glycerol; GPGPG, di(*sn*-glycero-3-phosphoryl) 1′,3′-*sn*-glycerol; TLC, thin-layer chromatography.

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INVESTIGATION of the polar lipids of bovine milk and mammary tissue has revealed a component that occurs at readily detectable levels in the tissue, but which appears to be virtually absent from milk. In two-dimensional thin-layer chromatography, the unknown component moved in the manner of cardiolipin (diphosphatidyl glycerol). It comprised 3–4% of the lipid phosphorus in the tissue, but its level in milk was nil (1). Morrison (2) found no cardiolipin in phospholipids of milk from seven species.

Cardiolipin is a distinctive and substantial lipid component of mitochondria (3, 4). Although this is not necessarily the only cellular locale of cardiolipin, the absence of this lipid from milk would imply that milk contains no mitochondria. Mitochondria are reasonably plentiful in lactating mammary tissue (5), and their failure to be transported into milk would be highly meaningful in terms of the mechanism of milk secretion. For example, it does not seem possible that milk fat droplets, which average several microns in diameter, could leave holes in the plasma membrane as a result of their secretion without some of the mitochondria (0.5–1.0 $\mu$) also passing into the milk.

Evidence regarding the cellular mechanism of milk secretion has been derived mainly from electron photomicrographs (5–10). While there is general agreement with the findings of Bargmann and Knoop (5) that milk fat is secreted by extrusion of droplets from the cell and milk protein is secreted by emptying of Golgi vacuoles, the extent to which these secretion processes disrupt the cell and its plasma membrane is somewhat conjectural. There is no direct evidence regarding the mechanism by which the dissolved substances of milk, such as lactose, are secreted, although transport from the cell via Golgi vacuoles has been proposed (11).

Bargmann and Knoop (5) interpret their micrographs as showing the milk fat droplet being smoothly enveloped in plasma membrane and pinched from the cell into the alveolar lumen without loss of continuity in the membrane. Biochemical data lend support to the contention that milk fat globules are coated with plasma membrane (11, 12). Other investigators (7, 9) speak of puncturing and tearing of the plasma membrane coincident to fat droplet secretion, and micrographs of secreted milk fat globules with adhering fragments of endoplasmic reticulum have been published (8, 9).

Despite allusions to rupturing of cells, the prevalent view of microscopy researchers is that very little cytoplasm is lost during milk secretion. We felt the matter might be further clarified if cardiolipin were used as a marker for possible cellular disintegration and release of mitochondria.

## METHODS

### *Materials*

Authentic cardiolipin (bovine heart) was obtained from Applied Science Laboratories Inc., State College, Pa. This material was homogeneous as judged by two-dimensional TLC (1); deacylation and paper chromatographic analysis (13) yielded one spot with the $R_f$ of GPGPG.

Lactating mammary tissue samples from two cows (Holstein) and two goats (Toggenburg) were obtained surgically or at slaughter. The tissue was cut into approximately 1.0 $cm^3$ pieces which were immediately cooled with crushed ice. At each step in dissecting and disintegrating the tissue for cellular studies, adipose and connective tissue were excluded so far as possible. We also took lactating mammary tissue from one pig (Berkshire), primarily to compare the degree of saturation of the fatty acids in its cardiolipin with that of cardiolipin from the cow and goat.

The total lipids of milk samples, both bovine and caprine, were extracted with chloroform–methanol 2:1 within 15 min after milking. Milk used as an incubation medium for mitochondria was also freshly drawn.

### *Tissue Fractionation*

All operations were carried out at 0–5°C. The tissue pieces were minced into small slivers, washed in ice-cold 0.25 M sucrose, and homogenized with 9 volumes of fresh ice-cold sucrose solution to yield 70–120 ml of total homogenate in a mechanically driven Potter–Elvehjem type homogenizer (Arthur H. Thomas Co., Philadelphia, Pa., model 4288 B). Mammary tissue is difficult to homogenize because of its network of connective tissue, and numerous strokes of the Teflon pestle were required.

Various pellets were obtained by centrifugation of the homogenate in an International preparative ultracentrifuge, model B-35, with swinging bucket head, as follows: 12 min at 1,000 *g* for debris, red blood cells, and nuclei; 20 min at 10,000 *g* for mitochondria; and 60 min at 100,000 *g* to sediment the microsomal fraction. All fractions were washed, resuspended, and resedimented at least once. On one occasion the nuclei were purified from the debris fraction by rejection of the material that sedimented at 400 *g* during 3 min and resedimentation of the remainder at 1,000 *g* for 12 min several times, with intermittent washes. A creamy layer formed in many of the tubes at various stages in the sedimentations. An investigation of this material, largely glyceride, will be reported subsequently.

Electron microscopy showed (for example, Fig. 1) that although these fractions were not pure they clearly represented concentrates of the organelles as designated; for techniques of electron microscopy see below.

### *Extraction of Lipids*

Tissue samples (2–10 g) were extracted by homogenization with 20 volumes of chloroform–methanol 2:1. Lipids were obtained from milk samples (25 or 100 ml) by chloroform–methanol extraction. For a 100 ml sample this involved three extractions: 200 ml of chloroform–methanol 1:1, 150 ml of the same mixture, and 100 ml of chloroform alone. The chloroform-rich layers from the three extractions were combined and the solvents evaporated at 40°C under reduced pressure (15–20 mm Hg). Polar lipids were separated from neutral lipids by silicic acid column chromatography (14). The diethyl ether used to elute neutral lipids from the column contained 0.25% (v/v) acetic acid to insure that free fatty acids were in the unionized, not salt, form. Otherwise, salts of fatty acids are eluted with the polar lipids, which complicates further analysis of the latter fraction.

Portions of all the cellular fractions (mitochondrial, microsomal, nuclear, supernatant, debris, cell cream, and whole homogenate) in 1–3 ml of sucrose solution were extracted three times in 12-ml centrifuge tubes with several volumes of chloroform–methanol 2:1. Phases were separated by low-speed centrifugation. The chloroform-rich layers were combined and evaporated to dryness, in a stream of nitrogen or under reduced pressure, at room temperature. The residues were immediately redissolved in an accurately measured volume (500 or 1,000 μl) of chloroform and stored (0–5°C) in tightly sealed vials for subsequent analyses.

### *Lipid Analysis*

Polar lipids were separated by two-dimensional TLC, detected on the plates, and analyzed for phosphorus by

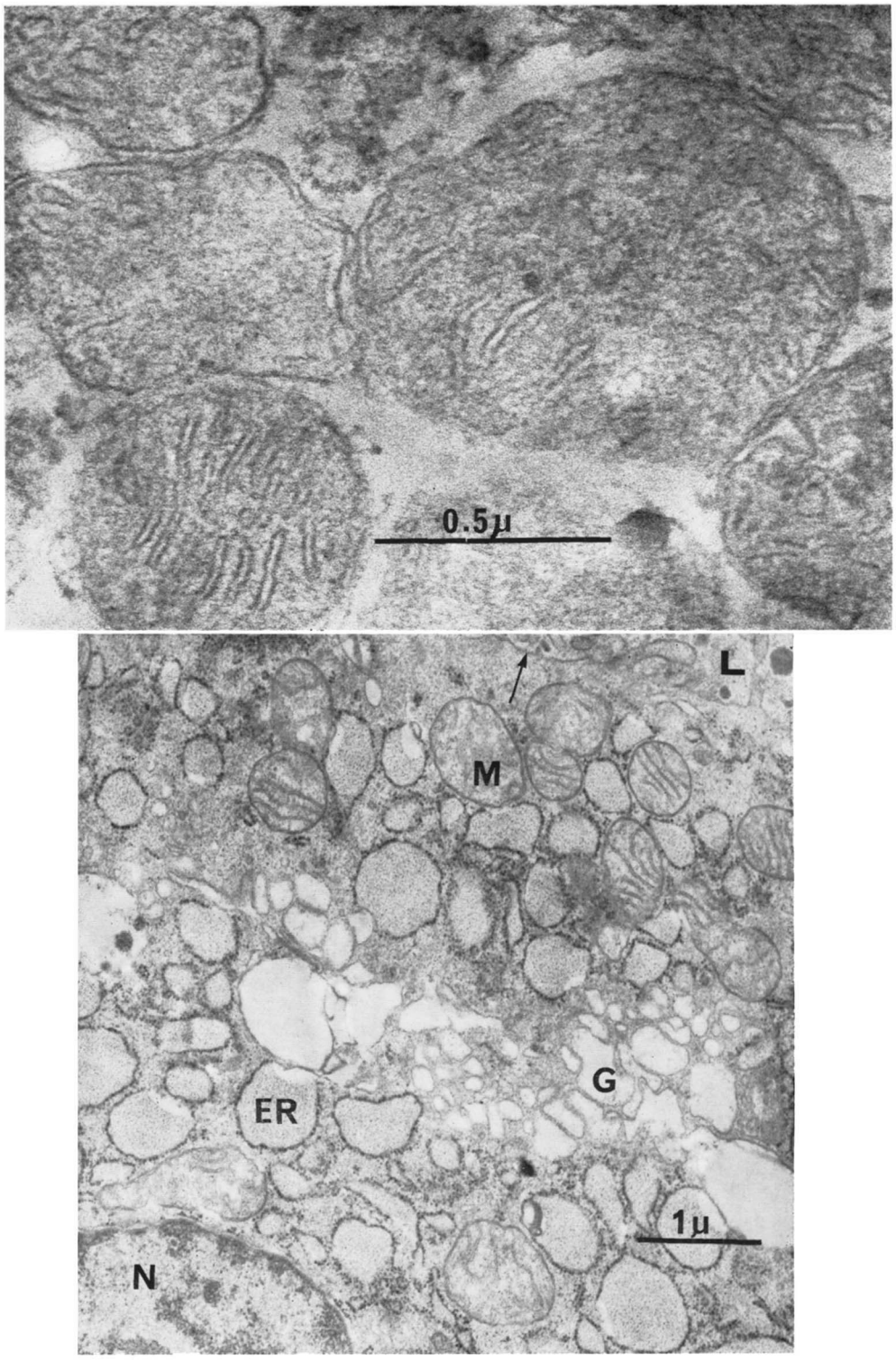


FIG. 1. *Above*. Mitochondrial preparation isolated from a homogenate of lactating mammary tissue.
*Below*. Mammary tissue from a lactating goat showing distribution of mitochondria (*M*) near apical plasma membrane (arrow) and just below lumen (*L*) containing secreted milk. Note also nucleus (*N*), endoplasmic reticulum (*ER*), Golgi region (*G*), and mitochondrion (dividing?) to the right of *M* and arrow.

the procedures of Rouser and his associates (15, 16) as adapted to milk and mammary tissue by Parsons and Patton (1). Because levels of cardiolipin in milk are minute, the sensitivity of the analytical system had to be

enhanced. The sample, equivalent to 100–200 μg of lipid phosphorus, was applied uniformly along one entire side (15 cm) of a thin-layer plate (bearing a layer, 0.4 mm thick, of Silica Gel HR, Brinkmann Instruments Inc., Great Neck, N. Y.); small spots of authentic cardiolipin (20 μl of 0.2% solution) were applied so as to run along the right and left margins adjacent to the sample; and the plate was developed in chloroform–methanol–water–28% aqueous ammonia 130:70:8:0.5. The cardiolipin standards were located by exposure of the plate to iodine vapor, and the band of silica gel between these two reference spots was scraped off and analyzed for phosphorus in duplicate, along with duplicate blanks from plates developed without sample. So far as is known, no phospholipid that may be in milk other than cardiolipin would be detected and determined by this procedure; in any event, for the purposes of this analysis, which was to determine the maximum possible level of cardiolipin in milk, the procedure seems satisfactory.

### *Characterization of Cardiolipin*

To obtain sufficient material for these experiments, we separated 700 mg of polar lipids from goat mammary tissue on 15 preparative thin-layer plates under the one-dimensional conditions described above for milk polar lipids. After solvent development, the reference spots of cardiolipin in the margins of the layers were located with Dittmer–Lester spray (17) while the main portion of the plate was shielded with a glass plate. The area of each plate adjacent to the spots of authentic cardiolipin was then scraped and the combined scrapings from all the plates were extracted with 30 ml of chloroform–methanol–acetic acid–water 40:80:20:2. The extraction mixture was shaken gently for 1 hr and then filtered through sintered glass to remove silica gel. The silica gel was washed with small portions of additional solvent. The resulting filtrate was evaporated at 40°C under reduced pressure (15–20 mm Hg).

The residue, 20 mg, dissolved readily in 180 μl of chloroform. This solution was used for various comparisons with a similar solution of authentic bovine heart cardiolipin. The two-dimensional system of Parsons and Patton (1) was employed for TLC comparisons. IR spectra between 2.5 and 12.5 μ (4000 and 800 $cm^{-1}$) were determined with a Perkin–Elmer Infracord Spectrometer, model 137, on 10% w/v solutions in chloroform.

In several similar experiments deacylation products of the supposed cardiolipin and the authentic sample were prepared by incubating 1–2 mg of the lipid in 200 μl of 0.5% KOH in methanol at 37°C for 30 min. The reaction mixtures were neutralized with 5–10 mg of Dowex 50 ($H^+$ form). These mixtures and 10 μl of 5% w/v sodium 2-glycerol phosphate (Calbiochem, Los Angeles, Calif.) in water were transferred to the origin (short axis) of a 15 × 57 cm paper (Whatman No. 1) chromatogram. The chromatogram was developed by allowing butanol–propionic acid–water 4:2:3 to descend and drip from the paper during 36 hr. After air drying, the paper was sprayed with a reagent (Hanes–Isherwood) composed of 5 ml of 60% perchloric acid, 10 ml of 1 N HCl, and 25 ml of 4% w/v ammonium molybdate made to 100 ml with water. The chromatogram was again dried and irradiated with a UV lamp to develop the blue color that is characteristic of the phosphorus-containing spots.

To further characterize cardiolipin of mammary

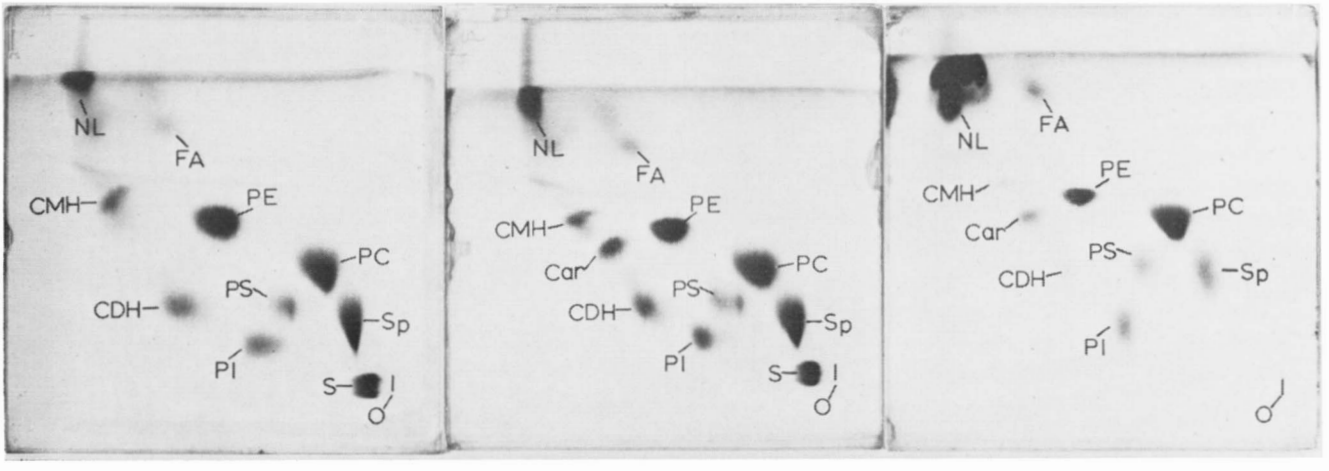


FIG. 2. Two-dimensional TLC of bovine milk polar lipids (left), the same lipids with cardiolipin added (center), and mammary tissue total lipids (right). The chromatograms were developed from right to left with chloroform–methanol–water–28% aqueous ammonia 130:70:8:0.5 and then upwards with chloroform–acetone–methanol–acetic acid–water 100:40:20:20:10. Spots were detected by chromic acid spray and charring. *O*, origin; *S*, carbohydrate (lactose) and protein; *Sp*, sphingomyelin; *PC*, phosphatidyl choline; *PS*, phosphatidyl serine; *PI*, phosphatidyl inositol; *PE*, phosphatidyl ethanolamine; *Car*, cardiolipin; *CDH*, ceramide dihexoside; *CMH*, ceramide monohexoside; *FA* free fatty acid; *NL*, neutral lipid.

tissue, we determined its fatty acid composition in samples from cow, goat, and pig by gas chromatography of the methyl esters. Approximately 1 mg of mitochondrial or total homogenate lipid was separated by two-dimensional TLC. The plate was sprayed with 0.2% 2′,7′-dichlorofluorescein in ethanol and the cardiolipin area of the thin layer was scraped into a small screw-cap vial with plastic gasket. 1 ml of 1% v/v $H_2SO_4$ in methanol was added to the vial, which was then flushed briefly with nitrogen, sealed, and held overnight at 40°C.

The vial contents were diluted with water (2 ml) and extracted three times with redistilled hexane (1 ml each time). The combined extract was applied to a column (2.45 m by 0.63 cm) containing 12% ethylene glycol succinate polyester on Gas-Chrom P (Applied Science Laboratories) in a Barber–Colman model 10 gas chromatograph. The described procedure for preparing methyl esters gave results for composition that were virtually identical with those obtained by extracting the silica gel with chloroform–methanol 1:2 and preparing methyl esters of the recovered lipids.

Oleic and linoleic acids together constituted 80–90% of the fatty acids in all the cardiolipin samples analyzed. Quantitative results for a reference standard (K 108, Applied Science Laboratories) containing these two acids among others indicated that they were being analyzed with a relative error of less than 10%.

### *Electron Microscopy*

Pieces of mammary tissue (largest dimension 1.0 mm) and suspensions of tissue fractions were fixed in 2% glutaraldehyde, postfixed in 1% $OsO_4$, dehydrated in ethanol, and embedded in Epon essentially as described by Stein and Stein (9). Thin sections were cut on a Porter–Blum ultramicrotome (model MT-2), placed on Formvar carbon-coated grids, stained with lead citrate (18), and examined in an RCA EMU-3H electron microscope.

## RESULTS

Two-dimensional TLC showed the absence of any component corresponding to cardiolipin in the milk polar lipids and the presence of such a component in the lipids of lactating mammary tissue (Fig. 2). This component represented 3.2 and 3.8% of the lipid phosphorus in the two bovine tissue samples, 3.4 and 4.9% of that from the goat samples. Like cardiolipin, this component gave a positive reaction in a test for phosphorus and a negative one for sugars and amino groups with selective spray reagents. As isolated by preparative TLC, the compound from goat mammary tissue yielded an IR spectrum very similar to that we obtained for authentic bovine heart cardiolipin and to that published (4) for bovine

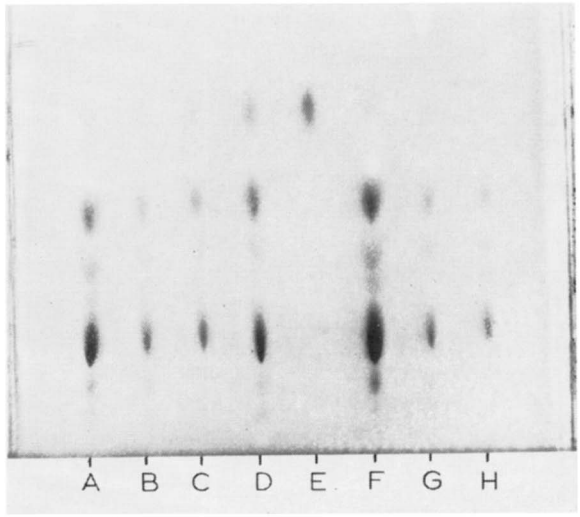


FIG. 3. One-dimensional TLC of lipids from subcellular fractions of lactating goat mammary tissue. The chromatogram was developed with the ammonia-containing solvent (see Fig. 2) and sprayed with Dittmer–Lester spray (17), which is specific for phospholipids. Fractions, with sample size (μg P) in parenthesis, were: *A*, total homogenate (6.5); *B*, debris (2.2); *C*, nuclei (1.9); *D*, mitochondria (5.5); *E*, reference cardiolipin (2.4); *F*, microsomes (16.9); *G*, "cell cream" (1.9); and *H*, supernatant fraction (1.6). Fractions *B*, *C*, *D*, and *F* were applied to the plate approximately in proportion to their occurrence in the tissue.

heart cardiolipin. The unknown from mammary tissue had all of the major and minor absorption maxima of the authentic cardiolipin, but showed greater absorption in the 6.2 and 7.2 μ regions. We attribute this to hydrocarbon contamination which could have occurred during the isolation process. The unknown also cochromatographed with authentic cardiolipin in the two-dimensional TLC system and yielded a deacylation product that appeared identical by paper chromatography with that (GPGPG) from reference cardiolipin. Both deacylation products yielded one blue spot which was detected at an $R_f$ of 0.25 with respect to 2-glycerol phosphate. The latter compound moved approximately 100 cm during the 36 hr development. Although the identification effort for bovine mammary tissue cardiolipin did not include characterization of its deacylation product or IR spectral analysis, its behavior on TLC and response to spray reagents were identical with those of the goat mammary tissue cardiolipin, and we conclude it is the same lipid. Mitochondria are characterized by high levels of cardiolipin (3, 4, 19), and the lipid in question is further identified as cardiolipin by its presence preferentially in the mitochondrial fraction of both goat and cow mammary tissue (Fig. 3 and Table 1).

Our mitochondrial preparations appeared to be concentrates of the organelle, although they were not completely free of other cellular components (Fig. 1). The cleanest mitochondrial fractions contained 10–14% cardiolipin in the phospholipids (Table 1). This is

TABLE 1 PHOSPHOLIPID COMPOSITION IN MITOCHONDRIAL PREPARATIONS FROM LACTATING TISSUE OF THE COW AND GOAT*

| | Cow | Goat |
|---|---|---|
| | % | |
| Phosphatidyl choline | 49.8 (±0.5) | 53.9 |
| Phosphatidyl ethanolamine | 31.4 (±0.5) | 24.2 |
| Cardiolipin | 10.1 (±0.5) | 13.6 (±0.2 ) |
| Others† | 8.8 (±0.4) | 9.0 |

* Data are phosphorus values for individual phospholipids (separated by TLC) expressed as a percentage of total lipid P. Data for duplicates show range.

† A composite of phosphatidyl serine, phosphatidyl inositol, and sphingomyelin in approximately equal amounts.

similar to the levels of 14% found by Getz, Bartley, Lurie, and Notton (19) for mitochondria of sheep organs and rat liver, but falls below the 17–20% cardiolipin content reported for mitochondrial lipids from bovine heart, liver, and kidney (4).

The fatty acids of cardiolipin from lactating mammary gland (cow, goat, and pig) are highly unsaturated (Table 2). In fact cardiolipin appears to be the most unsaturated lipid occurring anywhere within the ruminant. The fact that cardiolipin from whole tissue and mitochondrial fraction have very similar fatty acid compositions suggests a common origin or locus of synthesis (see data for goat, Table 2).

With the presence of cardiolipin in the tissue established, the extent of its presence in milk remained to be explored. Data for both the cow and goat indicate that it does not comprise more than 1% of the phospholipids and that it often occurs at much lower levels (Table 3). Occasionally we observed a faint spot corresponding to cardiolipin on thin-layer chromatograms of milk polar lipids. Usually no spot could be observed, and the low levels in the table were further supported by the following experiment. Milk phospholipids, 44.5 μg of lipid P, from a single goat were separated by one-dimensional thin-layer chromatography in the ammonia-containing solvent. Samples of authentic cardiolipin were separated on the same plate; as little as 2 μg (0.07 μg of P) could be detected with the Dittmer–Lester spray (17). No spot corresponding to cardiolipin was evident from the milk lipid sample under these conditions. This implies a level of less than 0.16% cardiolipin in the milk phospholipids of this sample of goat milk.

TABLE 2 FATTY ACID COMPOSITION* OF CARDIOLIPIN FROM LACTATING TISSUE OF COW, GOAT, AND PIG

| | Cow | Goat | Pig |
|---|---|---|---|
| | | *weight %* | |
| 14:0 | 2.4† | 1.4 (1.5) | (tr) |
| 16:0 | 4.4 | 3.3 (2.5) | (2.5) |
| 16:1 | 2.9 | 5.1 (2.9) | (5.5) |
| 18:0 | 1.8 | 3.6 (1.6) | (1.1) |
| 18:1 | 36.7 | 27.3 (29.9) | (13.5) |
| 18:2 | 49.6 | 57.3 (59.8) | (72.8) |
| 18:3 | 2.2 | 2.0 (2.0) | (4.7) |

* Based on gas–liquid chromatography of the methyl esters. Fatty acids are designated by chain length:no. of double bonds.

† Figures without parentheses are for cardiolipin from mitochondrial preparations; those in parentheses are for cardiolipin from whole homogenates of tissue. The two sets of data for the goat are derived from the same mammary gland.

TABLE 3 PERCENTAGE OF LIPID PHOSPHORUS CORRESPONDING TO CARDIOLIPIN IN THE PHOSPHOLIPIDS OF COW AND GOAT MILK

| | Cow | Goat |
|---|---|---|
| | % | |
| 1 | 0.14 (±0.00) | 0.22 (±0.03) |
| 2 | 0.97 (±0.06) | 0.29 (±0.02) |
| 3 | 0.87 (±0.05) | 0.58 (±0.06) |

Duplicate data for milk from two individual Holstein cows (Nos. 1 and 2); mixed herd milk, primarily Holstein (No. 3); and three individual Toggenburg goats.

The low percentage of cardiolipin in fresh milk might be explained on the basis of its instability in the medium. To check this, we incubated a suspension of freshly isolated mitochondria from lactating bovine tissue in freshly drawn bovine milk. A quantity of freshly isolated mitochondria (49.2 μg of lipid P, containing 5.0 μg of cardiolipin P) was added to each of two 25 ml milk samples (300 μg of lipid P). One sample was incubated for 18 hr at 2°C and the other for 2 hr at 40°C. TLC of lipids recovered from these samples and determination of P gave amounts of cardiolipin from the two incubated samples equivalent to 83% of the original amount of cardiolipin after incubation at 40°C and 100% after incubation at 2°C.

## DISCUSSION

This investigation establishes that cardiolipin occurs in lactating mammary tissue and is concentrated largely, if not exclusively, in the mitochondria of the tissue. Although the cardiolipin content of milk phospholipids may at times approach 1% (Table 3), on occasion, milk with extremely low levels of cardiolipin is obtained. Under normal conditions of lactation, the tissue phospholipids (3–5% cardiolipin) have 21–35 times as high a cardiolipin concentration as the milk phospholipids when the latter contain only 0.14%. However, the amount of phospholipid per gram of lactating tissue is normally 20–30 times that of milk[1] so the actual tissue:milk ratio for cardiolipin is of the order of several hundred to one. Since mitochondrial cardiolipin was ob-

[1] We have found 338 μg of lipid P per g of wet lactating tissue and 11.6 μg/g for milk. In earlier studies (20), phospholipids in goat and cow mammary tissue were found to comprise 20% of total lipids whereas those in milk are known (21) to range from 0.2 to 1.0% of the milk total lipids.

served to be quite stable in milk, we conclude that the normal mechanisms of milk secretion operate without transporting significant amounts of mitochondria from the cell. Milk secretion appears to result from biophysical mechanisms that preserve the structural–functional integrity of the cell, including its plasma membrane. Mitochondria are abundant in the apical (milk secreting) region of the cell (Fig. 1);[2] yet they remain in the cell. Moreover, it seems reasonable that there would be genetic selection for a mechanism for milk secretion that enables the lactating cell to retain indispensable organelles such as the mitochondria and nuclei.

While it is not certain that the small amounts of cardiolipin in milk are necessarily derived from mitochondria, this is a reasonable probability. Milk contains variable levels of leukocytes and sloughed epithelial tissue. These levels can be enhanced by mastitis, which is frequently a chronic condition in high-producing dairy cows. Hence, even the low levels of cardiolipin in milk may have little connection with the normal secretion mechanism.

The levels of 18:2 fatty acid we have found in mammary cardiolipin are high—50% or more of the total fatty acids. This high occurrence of the acid in the mitochondrion lipid may reflect essential fatty acid function.

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[2] That mitochondria occur in close proximity to fat globules in the apical region of the lactating cell is readily evident in micrographs from other studies (5–10).

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