Properties of Palmityl-CoA: L-α-Glycerolphosphate Acyl Transferase from Bovine Mammary Microsomes

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ABSTRACT

Palmityl-coenzyme A: L-α-glycerolphosphate acyltransferase is the most active acyltransferase of bovine mammary microsomes, with a specific activity ranging from 8-20 nmoles min^{-1} mg^{-1} protein. Corresponding acylation rates of 2.2, 1.4, 2.1, and 0.6 nmoles min^{-1} mg^{-1} were obtained for myristyl-, stearyl-, oleyl- and linoleyl-coenzyme A, respectively. Optimum pH of palmityltransferase was 7.7, and activity was not affected by buffer molarity in range 25-150 mM. Inhibitory effects of palmityl-coenzyme A (10 μM/0.1 mg microsomal protein) was relieved by bovine serum albumin. Sonication magnesium and ethylenediaminetetraacetic acid enhanced activity. Delipidation of microsomes reduced activity by 84%; restoration of extracted lipids achieved 70% of original activity. Apparent Km and Vmax values of 4.1 and 260 μM and 9.5 and 8.2 nmole min^{-1} mg^{-1} were determined for palmityl-coenzyme A and D,L-α-glycerolphosphate, respectively, using untreated microsomes. The enzyme was stable as lyophilized microsomes when stored at -30 C. Phosphatidic acid was the major product and marked quantities of diglycerides were formed, especially when microsomal protein was increased.

INTRODUCTION

The mammary gland of the lactating cow acylates ca. 1 kg fatty acids/day, indicating the possession of active acyl transferase(s). The activity of these enzymes markedly increases with the initiation of lactation which is apparently related to the synthesis of secretory triglycerides (1-3). Acyl transferases of bovine mammary microsomes may be unique, because they mediate, in a nonrandom manner, the acylation of predominantly saturated substrates having a wide range of mol wts (4). They specifically locate saturated acids (mostly palmitic and myristic) on position sn 2 (4-5), and they preferrentially locate endogenous short chain and oleic acid on position sn 3 of milk triglycerides (1,6). Bovine mammary acyl transferases also may perform the unique function of assembling heterogeneous (structural and compositional) triglycerides to ensure fluid milk fat droplets at physiological temperatures and facilitate secretion.

Whether different acyl transferases are involved in acylation of positions sn 1, sn 2, and sn 3 of milk glycerides is being investigated. Because of their importance, we studied mammary acyl transferases, particularly palmityl-CoA: L-α-glycerolphosphate (LαGP) acyl transferase, which is the most active in vitro (7).

MATERIALS AND METHODS

Preparation of microsomes: Mammary tissue excised from cows immediately after slaughter was minced in a meat grinder. This was homogenized in a regular Waring blender at top speed for 30 sec, after 1:2 dilution with phosphate bicarbonate buffer pH 7.4 (70 mM $KHCO_3$, 85 mM K_2HPO_4, and 9 mM KH_2PO_4), and rehomogenized in a Waring blender with a polytron assembly for 20 sec. All manipulations were carried out at 4 C.

The homogenate was centrifuged in a Sorvall refrigerated centrifuge using a G.S.A. rotor (r = 5.75 in.) at 1020 x g for 10 min to remove cellular debris. The supernatant was centrifuged at 14,600 x g for 15 min to remove mitochondria. This supernatant was centrifuged in a Beckman model L2-65 ultracentrifuge with type 21 rotor at 44,000 x g for 75 min. The microsomal pellets were pooled and freeze-dried, and small lots were stored in teflon sealed vials at -30 C.

Preparation of enzyme solution: Appropriate amounts of freeze-dried microsomes were dissolved in potassium phosphate buffer (66 mM, pH 7.4) using a tissue homogenizer with teflon pestle. The solution was sonicated for 1 min at 4 C in a model 8845-3 sonicator (Cole-Parmer Instrument Co., Chicago, Ill.), and the microsomes then were assayed for enzyme activity. Protein was determined by the method of Lowry (8) using crystalline bovine serum albumin as standard.

Enzymatic assay: Enzyme activity was measured spectrophotometrically (9) and by radioactive assay. Incubation media contained: acyl-CoA 5-20 μM; D,L-α-glycerolphosphate (DL-αGP) 400 μM; 5,5′ dithiobis-2-nitrobenzoic

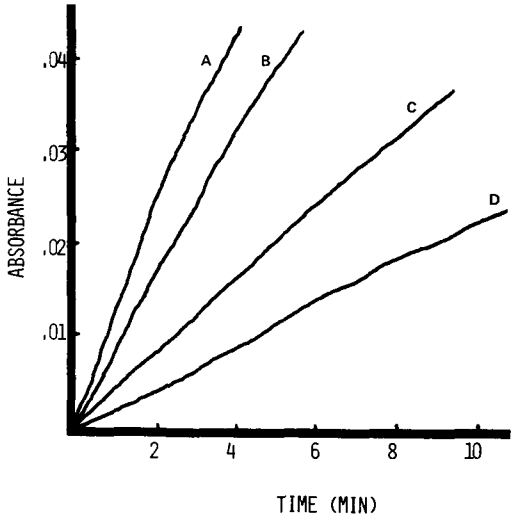

FIG. 1. Spectrophotometric tracing (at 412 nm) showing initial velocity of bovine mammary palmityl-CoA: L-α-glycerolphosphate acyl transferase as a function of various protein levels. Assay medium as in Table I. A = 0.3 mg protein, B = 0.2 mg protein, C = 0.1 mg protein, and D = 0.05 mg protein.

acid (DTNB) 1.0 mM; bovine serum albumin 3 mg, 0.1 mg microsomal protein in 1 ml Tris-HCl buffer (66 mM, pH 7.4). The incubation medium was equilibrated at 31 C for 3 min before acyl-CoA was added. The reference (control) sample lacked L-α-glycerolphosphate. Acyl-CoA: L-α-glycerolphosphate acyl transferase activity was quantified by the reaction of liberated CoASH with DTNB as measured continuously at 412 nm with a Perkin-Elmer model 356 spectrophotometer. A molar absorbance of 13,600 was used.

Delipidation: To determine the role of microsomal lipids on the activity of L-α-GP-acyl transferase, samples of microsomes were delipidated and assayed. Batches of freeze-dried microsomes (15 mg) were twice extracted with 20 ml acetone, acetone:water (9:1 v/v), or benzene. The extracted microsomes were dried carefully by nitrogen (4 C) and stored at -30 C. The extracted lipids, in ether solution, were stored for addition to the delipidated microsomes in restoration experiments.

Sonication of enzyme: Enzyme solutions were sonicated for a total of 60 sec at 4 C. One solution was sonicated using a bath sonicator model 8845-3 (Cole-Parmer Instrument Co.) Others were sonicated at different settings using a probe sonicator with a sonifer power supply (Bronson Sonic Power, 20,000 Hertz, Bronson Instruments, Danbury, Conn.). After sonication, the microsomes were assayed quickly for enzyme activity.

Analysis of products: To determine the products of the acyl transferase reactions, U-^{14}C-L-αGP or alternatively 1-^{14}C-palmityl-CoA or 1-^{14}C-stearyl-CoA was used in assays using similar conditions as described in the regular assay. The assay, containing appropriate amounts (0.1 mg) of microsomal protein, acyl-CoA, and DL-αGP, were incubated with continuous shaking at 30 C for 10 min, and the reaction was terminated by extracting the lipids according to the procedure of Folch, et al. (10). The solvent was evaporated, and the lipids dissolved in chloroform. The total radioactivity in each sample was determined. Lipids were fractionated by thin layer chromatography (TLC) using the system described by Lamb and Fallon (11), i.e. a solvent system of chloroform-methanol-3.5M NH_4OH (65:35:8). Standard lipid mixtures (Applied Science, State College, Pa.), including phosphatidic acid, were cochromatographed with these lipids. The lipid spots were located using iodine vapor. After evaporation of the iodine, the spots containing the identified lipids were transferred to scintillation vials, and 10 ml toluene base scintillation solution, containing 5.0 g 2,5-diphenyloxazole and 0.3 g 1,4-bis 2(4 methyl-5 phenyloxazolyl)benzene (Amersham/Searl Corp., Chicago, Ill.)/liter toluene, was used in each vial. Then radioactivity was quantified in a Packard TriCarb scintillation spectrometer. From these data, the extent of esterification into the glycerolipids was calculated.

Materials: All chemicals were reagent grade Bovine serum albumin (BSA) (fraction V powder), 5,5[1] DTNB, disodium DL-αGP, and CoA (trilithium salt) were purchased from Sigma Corp., St. Louis, Mo.). Stearyl-CoA, palmityl-CoA, oleyl-CoA, myristyl-CoA, linoleyl-CoA, and lecithin (bovine) were purchased from P.L. Biochemical (Milwaukee, Wisc.). L-α-glycerol 3-phosphate (U-^{14}C), 1-^{14}C-palmityl-CoA and 1-^{14}C-stearyl-CoA were purchased from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Palmityl-CoA; L-αGP acyl transferase was dependent upon the addition of active enzyme preparation, palmityl-CoA, and DL-αGP. Acyl transferase activity was linear over a range of protein concentrations (Fig. 1). The rate of acylation of palmityl-CoA was proportional to the microsomal protein up to 0.3 mg. All subsequent incubations containing 0.1 mg microsomal protein were assayed at 31 C.

The optimum pH of palmityl-CoA: L-αGP acyl transferase was 7.4-8.0. However, the enzyme was active over a pH range, i.e. 6.5-8.5.

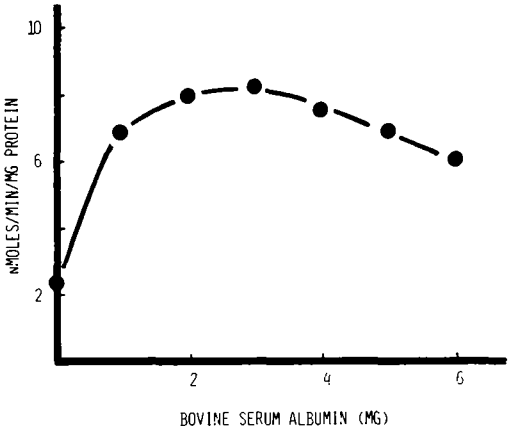

FIG. 2. Effect of bovine serum albumin upon specific activity of palmityl-CoA: L-α-glycerolphosphate acyl transferase from bovine mammary microsomes. Assay contained palmityl-CoA, 10 μM and D,L-α-glycerolphosphate, 450 μM. Other conditions as described in the text.

Increasing molarity of buffer in range 25-150 mM did not affect specific activity of the enzyme.

DTNB up to 1.0 mM stimulated release of CoA but inhibited the reaction above this level.

Magnesium ions enhanced activity, i.e. 0, 0.25, 0.5, and 1.0 mM (magnesium chloride) gave rates of 8.0, 9.2, 10.7 and 12.5 nmoles palmityl-CoA acylated min^{-1} mg^{-1} protein. The addition of ethylenediaminetetraacetic acid (EDTA) (2 mM) to the normal assay media doubled the rate of acylation, and the inclusion of magnesium ions (1.0 mM) in excess of the EDTA further accelerated the reaction 4-fold over untreated microsomes. This observation is being studied in detail.

Inhibition of palmityl-CoA: LαGP acyl transferase by acyl-CoA was relieved by the addition of BSA, with 3 mg/ml providing optimum activity (Fig. 2).

TABLE I

Effect of Sonication on Palmityl-CoA: L-α-Glycerolphosphate Acyl Transferase Activity from Bovine Mammary Microsomes[a]

Treatment	Acylation rate nmoles/min/mg protein
Normal	8.0
Sonicated (bath)	10.4
Sonicated (probe) 1	12.0
Sonicated (probe) 2	9.0
Sonicated (probe) 3	7.2

[a]In all cases, the enzyme solutions were sonicated for a total of 60 sec at 4 C. Complete assay contained: palmityl-CoA, 10 μM; dithiobis-2-nitrobenzoic acid, 1 mM; D, L-α-glycerolphosphate, 450 μM; 0.1 mg microsomal protein; Tris-HCl, 66 mM; and pH 7.4.

Sonication increased acyl transferase activity (33%) over that of a control, nonsonicated, enzyme preparation (Table I). The inclusion of small amounts of ethanol in the assay did not affect acyl transferase activity, whereas from 2-10% ethanol caused a gradual depression in activity, i.e. from 5-80% of normal.

Removal of microsomal-bound lipids reduced the specific activity of the acyl transferase. The effect was modified with the solvent used, i.e. acetone:water which removed more of the polar lipid components had the most pronounced effect (Table II). Addition of the extracted lipids even with sonication failed to restitute original activity; however, 70% of activity was restored by egg phospholipids, and phosphatidyl choline restored activity by 54%.

The enzyme became relatively saturated at low concentrations of palmityl-CoA, but increasing the acceptor, DL-αGP, to ca. 500 μM increased activity (Fig. 3). Using low levels of palmityl-CoA and saturating concentrations of

TABLE II

Effects of Solvent Extraction and Lipid Restoration upon Activity of Palmityl-CoA: L-α-Glycerolphosphate Acyl Transferase from Bovine Mammary Microsomes[a]

Treatment	Specific activity nmoles/min/mg protein
Normal microsomes	8.2
Acetone (100%) extracted microsomes	5.7
Acetone:water (9:1 v/v) extracted microsomes	1.3
Plus extracted lipids	2.1
Plus egg phospholipids	5.7
Plus bovine phosphatidylcholine	2.0
Benzene (100%) extracted microsomes	3.6
Plus extracted lipids	4.4

[a]Assay medium as in Table I.

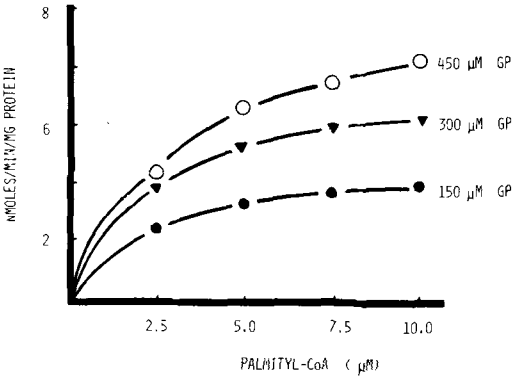

FIG. 3. Effects of increasing substrate concentrations upon acylation by palmityl-CoA: L-α-glycerolphosphate acyl transferase from bovine mammary microsomes. α-Glycerolphosphate denotes D,L-α-glycerolphosphate. Assay conditions as described in the text. GP = glycerolphosphate.

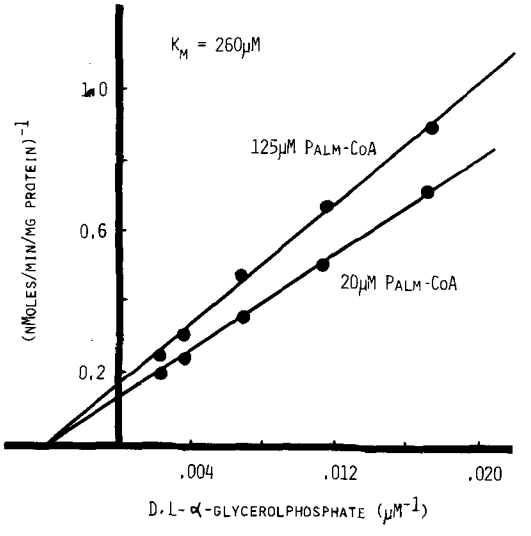

FIG. 4. Lineweaver-Burk plots showing effect of D,L-α-glycerolphosphate concentrations upon the rate of acylation at two levels of palmityl-CoA by palmityl-CoA: L-α-glycerolphosphate acyl transferase from bovine mammary microsomes. Assay medium contained bovine serum albumin, 6 mg; two fixed levels of palmityl-CoA, 20 and 125 μM; and varying levels of D,L-α-glycerolphosphate. All other conditions described in the text.

DL-αGP (500 μM), an apparent Km of 4.1 μM for palmityl-CoA was determined. The apparent Km of the palmityl-transferase for DL-αGP was 260 μM. Noteworthy was the inhibition of the enzyme by higher levels of palmityl-CoA (Fig. 4). At low levels of microsomal protein, i.e. 0.1 mg, the acyl transferase activity was inhibited by palmityl-CoA concentrations above 25 μM. This inhibition could be relieved by increasing

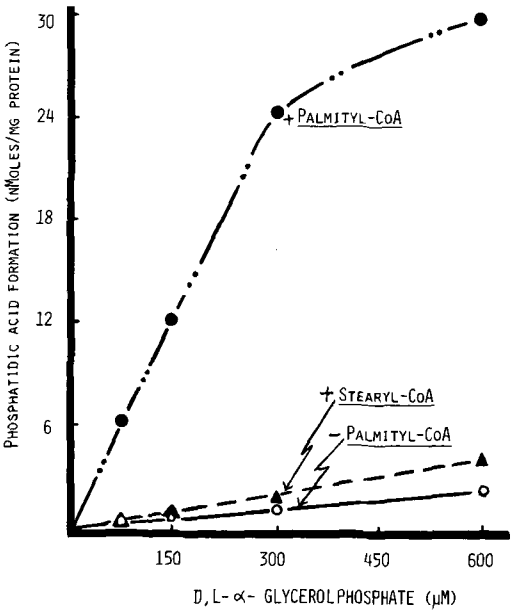

FIG. 5. The incorporation of L-α-glycerolphosphate (U-^{14}C) into phosphatidic acid in the absence, (○) and presence of (●) palmityl-CoA (20 μM) or (▲) Stearyl-CoA (20 μM) using 0.1 mg microsomal protein from bovine mammary microsomes incubated for 5 min at 35 C.

the microsomal protein or BSA content of the medium.

The apparent Vmax for the enzyme was 9.5 and 6.2 nmole/min/mg untreated microsomal protein for palmityl CoA and DL-αGP, respectively. The specific activity of palmityl-CoA: αGP acyl transferase from mammary tissue of different cows varied widely, i.e. from 1-22 nmoles palmityl-CoA acylated/min/mg protein. Most of the mammary tissue assayed was obtained from animals in late lactation, hence the values are probably lower than those obtaining in vivo at peak lactation. The enzyme used in the present study demonstrated remarkable stability, i.e. its specific activity decreased slightly, from 9.0-8.2 nmoles/min/mg protein, during storage for 9 months at -30 C as a freeze-dried powder.

Using labeled (U-^{14}C) DL-αGP and palmityl-CoA in normal assays and subsequent analysis by TLC, the principal product was phosphatidic acid (Table III). The presence of diglycerides probably reflects the activity of microsomal-bound phosphatidate phosphohydrolase, because acylation of monoglycerides was negligible in these preparations. Negligible radioactivity occurred in the triglycerides. When saturating levels of acyl-CoA were employed in the presence of 0.1 mg microsomal

TABLE III

Distribution of Radioactivity in Ester Lipids Following Incubation of U-^{14}C-L-α-Glycerolphosphate with Bovine Mammary Microsomes and Palmityl-CoA[a]

Lipid class	Incubation time 3 min	6 min
L-α-GP incorporated (nmoles/min)	3 ± 1.0	5.0 ± 1.0
	Distribution of radioactivity	
	Percent	
Lysophosphatidic acid	4 ± 1.0	3.5 ± 0.5
Phosphatidic acid	59 ± 2.1	63 ± 2.0
Diglycerides	25 ± 1.6	26 ± 3.0
Phospholipids	9.5 ± 1.5	5.0 ± 1.0

[a]Incubation tubes contained dithiobis-2-nitrobenzoic acid, 1 mM; bovine serum albumin, 3 mg; microsomal protein, 0.4 mg; palmityl-CoA 30 μM; and D, L-α-glycerolphosphate (U-^{14}C), 100 μM in 1 ml sodium phosphate buffer (66 mM, pH 7.2).

protein, phosphatidic acid formation increased with the levels of L-αGP provided (Fig. 5). The rate of phosphatidic acid synthesis observed using radioactive substrate was consistent with that observed in spectrophotometric assays. Incorporation was low when stearyl-CoA was used as substrate, and, in absence of acyl-CoA, phosphatidic formation acid was negligible, indicating a low concentration of endogenous acyl-CoAs in these bovine mammary microsomes.

Palmityl-CoA was the preferred acyl substrate under our assay conditions (Table IV). Acylation rates obtained with myristyl-CoA, stearyl-CoA, and oleyl-CoA were comparable but much less than with palmityl-CoA. The preference for palmityl-CoA was observed consistently with microsomal preparations of varying specific activities obtained from three different lactating animals.

TABLE IV

Comparative Rates of Acylation of D, L-α-Glycerolphosphate (450 μM) with Different Acyl-CoA Species by Acyl-CoA: L-α-Glycerolphosphate Acyl Transferase from Bovine Mammary Microsomes

Substrate	Rate nmoles/min/mg protein	
	Substrate concentration	
	10 μM	25 μM
Myristyl-CoA	1.7	2.2
Palmityl-CoA	8.2	9.0
Stearyl-CoA	1.0	1.4
Oleyl-CoA	1.4	2.1
Linoleyl-CoA	0.4	0.6

DISCUSSION

The involvement of acyl transferases in milk lipid synthesis has been reported for several species (7, 12-19). Our data indicate that microsomal enzymes are involved and that the properties of the enzymes from bovine mammary are quite similar to those from other animal tissues (11, 20-25).

The broad pH optimum with maximum activity around pH 7.5 for L-αGP acyl transferase agrees with the findings of others (21, 22, 26, 27). Lamb and Fallon (11) and Brandes, et al., (28) reported an optimum of 6.5 for the acyl transferase from rat and guinea pig liver, respectively. The negligible effects of altering the molarity of the buffer observed in this study corroborates the results of Jezyk and Lands (29) who showed that changes in concentrations of the buffer (8-80 mM) did not significantly affect the rate of αGP acylation by rat liver microsomes. However, Kuwahara (30) reported that acyl transferase of rat brain microsomes was affected by nature of buffer and ionic strength of the medium and that these effects could be ascribed to the state of hydration of the microsomes. However, it also could be related to the availability of free cations.

DTNB, a thiol reagent at low concentrations slightly stimulated acylation of L-αGP by bovine mammary microsomes in both spectrophotometric and radioactive assay systems. There are no previous reports of such stimulation. In fact, N-ethylmaleimide inhibited acyl transferase of pigeon liver microsomes by binding thiol groups (31), and Lands and Hart (32) reported that DTNB actually inhibited the acyl transferase mediating the initial acylation of αGP.

The effect of BSA in facilitating palmityl-CoA transferase has been well documented for this enzyme from other tissues and for other

enzymes which use acyl-CoA as substrates (20, 21, 28, 32,33). BSA reversibly binds palmityl-CoA and, at relatively low concentrations, prevents the formation of micelles, relieves inhibition of the acyl transferase, and conceivably ensures a monomolecular solution of substrate palmityl-CoA, as indicated by linearity of acylation rate. Furthermore, the BSA binds the free fatty acids released by the acyl-CoA hydrolase present in bovine mammary microsomes (1, 34). In the absence of BSA, nonlinear rate curves were obtained, as observed by Tanioka, et al. (15).

Mild sonication conceivably enhanced the acyl transferase by facilitating access of substrate to the enzyme via exposure of more microsomal surface area. It also may alter kinetic properties of these enzymes (36). Excessive sonication impaired activity, presumably as a result of protein denaturation. Similar effects were observed with bovine mammary microsomal stearyl-CoA desaturase (34).

The observation of Jezyk and Lands (29) that ethanol inhibited acyl transferase was confirmed in this study. It is probable that the ethanol solubilized some of the lipids required by this enzyme and thereby impaired function. This mechanism was corroborated by demonstration of the absolute lipid requirement for lipid, particularly polar lipids, by this enzyme. The observation that phosphatidyl choline was effective in restoring activity is consistent with the knowledge that this lipid is the major component of mammary microsomal membranes (35), and it confirms the report of Abou-Issa and Cleland (24) and Yamashita, et al., (23, 36) concerning the lipid requirement of this enzyme.

The apparent Km of DL-αGP for palmityl-CoA transferase (260 μM), while being lower, is within the general range, i.e. 0.2, 0.4, 0.5, and 0.67 mM, reported by Yamashita and Numa (36), de Jimenez and Cleland (37), Abou-Issa and Cleland, (24) and Lamb and Fallon (11) respectively, using L-αGP with rat liver microsomes but higher than the .05 mM reported by Husbands and Lands (31) for pigeon liver microsomes. It compares favorably with the Km of this enzyme of mammary microsomes from lactating rat and guinea pig. Mammary tissue of guinea pig and cow may contain limiting concentrations of L-αGP. Concentrations of ca. 90 and 80-250 μM L-αGP have been measured in guinea pig and bovine mammary (13, 38), respectively. These values vary depending upon nutritional and physiological status of these lactating animals. However, they indicate that availability of L-αGP may affect rate of glycerolipid synthesis in mammary tissue because of the apparent high Km of this substrate for the enzyme.

The Km for palmityl-CoA (4 μM) occurs at ca. the critical micellar concentration (cmc) for palmityl CoA (33) and is identical to Km for rat mammary microsomes which was measured at low substrate concentrations in the absence of BSA (15). Values of 10 and 50 μM were reported by Yamashita and Numa (36) and Lamb and Fallon (11). These are above the cmc, but these disparities can be explained by the difficulties involved in determining accurate Kms of amphipathic molecules, like long chain acyl-CoA species, as documented by several researchers (20, 27, 33, 34, 39) and discussed by Gatt, et al. (40).

In this respect, the problem of reconciling observations made on enzymes in vitro with the situation obtaining in vivo is challenging. In vitro low protein to substrate ratios usually are required for linearity, whereas in vivo higher protein to substrate ratios prevail. Okuyama and Lands (27) have studied this problem using the acyl transferases of rat liver microsomes. Relatively high concentrations of acyl-CoA exist in mammary tissue, e.g. 44 μM in guinea pig (13), and free fatty acids may attain concentrations of >4 mM (18) in cow mammary tissue. These facts should be considered when discussing specificity of acylation based upon in vitro observations.

The marked preference of the bovine mammary L-αGP-acyl transferase for palmityl-CoA has been discussed in relation to synthesis and the unique structure of bovine milk glycerides (7). The observed specificity provides an explanation for the nonrandom distribution of acyl groups in milk glycerides (4). Our preliminary data (7) and previous reports (5) would support the suggestion that a combination of partial segregation of fatty acids and specificity of acyl-CoA transferases accounts for the unique, nonrandom pattern of fatty acids in milk triglycerides.

The rapid, preferential acylation of position sn 1 and sn 2 of αGP with palmityl-CoA by these mammary microsomes is consistent with the events in vivo, wherein most of palmitic acid is located on sn 1 and sn 2 of milk glycerides (4). Furthermore, the observed preference of this enzyme for palmityl-CoA probably explains the preferential absorption and esterification of palmitic acid by bovine mammary cells (41) and its facile acylation by mammary homogenates (18). The acyl transferase from rat mammary showed a similar substrate preference, though not as exclusive as the bovine (15). Several other researchers have reported that palmityl-CoA is the most effec-

tive acyl donor in acylation of L-αGP by microsomes (11, 25, 26, 28, 42-44).

The rate of acylation of palmityl-CoA by mammary microsomes was ca. 10-fold greater than its rate of desaturation (34), whereas, by contrast, the respective activities for stearyl-CoA were similar. This observation provides an explanation for the greater generation of oleic compared to palmitoleic acid in bovine mammary microsomes and conceivably in lactating tissue also (34).

Phosphatidic acid was the principal product in these assays. Some workers reported that lysophosphatidic acid was the principal product (11, 28, 32, 45). However, several researchers have determined phosphatidic acid as the major product of acylation of L-αGP by microsomal preparations (13, 21-28, 31, 37, 39, 43-46). Significant amounts of radioactivity occurred in 1-sn-acylglycerolphosphate only when small amounts (50-100 mM) of (1-^{14}C) palmityl CoA were provided in our assays. The acylation of 1-acyl-sn-glycerolphosphate by bovine mammary microsomes (7) was more rapid (3-4 times) than the initial acylation, and the preference for palmityl-CoA was not as pronounced as in the acylation of DL-αGP. Our observations support the suggestion (41) that the L-αGP pathway is involved in bovine mammary glycerolipid synthesis and indicates that, in this respect, bovine mammary tissue is similar to mammary tissue of goat (16,17), rat (15), and guinea pig (12,13).

The phosphatidic acid formed by bovine mammary microsomes is dephosphorylated rapidly to sn-1,2 diglyceride and, in fact, only can be detected easily when low levels of microsomal protein (0.1-2 mg) are used and products are analyzed during the initial (linear) phase of the reaction. This indicates the presence of an active phosphatidate phosphohydrolase in bovine mammary tissue, as has been reported for mammary tissue of other species (15-17). It also validates the interpretation of precursor product curves obtained following the incubation of bovine mammary cells with radioactive acetate and glycerol in earlier separate experiments (47,48).

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