Fatty Acid Specificities of Microsomal Acyltransferases Esterifying Positions-1 and -2 of Acylglycerols in Mammary Glands from Lactating Rats

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The acyl specificities of several acyltransferases located in the microsomal fraction of lactating rat mammary gland have been investigated using palmitate and oleate as substrates along with CoA, ATP and Mg^{2+} , bovine serum albumin and NaF. With either *sn*-glycerol 3-phosphate or dihydroxyacetone phosphate (plus NADPH) as acyl acceptor, phosphatidic acid containing palmitate preferentially esterified at position-2 and oleate at position-1 was the major product. Dihydroxyacetone phosphate and *sn*-glycerol 3-phosphate competitively inhibited each other's acylations, suggesting that a single enzyme might be responsible for both esterifications and oleate was the preferred substrate for the formation of acyldihydroxyacetone phosphate. The specificities of the acyl-CoA-1-monoacyl-*sn*-glycerol 3-phosphate and the acyl-CoA-2-monoacyl-*sn*-glycerol 3-phosphate acyltransferases were also studied. The specificities observed combined with the relative velocities of these reactions suggest that phosphatidic acid is formed in the mammary gland with the first acylation occurring at position-1 favouring oleate followed by the second acylation at position-2 favouring palmitate. This is consistent with the unusual structure found in the triacylglycerols of rat milk. When a mouse liver microsomal fraction was used the opposite specificities were observed consistent with the structure of the triacylglycerols of mouse liver. The microsomal acylation of the monoacyl-*sn*-glycerol 3-phosphocholines was also investigated. Although no marked acyl specificity could be detected when the 2-monoacyl-*sn*-glycerol 3-phosphocholine was used as the acyl acceptor, both oleate and linoleate were esterified in preference to palmitate to the 1-monoacyl-*sn*-glycerol 3-phosphocholine.

Triacylglycerols from rat milk have an unusual intramolecular distribution of fatty acids. Palmitate is found mainly on the *sn*-2-position of the glycerol (Lin *et al.*, 1976; Grigor, 1977). This '2-saturated' structure is found in the milk of most species so far studied (Duncan *et al.*, 1966; Ackman *et al.*, 1968; Breckenridge *et al.*, 1969; Smith & Hardjo, 1974; Lin *et al.*, 1976; Grigor, 1977, 1979), and contrasts with the '2-unsaturated' structure of the triacylglycerols from most animal tissues (Brockerhoff *et al.*, 1966; Kuksis, 1972).

In a previous paper (Cooper & Grigor, 1978) we reported the synthesis of the '2-saturated' triacylglycerols *in vitro* studied in a cell-free preparation from mammary glands of lactating rats. The capacity to synthesize these milk-type triacylglycerols was absent in virgin mammary gland, but appeared at about the time of parturition.

The present paper reports the specificities and the

relative activities of several of the acyltransferases involved in the esterification of positions-1 and -2 of glycerol with microsomal preparations from lactating rat mammary gland. Because the triacylglycerols of mouse liver have the '2-unsaturated' structure, the specificity of the acyltransferases in microsomal fraction from mouse liver have also been investigated.

Fig. 1 shows the pathway of triacylglycerol synthesis and the acyltransferases we investigated. Schlossman & Bell (1976, 1977) have shown that in several tissues of the rat the acyl-CoA-dihydroxyacetone phosphate acyltransferase also catalyses the first esterification of *sn*-glycerol 3-phosphate. We have confirmed this for lactating rat mammary gland, and we have used this observation to infer the specificity of the acyl-CoA-*sn*-glycerol 3-phosphate acyltransferase.

During the course of this investigation we obser-

Fig. 1. Pathways of triacylglycerol synthesis

Key to acyltransferases: 1, acyl-CoA-*sn*-glycerol 3-phosphate 2-acyltransferase; 2, acyl-CoA-*sn*-glycerol 3-phosphate 1-acyltransferase (EC 2.3.1.15); 3, acyl-CoA-2-monoacyl-*sn*-glycerol 3-phosphate acyltransferase; 4, acyl-CoA-1-monoacyl-*sn*-glycerol 3-phosphate acyltransferase; 5, acyl-CoA-dihydroxyacetone phosphate acyltransferase (EC 2.2.3.42).

ved that the phospholipids from rat mammary gland have '2-unsaturated' structures. We have examined the specificities of acyl-CoA-1-monoacylglycerol-3-phosphocholine acyltransferase (EC 2.3.1.23) and acyl-CoA-2-monoacylglycerol-3-phosphocholine acyltransferase. These enzymes are not involved in phospholipid synthesis *de novo*, but may be involved in the rearrangement of fatty acids in preformed phospholipids.

Experimental

Animals

Pregnant rats of the Wistar strain and C57BL mice were purchased from the University of Otago Animal Breeding Station. All animals were given food and water *ad libitum*.

Materials

sn-Glycerol 3-phosphate, dihydroxyacetone phosphate (lithium salt), *L*- α -lysophosphatidylcholine (from soya beans and from egg yolk), palmitate, oleate, dihydroxyacetone, ATP, CoA, NADPH, phospholipase D (EC 3.1.4.4) from cabbage, snake (*Crotalus adamanteus*) venom and ATP-glycerol

phosphotransferase (EC 2.7.1.30) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Glycerol was obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. Radioactive fatty acids, [γ - 32 P]ATP, and [1(3)- 3 H]glycerol were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. 5-(Biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole was obtained from Ciba-Geigy (N.Z.), Wellington, New Zealand.

Methods

Preparation of microsomal fraction. Microsomal fractions were prepared from the mammary glands of rats in days 12–14 of lactation, and from mouse liver, as described previously (Cooper & Grigor, 1978).

Preparation of substrates. The 1-acyl-*sn*-glycerol 3-phosphate was prepared by treating *L*- α -lysophosphatidylcholine from soya bean or egg yolk with cabbage phospholipase D. About 20 mg of substrate was dissolved in 10 ml of 0.1 M-sodium acetate buffer, pH 5.6, containing 25 mM-CaCl₂, and 10 mg of phospholipase D was added. The solution was stirred gently at 23°C for 48 h. The digestion was stopped by adding 20 ml of chloroform/meth-

anol/acetic acid (1:1:0.01, by vol.). The lower (chloroform) phase, which contained the 1-acyl-*sn*-glycerol 3-phosphate, was removed and evaporated under N₂ and redissolved in chloroform. Total phosphorus was measured by the method of Bartlett (1969).

2-Acyl-*sn*-glycerol 3-phosphate was prepared from beef heart. Beef heart (300 g) was minced and homogenized in 1200 ml of chloroform/methanol (1:1, v/v), using a Sorvall Omni Mixer at half-speed for 30 s. After standing for 1 h the homogenate was filtered through cheesecloth and 0.5 vol. of chloroform and 0.3 vol. of water were added, giving chloroform/methanol/water (1:0.5:0.3, by vol.). The chloroform layer was dried and the lipid was redissolved in 10 ml of chloroform and 100 ml of acetone was then added. The acetone-insoluble lipid was dissolved in 15 M-acetic acid (10 mg/ml) and incubated in an atmosphere of N₂ for 16 h at 37°C to hydrolyse the plasmalogen to lysophosphatidylcholine and free aldehydes. The acetic acid was removed by adding 10 vol. of carbon tetrachloride and evaporating on a rotary evaporator between 35 and 40°C. This was repeated until all traces of acetic acid were removed. The product was redissolved in chloroform and the lysophosphatidylcholine obtained by preparative t.l.c. using chloroform/methanol/aq.NH₃ (sp.gr. 0.9) (13:6:1, by vol.) as the solvent. 2-Acyl-*sn*-glycerol 3-phosphate was prepared by digestion with phospholipase D. Table 1 lists the fatty acid compositions determined by g.l.c. of the monoacylglycerol-3-phosphocholines used as substrates for the phospholipase D digestions.

[1-(3-³H)]Glycerol 3-phosphate was synthesized by the method of Chang & Kennedy (1967). The product had a sp. radioactivity of 0.185 Ci/mmol. Dihydroxyacetone [³²P]phosphate was synthesized by the method of Schlossman & Bell (1976). The product had a sp. radioactivity of 15 mCi/mmol.

Enzyme assays. The standard assay contained 0.05 mM-bovine serum albumin (fatty acid-free), 10 mM-ATP, 4 mM-MgCl₂, 8 mM-NaF, 0.075 mM-CoA, 0.1 mM-oleate, 0.1 mM-palmitate, 0.1 mCi each

of [1-¹⁴C]palmitate (sp. radioactivity 59 mCi/μmol) and [9,10(n)-³H]oleate (sp. radioactivity 2.2 Ci/μmol) and an acyl acceptor in 0.25 ml of Tris/HCl buffer, pH 7.4. Microsomal protein (0.05–0.1 mg) was added and the mixture was incubated at 37°C with gentle shaking for the stated time. The reaction was terminated by the addition of 0.5 ml of chloroform/methanol/acetic acid (1:1:0.01, by vol.). The lower (chloroform) phase was removed, evaporated to dryness under N₂ at 40°C, and the lipids were redissolved in 0.1 ml of chloroform. The lipids from a portion of this solution were separated by t.l.c. with the basic solvent system. Fractions corresponding to lipid standards were scraped into scintillation vials and the incorporation of palmitate and oleate into the fractions was determined (Cooper & Grigor, 1978). In some experiments linoleate was substituted for oleate and [9,10-³H]palmitate (sp. radioactivity 50 mCi/mmol) and [1-¹⁴C]linoleate (sp. radioactivity 51 mCi/mmol) were used as the labelled acids.

An alternative assay for the acyl CoA-dihydroxyacetone phosphate and acyl CoA-*sn*-glycerol 3-phosphate acyltransferases omitted the radioactive fatty acids and used radioactive acyl acceptors (dihydroxyacetone [³²P]phosphate or [³H]glycerol 3-phosphate). This assay was used to test for competitive inhibition between the two substrates. The amount of product synthesized was measured by evaporating the chloroform layer and adding 5 ml of scintillant [0.55% (w/v) 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole in toluene/Triton X-100/water (20:10:3, by vol.)]. A portion of the original incubation mixture was also counted for radioactivity and the amount of acyl acceptor incorporated into the product was calculated.

Results and Discussion

Previously published analyses of triacylglycerols from rat milk (Lin *et al.*, 1976; Grigor, 1977) have shown that they have a '2-saturated' structure, with

Table 1. Fatty acid composition of monoacylglycerol-3-phosphocholines used for preparation of substrates

The percentage (by weight) of each fatty acid was determined by g.l.c. Samples of each lipid were treated directly with 2 ml of 1.6 M-HCl in methanol at 100°C for 20 min. The resultant methyl esters were extracted into hexane and then analysed by g.l.c. by using a column of 10% SP-2300 (Supelco Inc., Bellefonte, PA, U.S.A.) operated isothermally.

	Fatty acid (% of total, by weight)					
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	Other saturated	Other unsaturated
1-Monoacylglycerol-3-phosphocholine from egg yolk	62	23	8	2	1	4
1-Monoacylglycerol-3-phosphocholine from soya bean	36	13	31	14	2	4
2-Monoacylglycerol-3-phosphocholine from beef heart	10	17	31	18	8	16

the major fatty acid in the *sn*-2-position of the glycerol being palmitate. A preliminary study (M. R. Grigor, unpublished work) where the phosphatidylethanolamine and phosphatidylcholine fractions from rat mammary lipids were digested with snake venom phospholipase A₂ by the method of Wood & Harlow (1969) showed that the fatty acid distribution in these phospholipids was the opposite of that found in the triacylglycerols of the milk. In both phospholipids saturated fatty acids were found at the *sn*-1-position and unsaturated fatty acids at the *sn*-2-position. A similar difference in triacylglycerol and phospholipid structures has previously been noted for certain tissues of the pig (adipose tissue and kidney), both of which contain '2-saturated' triacylglycerols (Grigor *et al.*, 1971; Hagen, 1971).

In a previous paper (Cooper & Grigor, 1978) we reported that a postmitochondrial supernatant from lactating rat mammary gland could be used to synthesize triacylglycerols with the expected '2-saturated' structure of rat milk. However, the use of microsomal fractions and the addition of NaF resulted in synthesis of phosphatidic acid rather than triacylglycerols. From an equimolar mixture of palmitate and oleate in the incubation system approximately equal amounts of the two were incorporated into phosphatidic acid. When the phosphatidic acid was isolated and digested with phospholipase A₂ the distribution of palmitate and oleate on positions-1 and -2 (Table 2) is that expected for the triacylglycerol structure of the tissue (mammary gland or liver) from which the microsomal fraction was prepared. These observations are consistent with those from a similar experiment using a rat liver microsomal fraction (Possmayer *et al.*, 1969).

Table 2. *Stereospecificity of phosphatidic acid synthesis in microsomal preparations from lactating rat mammary gland and mouse liver*

Tissue	Molar ratio of palmitate to oleate		
	Phosphatidic acid	1-Monoacyl- <i>sn</i> -glycerol 3-phosphate (position-1)	Unesterified fatty acids (position-2)
Lactating rat mammary gland	1.24 ± 0.05	0.56 ± 0.08	1.52 ± 0.34
Mouse liver	0.94 ± 0.06	1.48 ± 0.20	0.27 ± 0.05

We used the mixture of two fatty acids in these experiments because it was considered that this represents a more physiological situation than the use of single fatty acids and comparison of the rates of esterification. In the cell the enzymes would be exposed to a mixture of fatty acyl-CoAs. The assay system also relied on the activity of the endogenous fatty acid-CoA ligase. Addition of acyl-CoAs as substrates can lead to detergent problems, which could affect the specificities observed.

Table 3 presents the fatty acid specificities of the acyltransferases involved in the esterification of the positions-1 and -2 of the glycerol. Schlossman & Bell (1976, 1977) have shown that in several tissues of the rat the microsomal acyl CoA-dihydroxyacetone phosphate acyltransferase and the acyl-CoA-*sn*-glycerol 3-phosphate acyltransferase are the same enzyme. We have carried out studies with microsomal preparations from the lactating mammary gland that suggest the same is likely to be true for this tissue. Both glycerol 3-phosphate and dihydroxyacetone phosphate inhibited each other's acylation in a competitive manner and high concentrations of dihydroxyacetone phosphate (130mM) were able to completely inhibit the acylation of 200μM-glycerol 3-phosphate. Two other acyltransferases have been described in mammalian cells; one in the mitochondria, which uses *sn*-glycerol 3-phosphate as acyl acceptor (Bjerve *et al.*, 1976; Halder *et al.*, 1979) and one localized in the peroxisomes and specific for dihydroxyacetone phosphate (Jones & Hajra, 1977). The kinetic data coupled with the relatively high forces used to pellet the mitochondria (225 000 *g*_{av}·min) suggest that any contamination of our microsomal samples by either of these activities is likely to be minimal. Assuming that the acyl specificity of the microsomal enzyme for both acyl acceptors is the same then the 1-monoacyl-*sn*-glycerol 3-phosphate synthesized from *sn*-glycerol 3-phosphate will contain mainly oleate.

Both acyl-CoA-1-monoacyl-*sn*-glycerol 3-phosphate acyltransferase and acyl-CoA-2-monoacyl-*sn*-glycerol 3-phosphate acyltransferase from rat mammary gland showed preference for palmitate, whereas the mouse liver enzymes showed preference for oleate. Thus the specificity for the second esterification appears to be for the fatty acid, irrespective of the position esterified. Previous studies with rat liver microsomal fractions have shown that in the second acylation unsaturated acyl-CoA molecules are esterified to position-2, whereas saturated acyl-CoA molecules are esterified to position-1 (Barden & Cleland, 1969; Yamashita *et al.*, 1973), although the relative specificity did, however, vary with the substrate concentrations used (Okuyama & Lands, 1972), being more marked at low concentrations of the mono-

Table 3. Specificities of the lactating rat mammary gland and mouse liver acyltransferases involved in acylhydroxyacetone phosphate and phosphatidic acid synthesis. Incubations were carried out as described for 20 min. Molar ratios of palmitate to oleate or palmitate to linoleate were calculated for each product. Results are means $\pm$ s.d. for the numbers of experiments indicated in parentheses.

Substrate	Source of substrate	Concn. of substrate (mM)	Product	Molar ratio of palmitate to oleate			
				With rat mammary gland as the enzyme source	With mouse liver as the enzyme source	With rat mammary gland as the enzyme source	With mouse liver as the enzyme source
Dihydroxyacetone phosphate		4.2	Acylidihydroxyacetone phosphate	0.62 $\pm$ 0.07 (5)	0.94 $\pm$ 0.2 (5)		
1-Monoacyl- <i>sn</i> -glycerol 3-phosphate	Egg yolk	0.66	Phosphatidic acid	5.37 $\pm$ 3.56 (6)	0.18 $\pm$ 0.05 (6)		
1-Monoacyl- <i>sn</i> -glycerol 3-phosphate	Soya bean	0.96	Phosphatidic acid	1.69 $\pm$ 0.51 (5)	0.51 $\pm$ 0.03 (5)		
2-Monoacyl- <i>sn</i> -glycerol 3-phosphate	Beef heart	0.15	Phosphatidic acid	2.11 $\pm$ 0.22 (4)	0.52 $\pm$ 0.09 (4)		
1-Monoacylglycerol-3-phosphocholine	Egg yolk	0.064	Phosphatidylcholine	0.44 $\pm$ 0.12 (5)	0.65 $\pm$ 0.08 (4)	0.66 $\pm$ 0.05 (4)	0.70 $\pm$ 0.08 (4)
1-Monoacylglycerol-3-phosphocholine	Soya bean	0.069	Phosphatidylcholine	0.78 $\pm$ 0.4 (6)	0.82 $\pm$ 0.21 (6)	0.33 $\pm$ 0.09 (3)	0.41 $\pm$ 0.17 (3)
2-Monoacylglycerol-3-phosphocholine	Beef heart	0.066	Phosphatidylcholine	0.99 $\pm$ 0.28 (4)	0.77 $\pm$ 0.20 (4)	0.68 $\pm$ 0.10 (4)	0.94 $\pm$ 0.18 (4)

acyl-*sn*-glycerol phosphate. In our studies saturating concentrations of substrates were used and the reaction rates were linear with time throughout the incubations. The specificities we report are lower than those of Okuyama & Lands (1972) for the rat liver enzymes when substrate-limiting concentrations were employed.

We have also measured $V_{\max}$ values for the different enzymes from lactating rat mammary gland (Table 4). The ratio of the rates of esterification of glycerol 3-phosphate to that of dihydroxyacetone phosphate is higher than that reported for other rat tissues (Schlossman & Bell, 1976, 1977), and the mouse mammary gland (Rao & Abraham, 1978), where values between 1 and 2 are common. Considerable confusion exists in the literature about the relative contributions of the acyldihydroxyacetone phosphate and glycerol 3-phosphate pathways to glycerolipid synthesis (Okuyama & Lands, 1970; Agranoff & Hajra, 1971; Manning & Brindley, 1972; Rognstad *et al.*, 1974; Geursen & Grigor, 1975; Pollock *et al.*, 1975, 1976). However, if the rat mammary gland contains a similar ratio of glycerol 3-phosphate to dihydroxyacetone phosphate to that found in the mouse mammary gland (i.e. 14) (Rao & Abraham, 1973), then the closeness of the K_m values reported here would imply that the favoured pathway is that involving the acylation of glycerol 3-phosphate.

The $V_{\max}$ values for acyl-CoA-1-monoacyl-*sn*-glycerol 3-phosphate acyltransferase and acyl-CoA-2-monoacyl-*sn*-glycerol 3-phosphate acyl-

Table 4. Kinetics of phosphatidic acid synthesis from different substrates with lactating rat mammary gland as the enzyme source

Incubations were carried out as described in the text for 10 min. The number of determinations is given in parentheses when there were more than two.

Substrate	Source of substrate	K_m (mM)	$V_{\max}$ (nmol of substrate/min per mg of protein)
<i>sn</i> -Glycerol 3-phosphate	—	0.58 $\pm$ 0.18 (6)	17.2 $\pm$ 5.1 (3)
Dihydroxyacetone phosphate	—	0.39 $\pm$ 0.12 (4)	4.4 $\pm$ 1.9 (4)
1-Monoacyl- <i>sn</i> -glycerol 3-phosphate	Egg yolk	—	2.1, 2.0
1-Monoacyl- <i>sn</i> -glycerol 3-phosphate	Soya bean	—	16.3, 16.0
2-Monoacyl- <i>sn</i> -glycerol 3-phosphate	Beef heart	—	13.0, 14.6

transferase, with soya bean and beef heart respectively as the substrate source, are approximately equal and are similar to the $V_{\max}$ value for the synthesis of phosphatidic acid from *sn*-glycerol 3-phosphate. The lower $V_{\max}$ for the acyl-CoA-1-monoacyl-*sn*-glycerol 3-phosphate acyltransferase with egg yolk as the substrate source compared with the other $V_{\max}$ values may be because of the high proportion of saturated fatty acids in the acyl acceptor.

From these observations we suggest that the formation of triacylglycerols in the lactating rat mammary gland proceeds with the initial acylation of *sn*-glycerol 3-phosphate occurring at position-1 rather than position-2. Although the kinetic data suggest that the second acylation occurs equally rapidly at both positions, the fatty acyl specificity of the 2-monoacyl-*sn*-glycerol 3-phosphate acyltransferase indicates that this reaction is not likely to be important for triacylglycerol synthesis. There is evidence which supports the concept that the first acylation in rat liver also occurs at position-1 (Yamashita *et al.*, 1972; Tamai & Lands, 1974).

Phosphatidic acid is a common intermediate in the synthesis of triacylglycerols and phospholipids. The '2-saturated' phosphatidic acid synthesized by the mammary gland microsomal fraction accounts for the structure of milk triacylglycerols, but not for the phospholipids. Two possibilities exist for the synthesis of these phospholipids. There may be different enzyme complexes within the microsomal membrane, each committed to the synthesis of either triacylglycerols or phospholipids. The relative concentrations of these lipid classes in the mammary gland are such that the amount of phosphatidic acid synthesized by the triacylglycerol system would be much greater than that synthesized by the phospholipid system and the latter would be unlikely to be detected. Alternatively, the phospholipid structure could arise from rearrangements of the fatty acids on the completed molecule. This requires the activity of phospholipases and acyltransferases, which have been shown to be present in many rat tissues (Gallai-Hatchard & Thompson, 1965; Bjornstad, 1966; Jezyk & Lands, 1968). The acyl-CoA-monoacylglycerol 3-phosphocholine acyltransferases could be involved in such a pathway. Those involved in acylating the 1-monoacyl-*sn*-glycero-3-phosphocholine appear to have the necessary specificity to produce the '2-unsaturated' phospholipids from either palmitate and oleate or palmitate and linoleate (Table 3), although the specificity in the acylation of the 2-monoacyl-*sn*-glycero-3-phosphocholine is less marked.

The acyl-CoA-1,2-diacyl-*sn*-glycerol acyltransferase of the lactating rat mammary gland has been described by Lin *et al.* (1976). They observed that the enzyme showed no specificity for the dia-

cylglycerol acceptor, but favoured medium-chain acyl-CoAs. These medium-chain acyl-CoAs were, however, poor substrates for the *sn*-glycerol 3-phosphate acyltransferases. These differences are consistent with the observation that the medium-chain fatty acids of rat milk tend to be confined to position-3. These observations, along with those presented in this paper, suggest that in the endoplasmic reticulum of the mammary cells of the lactating rat there is a set of acyltransferases that demonstrate unusual acyl specificities and are responsible for the synthesis of the unusual '2-saturated' triacylglycerols found in rat milk.

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