Stearyl CoA as a Precursor of Oleic Acid and Glycerolipids in Mammary Microsomes From Lactating Bovine: Possible Regulatory Step in Milk Triglyceride Synthesis

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ABSTRACT

The stearyl desaturase of lactating bovine mammary tissue is located in the microsomes and requires activated fatty acid and NADH for activity. Other enzymes, acyl-transferase(s) and deacylase which apparently compete with the desaturase for substrate are also present. Both the substrate 1-^{14}C-stearyl CoA and the oleic acid produced by desaturase are esterified into the various lipid classes. The oleic acid is preferentially acylated into position *sn*-3 of the triglycerides and *sn*-2 of the phosphatidylcholine. Experimental conditions causing reduced desaturase activity depressed triglyceride synthesis, and stimulation of desaturation by NADH, L$^{\alpha}$ GP, acidic pH, 5.6, was accompanied by increased incorporation of radioactive fatty acids into the triglycerides. These data indicated that desaturase and glyceride acyl transferase were located contiguously within the microsomal membranes. The possibility that desaturase activity might control triglyceride synthesis in vivo is discussed. It was observed that mammary tissue from nonlactating cows 1-2 weeks and 2 days prior to calving lacked or possessed very low stearyl desaturase activity.

Oleic acid is the principal unsaturated acid in bovine milk, and its concentration and intramolecular distribution may be very significant in reducing the melting point of bovine milk fat and maintaining it in a fluid condition at physiological temperatures (1). The quantity of *cis* octadecenoic acid in milk is variable ranging from 50-70% of the total octadecenoic acid present (2). It is estimated that 30-40% of the *cis* isomer, i.e., oleic acid, is synthesized in the mammary tissue by the action of the anzyme stearyl desaturase on absorbed exogenous stearic acid (1-5). The possible importance of exogenous stearic acid and its metabolism in lactating bovine has been alluded to by some authors (6-8,34) in that it may directly or indirectly regulate triglyceride synthesis. The possible essential role of oleic acid in milk fat synthesis has been discussed (1). In the present paper the finding that stearyl desaturase activity affects triglyceride synthesis by mammary microsomes in vitro corroborates the above intimations.

MATERIALS AND METHODS

Mammary tissue was obtained from lactating (30/40 lb. milk per day) Holstein cows following slaughter. The tissue was minced in a meat grinder, diluted 1:2 in 66 mmoles potassium phosphate buffer pH 7.4, and homogenized for 30 sec in Waring blendor. The homogenate was rehomogenized for 30 sec in a small mill (Polyscience Corp., Ill.) and filtered through cheese cloth. Temperature was kept at 4 C. The filtrate was sequentially centrifuged at 5000 g for 10 min, 10,000 g for 15 min, and 100,000 g for 60 min, to remove cell debris and mitochondria and other material and to recover microsomes in the final step (31). The microsomal pellet was washed with buffer and then freeze-dried and stored at -30 C.

The standard assay system consisted of 66 mmoles potassium phosphate buffer pH 7.4, 150 nmoles NADH, 50 nmoles 1-^{14}C-stearyl CoA and approximately 5 mg microsomal protein in 2 ml. Incubations were carried out at 37 C in a Rotamix shaker using capped culture tubes. The reaction was stopped after 15 min by extraction of lipids by the procedure of Folch et al. (9).

Nonlactating mammary tissue obtained by biopsy from pregnant cows, just prior to parturition, was minced in two volumes of tissue culture media 199 (Grand Island Biological Co., N.Y.) under aseptic conditions. Two milliliter aliquots of this homogenate were incubated with 50 nmoles Na-1-^{14}C-stearate (20 μc/μmole) for 2 hr after which the lipids were extracted (9).

The lipids were separated by thin layer chromatography (TLC) into the respective neutral lipid and phospholipid (PL) classes (1,10,11). The radioactivity in specific classes was determined by liquid scintillation counting (Packard Tri Carb). Radioactivity in the fatty acids was quantified by radio gas chromatography (1). Desaturase activity was measured by

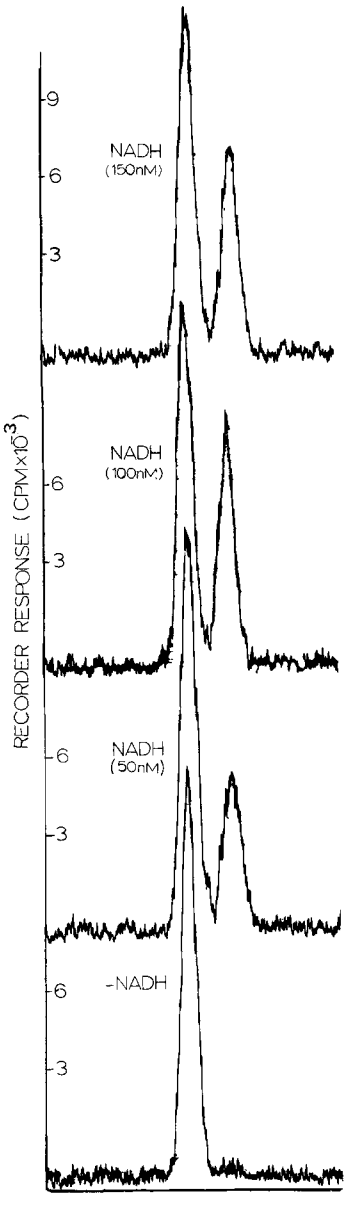

FIG. 1. Radiogas chromatograms of the methyl esters prepared from total lipid extracts of bovine mammary microsomes showing the effect of NADH on the desaturation of 1-^{14}C stearyl CoA to ^{14}C-oleic acid.

determining the quantity of the substrate 1-^{14}C-stearyl CoA (C18CoA) converted to labeled oleic acid (1), hereinafter referred to as endogenous oleic acid.

To determine the distribution of the labeled oleic acid (C18:1), the triglycerides (TG) and the phosphatidylcholine (Pc) isolated by TLC were enzymatically hydrolyzed by the methods of Luddy et al. (12) and Wells and Hanahan (13), respectively, and the products were analyzed as previously described (1). Protein was quantified by micro-Kjeldahl (14) or Lowry (15) method.

Prepared radiochemicals were purchased from New England Nuclear (Boston, Mass.) and reliable NADH was obtained from Sigma (Sigma Chemicals, St. Louis, Mo.).

RESULTS

Of the various subcellular components tested only the particulate fraction sedimented at 100,000 g, i.e., microsomal fraction, possessed stearyl desaturase activity. In the standard incubations used throughout this study desaturase activity varied among four different cows, but a mean value of 31±7% desaturation of substrate stearyl-CoA was obtained (20 incubations). In preliminary experiments when 1-C^{14}-stearyl CoA was replaced by 50 nmole Na-1-^{14}C-stearate and appropriate amounts of cofactors (ATP, CoASH, NADH Mg Cl_2) desaturation was only 7%, indicating low thiokinase activity in these microsomal preparations.

Chemically pure NADH was essential for stearyl desaturase (Fig. 1). The concentrations cited in Figure 1 are approximate. Under optimum standard conditions a maximum 38% of the substrate 1-^{14}C-stearyl CoA was desaturated to oleic acid.

Analyses of the microsomal lipids revealed that both the substrate ^{14}C stearyl CoA and the endogenous labeled oleic acid were mostly esterified. The TG and PL contained most of the radioactivity (Tables I and II). The data (Table I) indicate that the microsomes contained active acyl transferases and deacylase. Because of their apparent propinquity and concurrent activity both the desaturase and acyl transferase(s) enzymes were monitored, and the experimental results are tabulated together.

The distribution of radioactive stearic and oleic acid in various lipids from a representative standard incubation is shown in the radiogas chromatograms (Fig. 2) The observation that the TG and Pc contained the preponderance of the endogenous oleic acid is noteworthy. The 1,2 diglycerides and phosphatidylethanolamine (Pe) had low levels of labeled oleic acid. The ratio of stearic to oleic acid in the free fatty acids reflects that in the total lipid extract.

A number of experimental variables influenced the production of oleic acid and its incorporation into specific glycerolipids (Table II). The total extent of desaturation and acylation in the standard incubations were quite

TABLE I

The Distribution of Radioactivity in the Lipid Classes of Bovine Mammary Microsomes[a]

Lipids	Percentage distribution	
	Standard	Standard minus NADH
Phospholipids	48 ± 8.6	67.4
Diglycerides	4.6 ± 2.0	5.2
Free fatty acids	15 ± 3.1	11.0
Triglycerides	29 ± 8.2	16.3
Cholesterol ester	0.1 ± 0.1	0.1
Amount of substrate esterified, %	85	89

[a]Following incubation with 1-C^{14}-stearyl CoA under standard conditions with and without NADH.

consistent with exception of experiment IV. Replacement of 0.5 ml of buffer with an equal volume of supernatant caused a ca. 33% decrease in TG synthesis and an equal depression in extent of desaturation within the triglycerides. There was a concomitant increase in amount of radioactivity in the phospholipids though desaturation therein decreased slightly.

Omission of NADH, which eliminated desaturase activity (experiment II and Fig. 1), markedly reduced triglyceride labeling, whereas the labeling in the phospholipids was increased by 35%. L-α-Glycerolphosphate (20 nmole) stimulated both triglyceride synthesis and desaturase activity and enhanced acylation of endogenous oleic acid into the triglycerides, whereas PL synthesis was depressed.

Acidic incubation conditions reduced transacylase activity, especially with respect to the phospholipids. Desaturation was not impaired, and the quantity of endogenous oleic acid incorporated into the triglycerides actually increased slightly. Alkaline conditions decreased the activities of both desaturase and acyl transferase(s).

These data (experiments I-IV) revealed that the incorporation of radioactivity into the triglycerides was stimulated when desaturase activity was enhanced, whereas diminution or elimination of desaturase activity enhanced incorporation of label into the phospholipids.

Because the preponderance of the endogenous ^{14}C-oleic acid in the PL was associated with the Pc, the distribution of radioactivity in the PL in absence of endogenous oleic acid was examined (Table III). Significantly even though there was a marked increase in PL acylation (Table II), there was a diminution in Pc labeling and a marked increase of radioactive stearic acid in Pe and Pi. There two classes normally had low levels of endogenous oleic acid as shown (Fig. 2). These data may indicate that the availability of endogenous oleic acid may also influence the synthesis of Pc in mammary

TABLE II

The Effects of Experimental Factors on the Extent of Acylation and Desaturation of 1-^{14}C-Stearyl CoA[a]

Experimental conditions	Amount esterified, nmoles		Amount desaturated, nmoles	
	Triglycerides	Phospholipids	Triglycerides	Phospholipids
Experiment I				
Standard	15.0	19.5	8.7	7.4
+Supernatant	10.5	23.5	5.5	7.0
Experiment II				
Standard	14.1	24.0	8.1	6.9
-NADH	8.1	33.7	trace	0
Experiment III				
Standard	16.0	23.0	8.8	6.3
+L-α-Glycerolphosphate	21.5	16.1	12.9	5.3
Experiment IV				
Standard (pH 7.2)	12.5	18.0	7.2	7.4
pH 5.6	9.6	9.0	8.5	6.0
pH 8.3	5.0	10.0	3.3	3.4

[a]By bovine mammary microsomes incubated under standard conditions (see Methods).

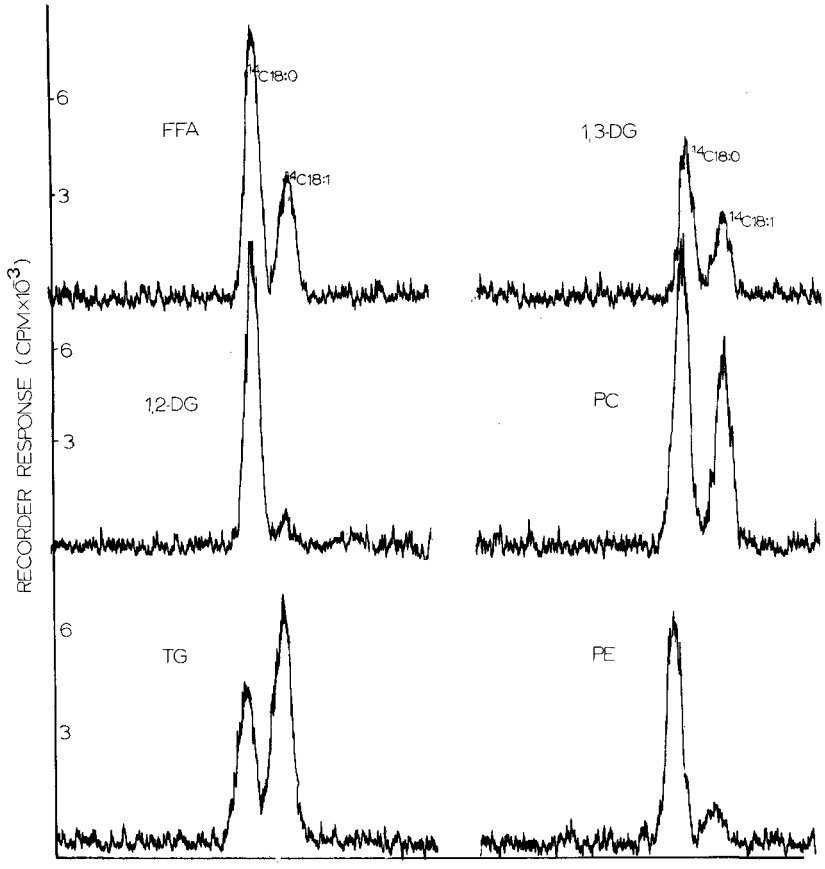

FIG. 2. Representative radiogas chromatograms showing the location and relative concentration of radioactivity in the various lipid classes, isolated following the incubation of bovine mammary microsomes with 1-^{14}C-stearyl CoA under standard conditions. The proportion of endogenous radioactive 1-^{14}C-oleic acid in these classes varied, i.e., free fatty acids (FFA) 32; 1,2 diglycerides (1,2DG) 13; 1,3 diglycerides 1,3DG) 27; triglycerides (TG) 60; phosphatidylcholine (Pc) 43; phosphatidylethanolamine (Pe) 16 and phosphatidylinositol (not shown) 5%, respectively.

tissue.

The intromolecular distribution of endogenous oleic acid was revealed following lipolysis (Fig 3). In the TG most of the ^{14}C-oleic acid was in the primary positions of the glyceride glycerol. Assuming that these triglycerides were synthesized from the 1,2 DG pool (Fig. 2), then conceivably most of the labeled oleic acid was on position *sn*-3 of the triglycerides. Endogenous oleic acid was concentrated in position *sn*-2 of the Pc.

Because stearyl desaturase, which is a mixed function oxidase (16), is inducible, it may be influenced by the endocrinological status of the cow. Hence we compared the desaturase activity in mammary tissue from pregnant nonlactating and lactating cows which were on similar diets. Because of the small amounts of tissue procured by biopsy, homogenates rather than microsomes were incubated with Na-1^{14}C stearate (Fig. 4). The homogenate from the lactating tissue possessed much more active desaturase and acyl transferase(s) than the tissues from the nonlactating cows. The mammary homogenates from the cow 1-2 weeks prepartum lacked desaturase activity completely.

DISCUSSION

These data demonstrate that the stearyl desaturase is located in the microsomes of lactating bovine mammary tissue, and its cofactor requirements are quite similar to the desaturase of rat and hen liver (17-23), rat adipose (24), goat mammary (2,5), fungal microsomes (25), plants and bacteria (16). These mammary microsomes also possessed active acyl trans-

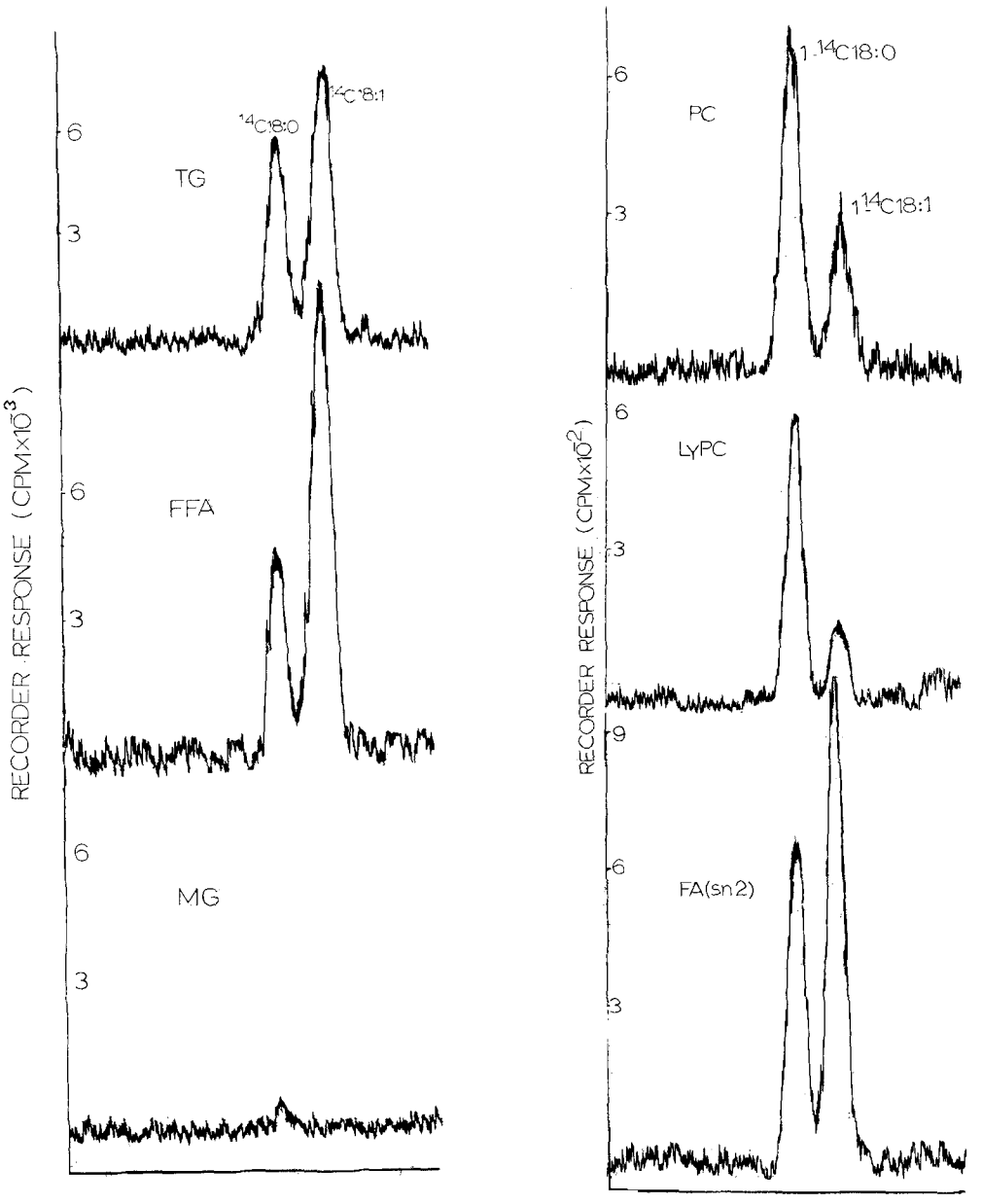

FIG. 3. Radio gas chromatograms showing the relative concentrations and positional distribution of radioactive stearic and oleic acid in triglycerides (TG) and phosphatidylcholine (Pc) isolated following the incubation of bovine mammary microsomes with 1-^{14}C-stearyl CoA. The original lipids, i.e., triglycerides (TG) and phosphatidyl-choline (Pc) were hydrolyzed with lipases (see methods) and the radioactive methyl esters of the products, separated by thin layer chromotography, were analyzed by radio gas chromatography.

ferases and deacylase. Probably these enzymes competed for available substrate as indicated by the consistent acylation and deacylation of stearyl CoA even when the acyl desaturase was inactivated. The apparent affinity of the transacylase enzymes for activated stearic and oleic acid differed with the glyceride acyl transferase preferring the endogenous oleic acid, whereas the phosphatide acyl transferase apparently preferred stearic acid.

The selectivity may be attributed to chemical specificity or to the juxtaposition of these enzymes in the microsomal membranes. Baker and Lynen (25) suggested some models

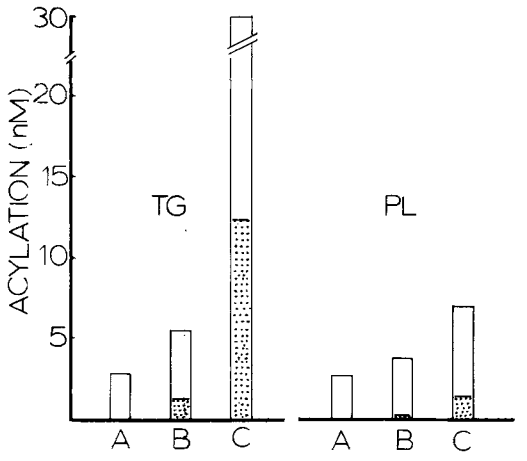

FIG. 4. Histogram showing the extent of esterification and desaturation (stippled areas) of Na-1-^{14}C-stearate by homogenates of bovine mammary tissue from non-lactating cows ca. 1-2 weeks (A) and 2 days (B) weeks prior to calving, and lacting cow (C) ca. 3 months after calving. TG and PL denote the triglycerides and phospholipids recovered from the incubated tissue.

pertaining to this with respect to fungal microsomal desaturase. In the latter the endogenous oleic acid is placed preponderantly in the PL, whereas in the bovine microsomes it is esterified into TG and PL and when more oleic acid is produced more TG is made. In the present context the negligible effect of acidic pH on desaturase and triglyceride acylation may be construed to suggest that the desaturase and glyceride acyl transferase enzymes are located close together in the interior of the mammary microsomal membranes, where because of its hydrophobicity the effects of H ion concentration on functional charged groups are minimized (26). Experimental evidence showing that lipids are necessary for desaturase activity (22) would support the above suggestion. Conceivably, as might be rationalized from surface chemistry requirements, the phosphatide acyl transferases are situated in the surface region of the membranes, and consequently they utilize the substrate stearyl CoA more effectively for PL synthesis. The closely parallel effects of various incubation conditions on desaturase activity and triglyceride synthesis in the present study are consistent with this suggestion. Attempts to isolate the individual enzymes from this complex are in progress to determine if this proposition is valid.

The depression of desaturation and glyceride synthesis by supernatant fractions was also observed in goat mammary microsomes (2). This could be explained by several factors in

TABLE III

Distribution of Radioactivity in the Phospholipids[a]

Lipid class	Percentage distribution of radioactivity: Standard incubation +NADH	Standard incubation -NADH
Lysophosphatidylcholine	1.1	1.2
Sphingomyelin	2.0	1.3
Phosphatidylcholine	46.5	31.1
Phosphatidylserine	4.2	3.6
Phosphatidylethanolamine	26.7	39.3
Phosphatidylinositol	19.5	23.5
Per cent of substrate esterified[b]	46	67

[a]Following the incubation of bovine mammary microsomes with 1-^{14}C-stearyl CoA in presence and absence of NADH.

[b]In phospholipids

the supernatant, i.e., added free fatty acids which inhibited the desaturase (2), additional PL-acyl transferase activity, and/or the progress of reactions which consumed the available NADH.

The finding that both desaturase activity and triglyceride labeling varied in a corresponding manner may be significant and help to explain some observations made with regard to milk glyceride synthesis in vivo (6,7). The relatively consistent structure of milk fat and nonrandom pattern of fatty acid acylation reflects an orderly assembly of milk glycerides in presence of the proper concentration and ratios of fatty acids. On the basis of experimental data a number of authors have concluded that mammary triglyceride synthesis is somehow controlled by the particular types and concentrations of long chain fatty acids available to the synthetic enzymes, and in the absence of these conditions triglyceride synthesis is depressed (6,7,34). Thus in cows on special concentrate rich diets, changes in the availability of stearic acid have been related to depressed milk fat production (7). Because in the present study triglyceride synthesis is reduced when desaturase activity is depressed, it is possible that in vivo the availability of a specific quantity of endogenous oleic acid may be the critical factor in facilitating or limiting triglyceride production. Thus any factor which influences the activity of desaturase, i.e., availability of activated precursor stearic acid, local NADH levels, and the inherent enzyme concentration may theoretically regulate triglyceride synthesis.

Steele et al. (27) have reported that feeding stearic acid to cows increased the secretion of oleic acid and milk fat yield, and Askew et al. (8) showed that stearic acid stimulated esterifi-

cation of fatty acids by homogenates of lactating mammary tissue. Both of these effects may have been mediated via the action of the stearyl desaturase.

The intramammary levels of NADH could act as a potent regulator of desaturase activity and thereby influence milk glyceride production. The enhanced incorporation of stearic acid into PL in absence of NADH (and hence desaturase activity) is consistent with the possible competition of the stearyl desaturase and PL acyl-transferase for the substrate. The concomitant depression in TG synthesis may be relevant to observations of changes in fat synthesis in certain in vivo situations. Conceivably under dietary conditions, e.g., high concentrate diets, and/or specific physiological conditions, e.g., ketosis, glucose and acetate concentrations in mammary tissue may be reduced with a subsequent drop in production of reducing equivalents (NADH, NADPH) (28,34). This would impair desaturase function and thereby limit milk triglyceride production.

Dietary and physiological factors, i.e. fasting and diabetes, may directly affect the level of desaturase in mammary tissue as occurs in the rat (24,29,30).

The effect of LαGP in stimulating desaturation and glyceride synthesis in the bovine microsomes may reflect the availability of extra acyl acceptors with consequent reduction of desaturase inhibition by the free fatty acids present in the microsomes (8). Goat mammary microsomes showed a similar response (2). The effect of LαGP on microsomal desaturase in vitro may obtain in vivo in mammary tissue of cows on high concentrate diets, though in these tissues LαGP concentration is quite high (34).

The intramolecular distribution of endogenous oleic acid in TG and Pc is similar to that obtained using mammary cells in vitro and that found in milk, and further indicates that mammary microsomes possess the enzymes required for milk fat synthesis (1,31,32).

The apparent absence of the stearyl desaturase in nonlactating tissue may reflect the inducible nature of this particular enzyme or of some of its components (24,29,22). The fact that stearyl desaturase of rat liver responds to insulin may indicate that the desaturase of bovine mammary tissue requires the lactogenic hormones for activity. However the difficulty in interpreting this type of data especially in bovine mammary tissue has been discussed by Baldwin (33).

ACKNOWLEDGMENTS

Financial support came from NSF Grant GB8430 and technical assistance from L. Ulmer, A. Rushmer and T. McDonald.

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[Received January 31, 1972]