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DIRECT TRANSFER OF FATTY ACIDS SYNTHESIZED 'DE NOVO' FROM FATTY ACID SYNTHETASE INTO TRIACYLGLYCEROLS WITHOUT ACTIVATION

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SUMMARY Medium chain fatty acids synthesized 'de novo' by lactating goat mammary gland fatty acid synthetase can be incorporated directly into triacylglycerols in the absence of a fatty acid activation system.

The synthesis of medium chain fatty acids by goat mammary synthetase was dependent on the removal of the medium chain product from the enzyme complex. The removal of the product from the enzyme could be excecuted by the microsomal triacylglycerol synthesizing system.

INTRODUCTION The synthesis of medium chain fatty acids ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$) by lactating rabbit and rat mammary gland is due to the presence of a specific medium chain thioesterase in the cytosol of these tissues (1, 2). This thioesterase changes the composition of the products synthesized by purified mammary gland fatty acid synthetases from predominantly long chain ($C_{14:0}$ and $C_{16:0}$) to mainly medium chain ($C_{8:0}$ - $C_{12:0}$) fatty acids.

Although goats milk contains about 10-12 moles % of $C_{10:0}$, it has not been possible to detect a similar thioesterase in the cytosol of lactating goat mammary gland (3, 4). However, the addition of the microsomal fraction to purified fatty acid synthetase from this tissue alters the fatty acids synthesized from short ($C_{4:0}$, $C_{6:0}$) and long chain fatty acids to that of short and medium chain fatty acids (5). This change in product composition is not due to the presence of a specific medium chain thioesterase in the microsomal fraction.

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As reported previously, goat mammary gland fatty acid synthetase exhibits both thioesterase and transacylase activity towards $C_{10:0}$ CoA (6, 7). This indicates that this synthetase has an inherent ability to terminate medium chain fatty acid synthesis by a thioesterase or transacylase reaction.

We have now investigated the effect of removing products *via* esterification into triacylglycerols on the induction of $C_{10:0}$ synthesis. This was to test the possibility of direct transfer of $C_{10:0}$ synthesized 'de novo' by goat mammary fatty acid synthetase into triacylglycerols without prior activation.

MATERIALS AND METHODS. NADPH, *rac*-glycerol 3-phosphate and dithiothreitol were purchased from Sigma Chemical Co., U.S.A. ATP and CoA were obtained from Boehringer Mannheim, West Germany. [1-^{14}C]Acetic anhydride and [2-^{3}H]-glycerol were supplied by the Radiochemical Centre Amersham, Bucks., U.K. [1-^{14}C]Acetyl-CoA and malonyl CoA were synthesized as described in (7). Palmitoyl-CoA was synthesized by the method of Sanchez *et al.* (8) and [2-^{3}H]-glycerolphosphate (specific radioactivity 9.3 Ci/mol) was synthesized enzymatically as described by Marshall and Knudsen (9).

Lactating goats, 2-3 weeks *post partum*, were used. Subcellular fractionation of mammary gland and purification of fatty acid synthetase were carried out as described by Knudsen (10). Fatty acid synthetase was incubated in 0.1 M-potassium phosphate buffer, pH 7.0, which contained 40 µM-[1-^{14}C]acetyl-CoA (specific radioactivity 3.8 - 6.5 Ci/mol), 1 mM-EDTA, 8 mM-$MgCl_2$ and 240 µM-NADPH. Malonyl-CoA was infused continuously (4 nmol/min) as described in (3).

Sodium hydroxide (0.5 ml, 5 M) was added to half of each incubation mixture to stop the reaction. Total fatty acid synthesis was measured, and the pattern of fatty acids synthesized was determined by radio-gas-liquid-chromatography (11). Chloroform-methanol (1:2 v/v, 3 ml was) added to the other half of the incubation mixture to stop the reaction and lipids extracted by the method of Bligh and Dyer (12) except that 0.1 M-HCl was used instead of water. Incorporation of [1-^{14}C]acetate into individual lipid classes was measured on an aliquot of the extract by analytical thinlayer chromatography as described in (9). The triacylglycerols in the remaining extract were isolated by preparative thin-layer-chromatography, using conditions identical to their separation on analytical plates. The fatty acids in the triacylglycerols were analyzed by radio-gas-liquid-chromatography. Microsomal-bound dipalmitoyl [2-^{3}H] glycerol was prepared essentially as described by Marshall and Knudsen (9) except that 85 µM palmitoyl-CoA was used. Furthermore, before phosphatidic acid was converted into diacylglycerol by adding 50 mM $MgCl_2$ the incubation mixture was centrifuged 1 hr. at 105,000 x g and the pellet resuspended in the original volume (17.5 ml) of 100 mM sodium phosphate buffer, pH 7.4, which contained 4 mg/ml of bovine serum albumin. This step was nescessary to prevent the conversion of diacylglyce-

rols into triacylglycerols. The amount of dipalmitoylglycerol synthesized was 31 nmole per mg microsomal fraction. ATP was measured by the method of (13).

Protein was precipitated with 15% (w/v) trichloroacetic acid and measured by the method of Lowry et al. with bovine serum as standard (14).

RESULTS Addition of substrates required for active triacylglycerol synthesis is needed in order to induce medium chain fatty acid synthesis by the microsomal fraction (Table 1). The omission of glycerol-3-phosphate plus ATP only slightly increases the amount of $C_{10:0}$ synthesized by fatty acid synthetase in the presence of the microsomal fraction. However, when glycerolphosphate, ATP and the microsomal fraction are all present, the proportion of $C_{10:0}$ synthesized increases from 8 to 24 moles %. Analysis of the individual fatty acids present in the triacylglycerol fraction shows that $C_{8:0}$, $C_{10:0}$ and $C_{12:0}$ are incorporated preferentially into triacylglycerols, compared with the incorporation of only 33% and 10% of $C_{14:0}$ and $C_{16:0}$, respectively.

This increased synthesis of medium chain fatty acids by triacylglycerol synthesis does not involve the hydrolysis of medium chain fatty acids from the fatty acid synthetase and subsequent reactivation (Table 2). Incubation of fatty acid synthetase with dipalmitoylglycerol bound to microsomal membranes increases the proportion of $C_{10:0}$ synthesized from 5 to 18 moles % even in the absence of ATP and glycerolphosphate. Increasing the amount of membrane-bound dipalmitoyl-glycerol increases both the amount of $C_{10:0}$ synthesized and its incorporation into triacylglycerols. The addition of ATP and glycerolphosphate reduces the induction of $C_{10:0}$ fatty acid synthesis by the membranes loaded with dipalmitoylglycerol (Table 2). Furthermore, the pattern of fatty acids incorporated into triacylglycerol changes from predominantly medium chain to equal proportion of medium and long chain fatty acids (Table 2). Almost all the $C_{10:0}$ synthesized in the presence and absence of ATP plus glycerol phosphate is recovered in triacylglycerols. This indicates

Table 1. The effect of triacylglycerol synthesis on medium chain fatty acid synthesis by lactating-goat mammary gland fatty acid synthetase. The incubation system was as described in the Method section. Incubations (1.0 ml) contained 100 μg fatty acid synthetase (specific activity 980 nmol NADPH oxidized/min per mg protein), lactating-goat mammary gland microsomal protein (0.4 mg) and 5 mM-ATP and 5 mM glycerol 3-phosphate as indicated. The values for total fatty acid and triacylglycerol synthesis are means ±S.D. of duplicates.

ADDITIONS			Percentage distribution of radioactivity in fatty acids (mol/100 mol)							nmol acetate incorporated from [1-^{14}C]acetyl-CoA into	
ATP plus glycerol 3-phosphate	Microsomal protein (mg)		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	Total fatty acids	Triacyl-glycerol
+	0	(TFA)*	25	4	2	5	12	34	18	6.4 ± 0.2	0
-	+	(TFA)	25	7	-	8	10	31	19	6.4 ± 0.2	0.6 ± 0.1
+	+	(TFA)	30	12	11	24	4	11	8	7.8 ± 0.2	3.2 ± 0.6
		(TG) **	12	11	15	41	10	9	2		

* TFA: Total fatty acids

** TG: Fatty acids in triacylglycerols

Table 2 Direct incorporation of 'de novo' synthesized medium chain fatty acids into triacylglycerol by microsomal fraction from lactating goat mammary gland with membrane-bound diacylglycerols as substrate

The incubation system is described in the Medthod section. Incubations (1.0 ml) contained diacylglycerol-loaded microsomal membranes from lactating goat mammary gland as indicated and 5 mM ATP and 5 mM glycerol 3-phosphate where shown. The values for total fatty acid and triacylglycerol synthesis are means ±S.D. of duplicates.

ADDITIONS			Percentage distribution of radioactivity in fatty acids (mol/100 mol)							nmol acetate incorporated from [1-^{14}C]acetyl-CoA into	
ATP plus glycerol 3-phosphate	Membrane bound diacylglycerols mg protein		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	Total fatty acid	Triacyl-glycerol
-	0	TFA*	44	9	5	5	11	23	3	4.8 ± 0.3	0
		TG**	-	-	-	-	-	-	-		
-	0.125	TFA	30	11	7	14	9	20	9	6.3 ± 0.1	2.0 ± 0.11
		TG	7	2	15	52	22	2	-		
+	0.125	TFA	22	9	5	11	12	31	10	6.4 ± 0.5	2.3 ± 0.25
		TG	8	-	-	27	27	38	-		
-	0.250	TFA	27	16	10	18	8	13	8	6.3 ± 0.6	3.1 ± 0.04
		TG	20	12	14	37	13	4	-		
+	0.250	TFA	26	13	7	15	9	17	13	5.1 ± 0.1	2.0 ± 0.4
		TG	10	1	8	32	20	26	3		

* TFA: Total fatty acids

** TG: Fatty acids in triacylglycerols

very strongly that the newly synthesized $C_{10:0}$ is incorporated directly into triacylglycerols without needing to be further activated. Since the microsomal membranes contain only very small amounts of ATP (0.08 nmol/ 250 mg compared with 1.1 nmol $C_{10:0}$ incorporated into triacylglycerol) reactivation of fatty acids by ATP associated with the microsomal fraction can be excluded.

DISCUSSION The results presented strongly indicate a direct transfer without hydrolysis and reactivation of medium chain fatty acids synthesized by goat mammary gland fatty acid synthetase into triacylglycerols. Since the ATP content of the microsomal fraction used was too low to account for reactivation of the incorporated fatty acids, the chain termination reaction must therefore involve a transacylase. This could be the transacylase which is present in the goat mammary gland fatty acid synthetase (7), which would then transfer the acyl chain to CoASH to form acyl CoA esters. Alternatively, fatty acid termination could involve a microsomal transacylase which transfers the acyl chain directly from the synthetase complex into triacylglycerols. Indications for a direct transfer of acyl groups from a bacterial fatty acid synthesizing system into glycerolipids has been reported (15).

Ruminant mammary fatty acid synthetases can terminate fatty acid synthesis at short chain ($C_{4:0}$, $C_{6:0}$) acids by transfering the acyl chain to CoASH, forming butyryl- and hexanoyl-CoA (16). Furthermore, both cow and goat mammary gland synthetases show high transacylase activity towards medium chain ($C_{8:0}$ - $C_{12:0}$) acyl-CoA esters (7). This also indicates that chain termination involves transfer by the transacylase of the synthetase complex of their acyl chains to CoASH.

Additional support for chain termination by a transacylase comes from recent experiments which indicate that CoASH is required for the termination reaction of rat liver fatty acid synthetase (17). However, further clarification of this point is needed.

The results presented also indicate that the removal or dissociation of the newly synthesized product from the synthetase is the rate-limiting step in the synthesis of $C_{10:0}$. Addition of the microsomal fraction alone only slightly increases $C_{10:0}$ synthesis, but when ATP and glycerolphosphate are also present the amount of $C_{10:0}$ synthesized increases due to its removal by esterification into triacylglycerol. The inhibitory effect of ATP and glycerolphosphate on the synthesis and removal of $C_{10:0}$ synthesis by the microsomal fraction with bound dipalmitoylglycerol is most likely due to competition by the newly activated long chain fatty acids at the level of triacylglycerol synthesis.

A similar change in the pattern of fatty acids synthesized brought about by the removal of product is found in Mycobacterium smegmatis (18), where a polysaccharide facilitates the removal (dissociation) of product from the fatty acid synthetase complex and thereby stimulates fatty acid synthesis. This results in an increased synthesis of $C_{24:0}$ and $C_{26:0}$-CoA esters compared with that of palmitoyl-CoA.

It is not known yet whether there is a similar binding factor in the mammary microsomal fraction. Even if there were a factor, it would have a limited binding capacity and the system would still need the enzymes and substrates for triacylglycerol synthesis to regenerate the binding factor by removing fatty acyl-CoA esters.

The results presented show for the first time in mammals the direct incorporation into triacylglycerol of a fatty acid formed by fatty acid synthetase without an activation step. A system in which the synthesis of a tissue-specific fatty acid (i.e. $C_{10:0}$ by goat mammary gland) is regulated by its removal by esterification into triacylglycerols may explain the very constant proportion of fatty acids which is found in milk triacylglycerols of different species (19).

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