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Triacylglycerol synthesis in goat mammary gland

Factors influencing the esterification of fatty acids synthesized *de novo*

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1. ATP alone had no effect on incorporation of fatty acids synthesized *de novo* and membrane-bound diacylglycerol into triacylglycerol. Combined addition of ATP and Mg^{2+} totally inhibits incorporation of fatty acids synthesized *de novo* and stimulated incorporation of membrane-bound diacylglycerol. 2. ATP, Mg^{2+} and glycerol 3-phosphate stimulate incorporation of fatty acids synthesized *de novo* into triacylglycerol, but inhibited the incorporation of membrane-bound diacylglycerol. 3. Diacylglycerol generated *in situ* was shown to be superior to diacylglycerols preloaded on the membrane as substrate for the diacylglycerol acyltransferase. 4. A model is proposed to explain the effect of absorbed exogenous fatty acid on fatty acid synthesis *de novo* in goat mammary gland.

We showed in the preceding paper (Hansen *et al.*, 1984) that ATP and ATP + Mg^{2+} inhibit triacylglycerol synthesis from endogenous diacylglycerol and fatty acids synthesized *de novo*. Furthermore, we showed that glycerol 3-phosphate partly relieved the ATP inhibition and that ATP, Mg^{2+} and glycerol 3-phosphate when added together stimulated triacylglycerol synthesis.

In the present paper we report evidence to explain the observed effects, and in addition we show that diacylglycerol generated *in situ* is superior to diacylglycerols preloaded on the membrane as substrate for the diacylglycerol acyltransferase.

Materials and methods

Materials

Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. [2-^{3}H]Glycerol was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Palmitoyl-CoA and decanoyl-CoA were synthesized by the method of Sánchez *et al.* (1973). [2-^{3}H]Glycerol 3-phosphate was synthesized as described by Marshall & Knudsen (1977). All other materials were obtained as described by Hansen *et al.* (1984).

Methods

Subcellular fractionation, enzyme preparation, protein determination and incubations were as described by Hansen *et al.* (1984).

Preparation of microsomal-bound dipalmitoyl[2-^{3}H]-glycerol 3-phosphate

The goat mammary-gland microsomal fraction was incubated at 37°C in 8.5ml of a mixture containing 100mM-sodium phosphate, pH 7.0, fatty acid-poor bovine serum albumin (2mg/ml), 72μM-palmitoyl-CoA, 300μM-[2-^{3}H]glycerol 3-phosphate (5.56Ci/mol) and 8.5mg of microsomal protein. After 10min the mixture was cooled to 0°C and centrifuged for 90min at 105000*g*. The microsomal pellet was suspended in 100mM-sodium phosphate buffer, pH 7.0, to a protein concentration of 1.4mg/ml. It was stored at −80°C. The amount of diacylglycerol phosphate synthesized was in the range of 28–38nmol/mg of microsomal protein.

Dipalmitoyl[2-^{3}H]glycerol was prepared as described above, except that the incubation mixture was made 8mM with $MgCl_2$ after 10min and then incubated for 10min more before centrifugation. The yield of dipalmitoyl[2-^{3}H]glycerol was about 25nmol/mg of microsomal fraction.

Results

Incubation of goat mammary fatty acid synthetase with diacylglycerol-loaded microsomal fraction resulted, as reported by Grunnet & Knudsen (1981), in synthesis of medium-chain fatty acids (Table 1). Incorporation of fatty acids synthesized *de novo* into triacylglycerols could be observed, along with the conversion of labelled diacylglycerol

Table 1. *Fatty acid synthesis and incorporation of fatty acids synthesized de novo into triacylglycerol by the microsomal fraction loaded with membrane-bound diacylglycerol*
The incubation system is described in the Materials and methods section. The incubation (1 ml) contained fatty acid synthetase (174 μg; specific activity 832 nmol of NADH oxidized/min per mg of protein) and diacylglycerol-loaded microsomal fraction (200 μg; 25 nmol of diacylglycerol/mg of protein); 5 mM-ATP, 5 mM-glycerol 3-phosphate and 8 mM-$MgCl_2$ were added as indicated. The values for total fatty acid synthesis and triacylglycerol synthesis are means $\pm$ half the difference between duplicates. The results shown are typical results from at least three independent experiments, with microsomal preparations and fatty acid synthetase from at least two separate goats. Abbreviations: TG, triacylglycerol; TFA, total fatty acid.

Additions				Distribution of radioactivity in fatty acids (%)							Acetate incorporated (nmol) from [1-^{14}C]acetyl-CoA into:		[^{3}H]Diacylglycerol (nmol) incorporated into:
ATP	Glycerol 3-phosphate	$MgCl_2$		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	Total fatty acids	Triacylglycerol	Triacylglycerol
–	–	–	TG	14	10	9	35	22	9	–			
			TFA	46	11	5	9	6	11	10	3.8 $\pm$ 0.20	0.85 $\pm$ 0.08	1.0 $\pm$ 0.20
+	–	–	TG	13	9	9	33	28	7	–			
			TFA	41	10	5	9	10	17	8	4.6 $\pm$ 0.10	1.03 $\pm$ 0.19	1.1 $\pm$ 0.38
+	–	+	TG	–	–	–	22	21	57	–			
			TFA	55	10	4	4	3	12	12	3.5 $\pm$ 0.28	0.12 $\pm$ 0.01	1.4 $\pm$ 0.07
+	+	+	TG	21	14	12	39	12	2	–			
			TFA	45	11	6	13	7	12	7	4.5 $\pm$ 0.01	1.39 $\pm$ 0.01	–0.2 $\pm$ 0.2
+	+	–	TG	19	12	12	39	14	4	–			
			TFA	43	11	6	10	8	13	10	4.0 $\pm$ 0.04	0.82 $\pm$ 0.01	1.0 $\pm$ 0.45

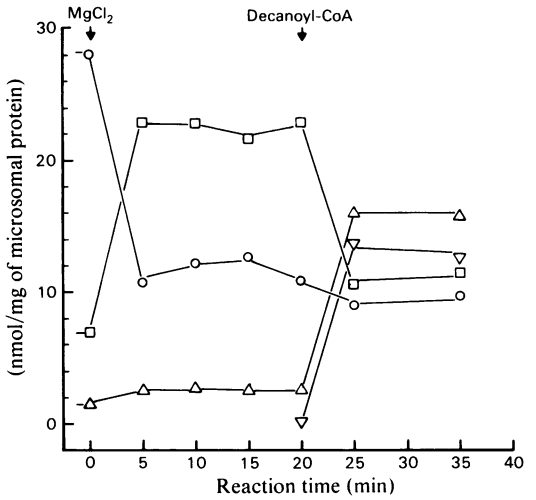

Fig. 1. *Time course for the breakdown of microsomal-bound diacylglycerol phosphate and reaction of the diacylglycerol formed with decanoyl-CoA*

The incubation mixture (1.0ml) contained 100mM-sodium phosphate buffer, pH7.0, and 125μg of diacylglycerol phosphate-loaded microsomal fraction (28nmol/mg of protein). $MgCl_2$ (8mM) was added at the start of the incubation and 30μM-decanoyl-CoA was added after 20min incubation. Samples (100μl) were removed at the times indicated, and radioactivity in the different lipid classes was determined as described in the Materials and methods section. The results shown are typical results from at least three independent experiments, with microsomal preparations and fatty acid synthetase from at least two separate goats. ○, Diacylglycerol phosphate; □, diacylglycerol; △, triacylglycerol (^{3}H-labelled); ▽, triacylglycerol (^{14}C-labelled).

into triacylglycerol. The addition of ATP alone to this system decreased the relative amounts of $C_{10:0}$ to $C_{12:0}$ synthesized and incorporated into triacylglycerol, but had no significant effect on the total incorporation of fatty acids synthesized *de novo* and ^{3}H-labelled diacylglycerol into triacylglycerol (Table 1).

Combined addition of ATP and Mg^{2+} strongly inhibited synthesis *de novo* of medium-chain fatty acids and incorporation of these acids into triacylglycerol. Only small amounts of medium- and longer-chain fatty acids synthesized *de novo* could be detected in the triacylglycerol fraction. Despite the inhibited triacylglycerol synthesis from fatty acids synthesized *de novo*, the incorporation of ^{3}H-labelled diacylglycerol was slightly stimulated by the addition of ATP and Mg^{2+} (Table 1).

The conversion of diacylglycerol into triacylglycerol without the concomitant incorporation of labelled fatty acids made it necessary to test the possibility of an acyl-CoA-independent conversion of ^{3}H-labelled diacylglycerols into triacylglycerols; this was done as shown in Fig. 1. When phosphatidic acid is converted into diacylglycerol by the addition of Mg^{2+}, a stable concentration of diacylglycerol, as well as triacylglycerol, is obtained for at least 15min. Addition of acyl-CoA to this system immediately resulted in a stoichiometric increase in the membrane content of ^{3}H-labelled and ^{14}C-labelled triacylglycerol, indicating that no unspecific transacylase reactions occurred in the membrane. Addition of ATP did not have any effect on the system described above (results not shown). The explanation why only small amounts of fatty acids synthesized *de novo* were incorporated into triacylglycerols in the experiment with added ATP and Mg^{2+} in Tables 1–3 could be competition from activated endogenous fatty acids formed in the membrane as a consequence of addition of ATP and Mg^{2+}. When the lipid contents of the microsomal membranes were analysed, they contained at least 5nmol of free fatty acids/mg of protein, with $C_{16:0}$ and $C_{18:0}$ as the dominant species (results not shown). The ATP- and Mg^{2+}-dependent enzyme catalysing fatty acid activation in mammary gland is reported to be present in the microsomal fraction (Bickerstaffe & Annison, 1971) and the free CoA needed for this reaction could be delivered by the fatty acid synthetase reaction, where the consumption of malonyl-CoA constantly liberates free CoA. Further support for inhibition of incorporation of fatty acids synthesized *de novo* being due to competition from activated endogenous fatty acids is that ATP and Mg^{2+} stimulate conversion of [^{3}H]diacylglycerol into triacylglycerol.

The addition of glycerol 3-phosphate to the incubation containing ATP and Mg^{2+} restored synthesis *de novo* of medium-chain fatty acids and enhanced incorporation of short- and medium-chain fatty acids synthesized *de novo* into triacylglycerols. Furthermore the combined addition of ATP, Mg^{2+} and glycerol 3-phosphate totally inhibited incorporation of ^{3}H-labelled diacylglycerols into triacylglycerols (Table 1). Addition of glycerol 3-phosphate in the presence of ATP, but in the absence of Mg^{2+}, only slightly changed the pattern of fatty acids synthesized and incorporated into triacylglycerol, and had no effect on the total amount of fatty acids or diacylglycerols incorporated into triacylglycerol (Table 1). This indicates that the glycerol 3-phosphate effect is dependent on the presence of ATP as well as Mg^{2+} in the system. This phenomenon is further illustrated in the experiment shown in Table 2, where incorporation of [^{3}H]glycerol 3-phosphate into tri-

Table 2. *Effect of ATP, Mg^{2+} and glycerol 3-phosphate on triacylglycerol synthesis when goat mammary-gland fatty acid synthetase is incubated in the presence of goat mammary-gland microsomal fraction*

The incubation system was as described in the Materials and methods section. Incubations (1.0ml) contained 160μg of fatty acid synthetase (specific activity 1135nmol of NADPH oxidized/min per mg of protein), 2mg of microsomal fraction, and 30μM-[1-^{14}C]acetyl-CoA (sp. radioactivity 12.2 Ci/mol); malonyl-CoA was infused at a rate of 2.0nmol/min for 15min. ATP (2.5mM), $MgCl_2$ (8mM) and [2-^{3}H]glycerol 3-phosphate (2.5mM; sp. radioactivity 5.56 Ci/mol) were added as indicated. The results shown are typical results from at least three independent experiments, with microsomal preparations and fatty acid synthetase from at least two separate goats. Abbreviations: n.d., not detected.

Additions	Acetate incorporated (nmol) from [1-^{14}C]acetyl-CoA into triacylglycerol	Glycerol 3-phosphate (nmol) incorporated into triacylglycerol
None	1.00±0.28	–
ATP, $MgCl_2$	0.08±0.01	–
[2-^{3}H]Glycerol 3-phosphate, $MgCl_2$	1.01±0.10	n.d.
ATP, $MgCl_2$, [2-^{3}H]glycerol 3-phosphate	2.67±0.60	7.57±1.01

acylglycerol by the microsomal fraction was measured, when incubated with fatty acid synthetase.

In these experiments glycerol 3-phosphate is not incorporated into triacylglycerol unless both ATP and Mg^{2+} are present in the incubation medium.

The incorporation of 1-^{14}C-labelled fatty acids into triacylglycerol was enhanced when glycerol 3-phosphate was added together with ATP and Mg^{2+} (Table 1). The short- and medium-chain fatty acids synthesized by goat mammary fatty acid synthetase are released as acyl-CoA compounds (Hansen & Knudsen, 1980; Knudsen & Grunnet, 1982); the stimulatory effect of ATP and Mg^{2+} on glycerol 3-phosphate incorporation therefore cannot be caused by activation of these acids. The effect must be caused by activation of endogenous fatty acids in the membrane giving rise to increased synthesis of the co-substrate for triacylglycerol, diacylglycerol. These results indicate that activated exogenous long-chain fatty acids in goat mammary gland will stimulate incorporation of short- and medium-chain fatty acids synthesized *de novo* into triacylglycerol when supplied in the presence of non-rate-limiting glycerol 3-phosphate.

Consequently addition of palmitoyl-CoA should therefore mimic the effect of ATP plus Mg^{2+}. The addition of palmitoyl-CoA to the incubation system resulted, as expected, in a decreased synthesis of medium-chain fatty acids and incorporation of short- and medium-chain fatty acids into triacylglycerol (Table 3). The inhibitory effect of palmitoyl-CoA could be relieved by glycerol 3-phosphate, and addition of Mg^{2+} further stimulated triacylglycerol synthesis significantly, owing to activation of phosphatidate phosphohydrolase. The combined addition of palmitoyl-CoA, glycerol 3-phosphate and Mg^{2+} gives maximal induction of medium-chain fatty acid synthesis.

The inhibition of ^{3}H-labelled diacylglycerol incorporation into triacylglycerol in the presence of ATP, Mg^{2+} and glycerol 3-phosphate (Table 1) indicated that newly synthesized diacylglycerol from activated endogenous fatty acids and added glycerol 3-phosphate is preferred as substrate by the diacylglycerol acyltransferase, rather than the diacylglycerol synthesized on the membrane before the incubation. Further support for this hypothesis was obtained in the following experiment. Fatty acid synthetase was incubated with the microsomal fraction loaded with ^{3}H-labelled phosphatidic acid with addition of Mg^{2+} at the start of the incubation. The results presented in Table 4 show that triacylglycerol synthesis on the microsomal fraction loaded with ^{3}H-labelled phosphatidic acid is more than doubled by addition of Mg^{2+}. The background incorporation into triacylglycerol before Mg^{2+} addition is due to the presence of a limited pool of endogenous diacylglycerol in the membrane, combined with a small amount of diacylglycerol synthesized during the preparation of the diacylglycerol phosphate-loaded microsomal reaction. The fatty acids that are synthesized *de novo* incorporated into triacylglycerol in the absence of Mg^{2+} are mainly medium-chain fatty acids, with $C_{12:0}$ as the dominant species. The increased incorporation of fatty acids synthesized *de novo* observed after addition of Mg^{2+} (Table 4) is mainly due to increased esterification of short-chain fatty acids; a similar pattern is observed when fatty acid synthetase is incubated with the microsomal fraction in the presence of ATP, Mg^{2+} and glycerol 3-phosphate (Hansen *et al.*, 1984).

Addition of both ATP and Mg^{2+} to the incuba-

Table 3. *Effect of palmitoyl-CoA on triacylglycerol synthesis from fatty acids synthesized de novo by goat mammary-gland fatty acid synthetase*

The incubation system was as described in Table 2, except that unlabelled glycerol 3-phosphate was used. Palmitoyl-CoA was added as indicated. The results shown are typical results from at least three independent experiments, with microsomal preparations and fatty acid synthetase from at least two separate goats. Abbreviations: TG, triacylglycerol; TFA, total fatty acid.

Additions				Distribution of radioactivity in fatty acids (%)							Acetate incorporated (nmol) from [1-^{14}C]acetyl-CoA into:	
Palmitoyl-CoA (20 μM)	Glycerol 3-phosphate (5 μM)	$MgCl_2$ (10 μM)		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	Total fatty acids	Triacylglycerol
–	–	–	TG	8	6	10	42	29	5	–		
			TFA	33	7	5	15	12	20	8	6.28±0.23	1.00±0.28
+	–	–	TG	–	–	–	17	54	30	–		
			TFA	28	9	5	8	15	26	10	6.74±0.07	0.52±0.03
+	+	–	TG	15	12	15	37	21	1	–		
			TFA	27	9	7	14	15	17	12	6.22±0.22	0.92±0.05
+	+	+	TG	15	12	15	34	20	4	2		
			TFA	27	12	9	17	13	15	7	8.77±0.56	1.99±0.26

Table 4. *Fatty acid synthesis and incorporation of fatty acids synthesized de novo into triacylglycerol by the microsomal fraction loaded with membrane-bound diacylglycerol phosphate*

The incubation conditions were as described in the Materials and methods section. The incubations (1.0 ml) contained fatty acid synthetase (176 μg; specific activity 1135 nmol of NADPH oxidized/min per mg of protein), diacylglycerol phosphate-loaded microsomal fraction (300 μg; 28 nmol of diacylglycerol phosphate/mg of protein), 8 mM-$MgCl_2$ and 5 mM-ATP added as indicated. The values for total fatty acid synthesis and triacylglycerol synthesis are means ± half the difference between duplicates. The results shown are typical results from at least three independent experiments, with microsomal preparations and fatty acid synthetase from at least two separate goats. Abbreviations: TG, triacylglycerol; TFA, total fatty acid.

Additions				Distribution of radioactivity in fatty acids (%)							Acetate incorporated (nmol) from [1-^{14}C]acetyl-CoA into:		[^{3}H]Diacylglycerol phosphate incorporated (nmol) into:
ATP (5 mM)	Mg^{2+} (10 mM)	Microsomal fraction*		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	Total fatty acids	Triacylglycerol	Triacylglycerol
–	–	–	TG	–	–	–	–	–	–	–			
			TFA	55	12	3	4	5	20	2	3.27±0.87	–	–
–	–	+	TG	7	11	9	29	32	13	5			
			TFA	46	7	3	8	11	18	8	5.64±0.04	0.64±0.11	0.76±0.16
–	+	+	TG	17	14	15	34	15	5	–			
			TFA	46	10	8	14	7	11	3	5.27±0.38	1.69±0.24	1.66±0.16
+	+	+	TG	–	–	–	20	41	39	–			
			TFA	44	8	3	5	8	25	7	4.02±0.23	0.14±0.01	6.65±0.15

* Loaded with diacylglycerol phosphate (0.4 mg).

tion of fatty acid synthetase with phosphatidic acid-loaded microsomal fraction (Table 4) resulted in inhibition of short- and medium-chain fatty acid esterification into triacylglycerol, as could be observed when diacylglycerol-loaded membranes were used (Table 1). The conversion of [^{3}H]-phosphatidic acid into triacylglycerol in the presence of Mg^{2+} and ATP was much larger than the conversion of [^{3}H]diacylglycerol into triacylglycerol under similar conditions, indicating that activated endogenous fatty acids are much more efficiently esterified into triacylglycerol when the diacylglycerol co-substrate is generated *in situ*.

Discussion

The ATP inhibition of triacylglycerol synthesis shown in the present experiment is Mg^{2+}-dependent (Table 1). The difference between the present experiments and the results reported previously, where addition of Mg^{2+} was not required for observation of the ATP effect (Hansen *et al.*, 1984), is most probably due to the difference in the treatment of the microsomal fraction. Incubation with EDTA-containing buffers during preparation of phosphatidate- and diacylglycerol-loaded membranes could remove Mg^{2+} embedded in the membrane. It should also be considered that incubations with the untreated microsomal fraction contained 10 times more protein than do incubations with treated membranes. The inhibitory effect of combined addition of ATP plus Mg^{+} on fatty acid synthesis *de novo* and subsequent incorporation into triacylglycerol, and the observation that this effect can be relieved by glycerol 3-phosphate, are consistent with previous results (Hansen *et al.*, 1984).

The present results strongly indicate that endogenous fatty acids in the microsomal fraction are activated and incorporated into glycerolipids when the necessary cofactors are present. Furthermore, the results show that the endogenously activated fatty acids are incorporated into triacylglycerols in preference to medium- and short-chain fatty acids synthesized *de novo* when membrane-bound diacylglycerol is the main acyl acceptor (Tables 1 and 4). However, in the presence of glycerol 3-phosphate, activated long-chain fatty acids seem to be channelled into the *sn*-1 and *sn*-2 positions of the glycerol backbone, as shown in Table 2. The increased incorporation of activated endogenous fatty acids in the presence of glycerol 3-phosphate increases the amount of diacylglycerol available for triacylglycerol synthesis, allowing short- and medium-chain fatty acids to be esterified in the *sn*-3 position. The use of added palmitoyl-CoA gives rise to effects similar to those with activated endogenous fatty acids (Table 3), underlining the dual effect of long-chain acyl-CoA on triacylglycerol synthesis from short- and medium-chain fatty acids synthesized *de novo*. The fact that formation of diacylglycerol *in situ* from phosphatidic acid during the experiment provides a better substrate for triacylglycerol synthesis than preformed diacylglycerol (Tables 1 and 3) is consistent with the observation that ATP, Mg^{2+} and glycerol 3-phosphate incubated with untreated microsomal fraction stimulate triacylglycerol synthesis, especially from short-chain fatty acids (Hansen *et al.*, 1984). The present results underline the importance of the rate of activation of exogenous fatty acids in the mammary gland relative to the rate of fatty acid synthesis *de novo* and the glycerol 3-phosphate concentration. Thus, if the supply of exogenous fatty acids to the mammary gland is lowered, the relative concentration of short- and medium-chain fatty acids could be increased in the secreted triacylglycerol. Although the relative content of fatty acids synthesized *de novo* is increased, total synthesis *de novo* could be decreased, owing to decreased formation of l-acylglycerol phosphate and diacylglycerol necessary for the removal of medium-chain fatty acyl-CoA from the fatty acid synthetase. On the other hand, if the supply of long-chain fatty acids is greatly enhanced, it could inhibit incorporation of fatty acids synthesized *de novo* into triacylglycerol, owing to competition for the diacylglycerol acyltransferase (Marshall & Knudsen, 1980), and decrease fatty acid synthesis *de novo*. Limitation of glycerol 3-phosphate supply in the above-described model would decrease the formation of diacylglycerol relative to the formation of long-chain acyl-CoA by activation of exogenous fatty acid, resulting in an inhibition of incorporation and synthesis of short- and medium-chain fatty acids synthesized *de novo*. Therefore a very simple explanation for the unique synthesis of short- and medium-chain fatty acids in mammary gland could be that glycerol 3-phosphate is rate-limiting for triacylglycerol synthesis in all ruminant tissues except mammary gland.

The hypothetical model described above is supported by experimental results (Kinsella & Gross, 1973) which showed that palmitoyl-CoA was the preferred substrate for the initial acylation of glycerol 3-phosphate by the acyltransferase of bovine mammary microsomal fraction. These authors concluded that palmitic acid is a key substrate for initiation of mammary glycerolipid synthesis via phosphatidic acid.

Mayorek & Bar-Tana (1983) showed that the model to some extent is applicable to rat liver hepatocytes. They found that palmitate at low con-

centration stimulated the incorporation of octanoate into triacylglycerol; at higher concentrations of palmitate, triacylglycerol synthesis from octanoate was inhibited, whereas the glycerol incorporation was stimulated. These results indicated that triacylglycerol synthesis from glycerol 3-phosphate has to be initiated with a long-chain fatty acid at the *sn*-1 position of the glycerol backbone, and that long-chain acyl-CoA species, e.g. palmitoyl-CoA, compete with medium-chain acyl-CoA for the *sn*-3 position, an effect similar to that observed in the present work. Rao & Abraham (1983) reported that decanoic acid synthesized *de novo* by mouse mammary-gland tissue slices were preferentially esterified to the *sn*-3 position of triacylglycerols, as it is found in milk. On the other hand, when decanoate was added to the incubation mixture, this acid was found to be esterified equally at the *sn*-1, *sn*-2 and *sn*-3 positions of glycerol. The conclusion of this experiment was that decanoyl-CoA synthesized *in vivo* in mouse mammary gland never accumulated in the cell to such an extent that it could compete with activated exogenous long-chain fatty acids for acylation of the *sn*-1 position of the glycerol backbone, and therefore mainly was incorporated into the *sn*-2 and -3 positions, a situation which also is seen in the present experiment.

Finally, Insull *et al.* (1959) showed that changing the amount and the nature of the fat in human diet greatly influenced the amount of medium-chain fatty acids in human milk.

The stimulatory role of diacylglycerol generated *in situ* over that of preformed diacylglycerol seen in the present study may be due to structural factors. Polokoff & Bell (1980) reported that the microsomal phosphatidate phosphatase from rat liver co-purified with diacylglycerol acyltransferase, indicating a close physical association between these two enzymes. *In vivo*, the breakdown of phosphatidate by phosphatidate phosphatase, may therefore create the substrate for the diacylglycerol acyltransferase, presenting the substrate in the right place at the right time. Thus the potential for esterification *in vivo* of short-chain fatty acids into the *sn*-3 position of triacylglycerols may be much more favourable than has been assumed on the basis of experiments *in vitro* (Marshall & Knudsen, 1977).

Finally, the present results, showing preference for diacylglycerol generated *in situ* over membrane-bound diacylglycerol as substrate for diacylglycerol acyltransferase, raises serious doubt about the validity of the values for the activity of microsomal diacylglycerol transferase based on the use of endogenous or exogenous added diacylglycerols.

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