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Medium-Chain Fatty Acid Synthesis in Lactating-Rabbit Mammary Gland

INTRACELLULAR CONCENTRATION AND SPECIFICITY OF MEDIUM-CHAIN ACYL THIOESTER HYDROLASE

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The concentration of medium-chain acyl thioester hydrolase and of fatty acid synthetase was determined by rocket immunoelectrophoresis in nine different particle-free supernatant fractions from lactating-rabbit mammary gland. The molar ratio of the hydrolase to fatty acid synthetase was 1.99 ± 0.66 (mean $\pm$ S.D.). A rate-limiting concentration of malonyl-CoA was required to ensure the predominant synthesis of medium-chain fatty acids when 2 mol of the hydrolase was added per mol of fatty acid synthetase. The interaction of the hydrolase with fatty acid synthetase was concentration-dependent, though an optimum concentration of hydrolase to synthetase could not be obtained. The lactating-rabbit mammary gland hydrolase altered the pattern of fatty acids synthesized by fatty acid synthetases prepared from cow, goat, sheep and rabbit lactating mammary glands, rabbit liver and cow adipose tissue.

Lactating-rabbit and lactating-rat mammary gland contain a medium-chain acyl thioester hydrolase (mol.wt. 29000–33000) which modifies the pattern of fatty acids synthesized by fatty acid synthetase from long- to medium-chain fatty acids (Knudsen *et al.*, 1976; Libertini & Smith, 1978). Immunological studies have shown that the hydrolase appears in rabbit mammary gland between days 17 and 22 of pregnancy, which coincides with the onset of milk-fat synthesis (Chivers *et al.*, 1977). In the present paper the concentrations of the hydrolase and fatty acid synthetase in lactating-rabbit mammary gland have been determined by rocket immunoelectrophoresis. The specificity for, and the interaction of the hydrolase with, different fatty acid synthetases have also been investigated.

Materials and Methods

Materials

New Zealand White rabbits and Wistar rats at 14 days *post partum*, red Danish dairy cows at 6–7 months *post partum* and goats and sheep of mixed breeds at 14 days *post partum* were used. CoA was obtained from Boehringer, Mannheim, West Germany, and [1-^{14}C]acetic anhydride was from The Radiochemical Centre, Amersham, Bucks., U.K. Dithiothreitol and bovine serum albumin (fraction V, fatty acid-poor) were from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of analytical purity and were obtained from E. Merck, Darmstadt, West Germany.

Methods

Acetyl-CoA was prepared from acetic anhydride as described by Stadtman (1957). Malonyl-CoA was synthesized by the method of Eggerer & Lynen (1962) and dodecanoyl-CoA was synthesized by the method of Sanchez *et al.* (1973).

Fatty acid synthetase was purified from lactating mammary glands from cow, goat, rabbit and sheep and from rabbit liver as described by Knudsen (1972). Fatty acid synthetase from adipose tissue was purified by the same method, except that the adipose tissue was homogenized at room temperature in 2 vol. of 100 mM-potassium phosphate buffer (pH 7.0)/250 mM-sucrose/4 mM-EDTA/1.0 mM-dithiothreitol in a Waring blender at full speed for 30 s.

Purification of medium-chain acyl-thioester hydrolase from lactating-rabbit mammary gland. The particle-free supernatant fraction from 14-day-lactating-rabbit mammary gland was prepared as described by Knudsen *et al.* (1976). Solid $(NH_4)_2SO_4$ was added continuously to the particle-free supernatant. The protein precipitated between 245 and 390 g of $(NH_4)_2SO_4$/litre initial volume was collected by centrifugation at $10000 g_{av.}$ for 10 min. The precipitate was dissolved in 100 ml of 1.0 mM-potassium phosphate buffer, pH 7.0, containing 1.0 mM-EDTA and 0.5 mM-dithiothreitol and dialysed against several 2-litre portions of distilled water to reduce the ionic strength to about 1.0 m ohm^{-1} at 4°C. The dialysed sample was loaded on a column (2.5 cm × 25 cm) of DEAE-cellulose previously equilibrated with 1.0 mM-potassium phosphate buffer, pH 7.0, contain-

ing 1.0mM-EDTA and 0.5mM-dithiothreitol. The adsorbed protein was eluted with a linear gradient consisting of 500ml of each of 10mM- and 175mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 0.5mM-dithiothreitol and 20% (v/v) glycerol. The fractions that contained enzyme activity were pooled, and the protein was precipitated by addition of 470g of $(NH_4)_2SO_4$/litre. The precipitate was suspended in 5ml of 100mM-potassium phosphate buffer, pH7.0, containing 1.0mM-EDTA and 0.5mM-dithiothreitol and dialysed against the same buffer until it was completely dissolved, and then applied to a column (2.5cm × 80cm) of Sephadex G-75. The column was eluted with 100mM-potassium phosphate buffer, pH7.0, containing 1.0mM-EDTA and 0.5mM-dithiothreitol (25ml/h), and the fractions (7ml) containing enzyme activity were pooled. The enzyme was concentrated by adding 470g of $(NH_4)_2SO_4$/litre. The precipitate was dissolved in 5ml of 60mM-Tris buffer adjusted to pH6.9 with conc. H_3PO_4 and dialysed at 4°C against 1 litre of the same buffer for 4h. The dialysed sample was made to 15% (v/v) with glycerol and applied to a preparative electrophoresis gel (7.5mm × 13.7cm; Ultraphore Colora, Messtechnik G.m.b.H., 7073 Lorch/Wurtt. 1, West Germany). The gel was 8cm high and consisted of 2cm of 2.5% (w/v) polyacrylamide (2.5% cross-linked) stacking gel in 60mM-Tris buffer adjusted to pH6.9 with conc. H_3PO_4 and 6cm of 15% (w/v) polyacrylamide (2.7% cross-linked) separation gel in 280mM-Tris/HCl buffer, pH8.9.

The upper and lower buffer compartments contained 50mM-Tris buffer containing 400mM-glycine, pH8·5. The electrophoresis was carried out at 2°C with a constant power of 25W during the stacking period and 50W during the separation period. The protein bands were continuously eluted with 124mM-Tris/HCl buffer, pH8.1, at a flow rate of 40ml/h. Fractions (5ml) containing enzyme activity were pooled and stored at −60°C.

The enzyme purified by this procedure was homogeneous on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate by the method described by Knudsen *et al.* (1976).

Spectrophotometric assay of medium-chain acyl thioester hydrolase activity. Throughout the purification procedure, medium-chain acyl thioester hydrolase activity was assayed by measuring the release of thiol groups from dodecanoyl-CoA as model substrate (Knudsen *et al.*, 1976).

Protein determination. Proteins were precipitated with 15% (w/v) trichloroacetic acid and determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Incubation conditions. Fatty acid synthetase was incubated at 37°C in 100mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 0.24mM-NADPH and 42μM-[1-^{14}C]acetyl-CoA. Rate-limiting concentrations of malonyl-CoA for fatty acid synthesis were obtained by infusing 2–3μl of various malonyl-CoA solutions/min. This resulted in the indicated infusion rates, which are expressed in nmol/min. A Harvard infusion pump, model 971, was fitted with eight 1.0ml disposable syringes. To ensure efficient mixing of the infused malonyl-CoA and the incubation medium, the infusion tube was fixed near the bottom of the incubation vessel, which was shaken with a rotary motion by using an Evapomix (Buckler Instruments, Fort Lee, NJ, U.S.A.) at shaker speed 6. The hydrolase was added as indicated. All incubations were done in duplicate, and each incubation mixture was analysed for the incorporation of [1-^{14}C]acetate into C_4–C_{18} fatty acids and for the distribution between the fatty acids as described by Knudsen (1976). This method quantitatively separates [^{14}C]acetate from longer-chain fatty acids. The recovery of $C_{4:0}$ and $C_{16:0}$ (mean ± S.D. for 12 extractions) was 94.0±3.9% and 97.0±2.3% respectively. Some 90% of the column effluent from the radio-gas chromatograph was combusted to CO_2, and counted in an 80ml flow-through counter with a total flow of 70ml/min, and a counting efficiency of 95%. All values for the molar percentage of fatty acids synthesized are means of duplicate incubations.

Rocket immunoelectrophoretic estimation of medium-chain acyl thioester hydrolase and fatty acid synthetase. Antibodies to fatty acid synthetase and to medium-chain acyl thioester hydrolase were raised in goats. The antisera were processed as described by Harboe & Ingild (1973) to give fractions that contained immunoglobulins. The immunoglobulin preparation was shown to be monospecific by cross immunoelectrophoresis in the Svendsen buffer system (Veeke, 1973). Rocket immunoelectrophoresis was carried out as described by Veeke (1973), except that 1.0% (v/v) Triton X-100 was added to the gel solution when the medium-chain acyl thioester hydrolase was assayed. All enzyme solutions and dilution buffers used in the assay of the hydrolase were made 1mg/ml with respect to bovine serum albumin to prevent adsorption of the enzyme on glassware. The final concentration of potassium phosphate in all the hydrolase dilutions was adjusted to 50mM to standardize the effect of potassium phosphate on peak height. All assays were done in duplicate.

Calculation of the rate of incorporation of malonyl-CoA

Malonyl-CoA utilization was calculated from the incorporation of [1-^{14}C]acetate by the following formula, where n represents the number of carbon atoms in the individual fatty acids:

$$\sum_{n=4}^{16}[(\text{molar \% of } C_n \times n) - 2] \times \text{nmol of [1-}^{14}\text{C]acetate incorporated}$$

This formula takes into account the fact that there is no synthesis of butyrate from acetyl-CoA by fatty acid synthetases from lactating mammary glands of rabbit, cow and goat (J. Knudsen, unpublished work).

Results

Rocket immunoelectrophoresis

Medium-chain acyl thioester hydrolase. The results of typical experiments showing the effect of albumin on peak heights are shown in Fig. 1. The addition of 0.2mg of albumin/ml to two different particle-free supernatant fractions with 54 and 44 μg of enzyme/ml increased the peak height by about 20 and 25% respectively. Increasing the albumin concentration to 1 mg/ml further increased the peak height slightly, but no further effect on peak height was seen above this concentration of albumin. The results in Fig. 2 show that the effect of albumin can be explained as decreased adsorption of enzyme on glassware. In these experiments the purified enzyme was diluted in two different ways both in the presence and absence of 1.0mg of albumin/ml. In dilution method (*a*), each dilution step was obtained by diluting 100 μl of enzyme stock solution with a calculated amount of buffer. Dilution method (*b*) used successive dilution of the previous dilution and so on. With albumin present, both dilution methods gave similar results. Without albumin present, both methods gave a lower value, but this was most pronounced for dilution method (*b*) at high dilution. The effect of albumin on the adsorption of enzyme on glassware might also explain the stimulating effect of albumin on chain-length termination (see below).

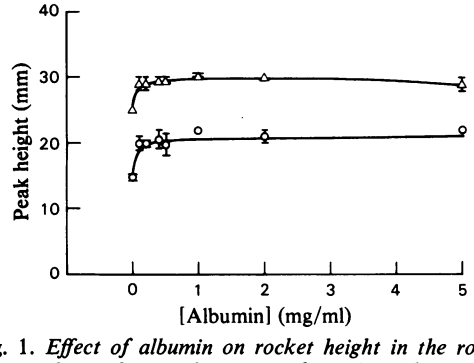

Fig. 1. *Effect of albumin on rocket height in the rocket immunoelectrophoretical assay of medium-chain acyl-thioester hydrolase*

For analytical details, see the Materials and Methods section; 54 μg (△) and 44 μg (○) of medium-chain acyl thioester hydrolase/ml in the solution were assayed. The error bars represent ± half the difference between duplicates.

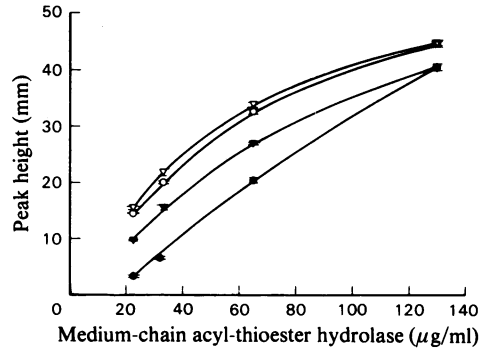

Fig. 2. *Effect of mode of dilution and bovine serum albumin on rocket height in the rocket immunoelectrophoretic assay of medium-chain acyl thioester hydrolase*

For analytical details, see the Materials and Methods section. ▽, ▼, Results from dilution method (*a*) with and without albumin (1.0mg/ml) respectively; ○, ●, results from dilution method (*b*) with and without albumin (1 mg/ml) respectively. Error bars represent ± half the difference between duplicates. Duplicate samples of four standards were used on each plate.

The concentration of the hydrolase in nine different particle-free supernatant fractions from lactating-rabbit mammary glands varied from 86 to 480 μg/ml (Table 1).

Fatty acid synthetase. The concentration of fatty acid synthetase in the nine particle-free supernatant fractions assayed for the hydrolase varied between 0.95 and 2.97mg/ml (Table 1). The large variation in enzyme content is partly caused by different content of milk in the gland at the time of killing the animals.

Assuming a mol.wt. of 475000 for fatty acid synthetase (Grunnet & Knudsen, 1978) and 29000 for that of the hydrolase (Knudsen *et al.*, 1976), the molar ratio of hydrolase/fatty acid synthetase is 1.99 ± 0.66 (mean ± S.D. for nine preparations).

Effect of albumin on fatty acid chain termination by medium-chain acyl thioester hydrolase from lactating-rabbit mammary gland

Bovine serum albumin stimulates fatty acid chain termination by the hydrolase, and a high proportion of this enzyme in the presence of albumin decreases the rate of incorporation of [1-^{14}C]acetate (Knudsen *et al.*, 1976). In the assays used in these previous experiments, malonyl-CoA concentrations that were rate-limiting for fatty acid synthesis were generated from [1-^{14}C]acetyl-CoA by acetyl-CoA carboxylase. Therefore the effect of bovine serum albumin on chain termination by the hydrolase and on the rate of acetate incorporation could not be distinguished from a possible indirect effect through acetyl-CoA

Table 1. *Concentration of medium-chain acyl thioester hydrolase and of fatty acid synthetase in the particle-free supernatant fraction from lactating-rabbit mammary gland, as determined by rocket immunoelectrophoresis*

For details of the assay system see under 'Methods'. The values for the μg of enzyme protein are mean values ± S.D. for the numbers of individual determinations given in parentheses.

Experiment	Fatty acid synthetase (μg/ml)	Medium-chain acyl thioester hydrolase (μg/ml)	Molar ratio of medium-chain acyl thioester hydrolase/ fatty acid synthetase
1	1734 ± 171 (6)	181 ± 7 (2)	1.71
2	2939 ± 249 (5)	237 ± 1 (2)	1.32
3	1205 ± 120 (4)	155 ± 11 (6)	2.11
4	1069 ± 107 (4)	138 ± 6 (5)	2.12
5	2982 ± 54 (3)	480 ± 78 (2)	2.64
6	947 ± 130 (4)	86 ± 9 (5)	1.49
7	2974 ± 267 (4)	176 ± 13 (4)	0.97
8	1272 ± 8 (3)	206 ± 9 (4)	2.65
9	1118 ± 64 (3)	198 ± 8 (4)	2.90
			Mean ± S.D. 1.99 ± 0.67

Table 2. *Effect of bovine serum albumin on fatty acids synthesized by lactating-rabbit mammary-gland fatty acid synthetase in the absence and presence of medium-chain acyl thioester hydrolase*

The incubation conditions are described in the Materials and Methods section. The incubation mixtures (0.50ml) contained 240μg of fatty acid synthetase from lactating-rabbit mammary gland (specific activity 743nmol of NADPH oxidized/min per mg of protein), 20μg of medium-chain acyl thioester hydrolase (specific activity 293nmol of dodecanoyl-CoA hydrolysed/min per mg of protein), bovine serum albumin as indicated and 42μM-[1-^{14}C]acetyl-CoA (specific radioactivity 3.8μCi/μmol). Malonyl-CoA was infused at a rate of 2.64nmol/min for 15min. The values for total acetate incorporation are means ± half the difference between duplicates. Total nmol of malonyl-CoA infused 39.6nmol.

Addition	Percentage distribution of radioactivity in fatty acids (mol/100mol)							Calculated malonyl-CoA utilization (nmol)	Total acetate incorporation from [1-^{14}C]acetyl-CoA (nmol)
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$		
None	34	8	2	3	6	41	6	11.4	2.95 ± 0.01
0.52mg of albumin	33	12	2	6	7	37	3	10.8	2.93 ± 0.04
5.2mg of albumin	39	15	4	2	3	31	6	11.1	3.36 ± 0.02
Hydrolase	33	10	6	21	13	17	—	10.3	3.17 ± 0.08
Hydrolase + 26μg of albumin	17	6	7	23	15	24	8	22.4	5.47 ± 0.04
Hydrolase + 52μg of albumin	16	5	11	31	17	17	3	19.3	5.05 ± 0.04
Hydrolase + 130μg of albumin	21	4	14	36	16	9	—	16.8	4.77 ± 0.05
Hydrolase + 502μg of albumin	14	5	11	35	17	16	2	20.3	5.21 ± 0.04
Hydrolase + 5.02mg of albumin	15	7	14	36	15	12	1	20.7	5.57 ± 0.18

carboxylase. To overcome this problem in the present experiments, rate-limiting concentrations of malonyl-CoA for fatty acid synthesis were created by infusing malonyl-CoA at low rate throughout the experimental period (see under 'Methods').

The effect of albumin on the chain lengths of the fatty acids synthesized by lactating-rabbit mammary-gland fatty acid synthetase in the presence and absence of the hydrolase is shown in Table 2. Low concentrations of bovine serum albumin (1mg/ml) only had a slight effect on the pattern of fatty acid synthesized by purified fatty acid synthetase alone. However, high concentrations of albumin (10mg/ml) increased the proportion of short-chain fatty acid synthesized. The calculated rate of incorporation of malonyl-CoA was unchanged by adding bovine serum albumin. Therefore the increased rate of incorporation of [1-^{14}C]acetate only represents a larger number of chain initiations caused by the change in chain length.

The addition of 1.36mol of hydrolase per mol of fatty acid synthetase resulted in high proportions of medium-chain fatty acids being synthesized without changing the calculated amount of malonyl-CoA utilized (Table 2). The addition of 52μg of albumin per ml greatly increased the rate of [1-^{14}C]acetate incorporated and the calculated rate of malonyl-CoA incorporation. This resulted in an increased synthesis of medium- and long-chain fatty acids with only slight change in the synthesis of $C_{4:0}$.

Increasing the albumin from 52 to 104 or 260 μg/ml did not increase the rate of incorporation of malonyl-CoA, but it further altered the pattern of fatty acids, synthesized, so that $C_{10:0}$ became the dominating fatty acid. Even up to 10 mg/ml, albumin did not further increase the incorporation of malonyl-CoA or increase the synthesis of medium-chain fatty acids. This effect of albumin has been repeated in three independent experiments. It is interesting that the effect of albumin on both the rate of malonyl-CoA utilization and the pattern of fatty acids synthesized (Table 2) is seen in the same concentration range as when albumin affected rocket height in the rocket immunoelectrophoretic assay. Only 27–56% of the infused malonyl-CoA was used for fatty acid synthesis, although the capacity of the added fatty acid synthetase to utilize malonyl-CoA was 34 times the infusion rate. However, the calculation of the malonyl-CoA concentration at the end of the incubation period is complicated because of a possible but unknown rate of malonyl-CoA decarboxylation during the incubation period.

Effects of rate of malonyl-CoA infusion and amount of medium-chain acyl thioester hydrolase on fatty acid synthesis

Increasing the rate of malonyl-CoA infusion from 0.31 to 8.35 nmol/min gradually increased the chain length of fatty acids synthesized by fatty acid synthetase in the presence of 1.36 mol of the hydrolase/mol of synthetase (Table 3). At the highest rate of infusion (where the capacity of fatty acid synthetase to convert malonyl-CoA to fatty acids is still three times higher than the infusion rate), $C_{14:0}$ and $C_{16:0}$ became the predominant fatty acids synthesized. At the two lowest infusion rates, no long-chain

Table 3. *Effects of different rates of malonyl-CoA infusion on the pattern of fatty acids synthesized by lactating-rabbit mammary gland fatty acid synthetase in the presence of medium-chain acyl thioester hydrolase*

The incubation system is described in the Materials and Methods section. The incubation mixture (0.5 ml) contained 240 μg of lactating-rabbit mammary-gland fatty acid synthetase (specific activity 743 nmol of NADPH oxidized/min per mg of protein), 260 μg of bovine serum albumin, 20 μg of medium-chain acyl thioester hydrolase (specific activity 255 nmol of dodecanoyl-CoA hydrolysed/min per mg of protein) and 42 μM-[1-^{14}C]acetyl-CoA (specific radioactivity 3.8 μCi/μmol). Malonyl-CoA was infused at the indicated rates for 15 min. Values for total acetate incorporation are means ± half the difference between duplicates.

Malonyl-CoA infused/min (nmol)	Percentage distribution of radioactivity in fatty acids (mol/100 mol)							Calculated malonyl-CoA utilization (nmol)	Total acetate incorporation from [1-^{14}C]acetyl-CoA (nmol)
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$		
0.31	49	12	20	19	—	—	—	2.7	1.31 ± 0.01
0.68	43	8	23	19	7	—	—	6.8	2.25 ± 0.08
1.36	20	4	13	34	16	11	2	9.5	3.15 ± 0.02
2.72	7	—	6	26	19	29	13	11.8	4.55 ± 0.03
5.43	14	1	3	24	13	27	18	11.5	4.92 ± 0.04
8.35	8	2	4	13	10	24	39	12.8	4.64 ± 0.03

Table 4. *Effect of increased ratio of medium-chain acyl thioester hydrolase/fatty acid synthetase*

The incubation system is described in the Materials and Methods section. The incubation mixture (0.5 ml) contained 240 μg of lactating-rabbit mammary-gland fatty acid synthetase (specific activity 743 nmol of NADPH oxidized/min per mg). Bovine serum albumin (260 μg) and medium-chain acyl thioester hydrolase (specific activity 225 nmol of dodecanoyl-CoA hydrolysed/min per mg) were added as indicated. [1-^{14}C]Acetyl-CoA (43.4 μM; specific radioactivity 5.95 μCi/μmol) and malonyl-CoA were infused at a rate of 2.6 nmol/min for 15 min. The values for total acetate incorporation are means ± half the difference between duplicates.

Molar ratio of medium-chain acyl thioester hydrolase/fatty acid synthetase	Percentage distribution of radioactivity into fatty acids (mol/100 mol)							Calculated malonyl-CoA utilization (nmol)	Total acetate incorporated from [1-^{14}C]acetyl-CoA (nmol)
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$		
None	19	6	2	2	3	43	25	17.3	3.50 ± 0.09
0.44	16	6	8	23	14	24	9	16.0	3.80 ± 0.16
1.0	13	3	12	36	18	14	4	16.5	4.12 ± 0.06
2.0	10	6	20	40	16	7	1	16.6	4.56 ± 0.06
3.1	9	5	24	43	14	5	—	18.5	5.10 ± 0.06
5.0	7	4	35	44	8	2	—	18.4	5.36 ± 0.15

Table 5. *Fatty acids synthesized by fatty acid synthetases (prepared from different tissues) in the presence and absence of medium-chain acyl thioester hydrolase from lactating-rabbit mammary gland*

The incubation system is described in the Materials and Methods section. The incubation mixture (0.5ml) contained 19.7μg of medium-chain acyl thioester hydrolase (specific activity 273nmol of dodecanoyl-CoA hydrolysed/min per mg of protein) as indicated, bovine serum albumin (260μg) and fatty acid synthetases from lactating-cow mammary gland (248μg; specific activity 443nmol of NADPH oxidized/min per mg of protein), lactating-cow adipose tissue (106μg; specific activity 430), lactating-goat mammary gland (240μg; specific activity 1347), lactating-sheep mammary gland (250μg; specific activity 1077). lactating rabbit mammary gland (240μg; specific activity 743) and lactating-rabbit liver (52μg; specific activity 690). [1-^{14}C]Acetyl-CoA (42μM; specific radioactivity 3.8μCi/μmol)/malonyl-CoA was infused at a rate 1.38nmol/min for 15min. The values for total acetate incorporation are means ± half the difference between duplicates.

Source of fatty acid synthetase	Medium-chain acyl thioester hydrolase	Percentage distribution of radioactivity in fatty acids (mol/100mol)							Total acetate incorporation from [1-^{14}C]acetyl-CoA (nmol)
		$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	
Cow mammary gland	−	32	10	2	2	6	36	12	2.28 ± 0.09
Cow mammary gland	+	13	6	5	37	20	19	—	2.86 ± 0.03
Cow adipose tissue	−	46	9	—	—	6	29	10	3.01 ± 0.27
Cow adipose tissue	+	39	7	9	22	15	8	—	3.56 ± 0.05
Goat mammary gland	−	7	6	—	—	11	52	24	2.15 ± 0.05
Goat mammary gland	+	12	5	11	38	24	10	—	2.97 ± 0.05
Sheep mammary gland	−	35	10	4	5	8	30	8	3.15 ± 0.10
Sheep mammary gland	+	31	6	16	37	10	—	—	4.04 ± 0.08
Rabbit mammary gland	−	35	10	4	5	8	30	8	3.68 ± 0.17
Rabbit mammary gland	+	24	8	27	29	9	3	—	3.93 ± 0.00
Rabbit liver	−	42	8	—	—	—	25	25	3.14 ± 0.22
Rabbit liver	+	28	5	16	31	13	7	—	4.26 ± 0.12

fatty acids were synthesized. As in the previous experiment, only a fraction (at most 25nmol) of the malonyl-CoA infused was converted to fatty acids. This indicates a feedback inhibition of the fatty acid synthetase, possibly by CoA. The final concentration of CoA in the present experiment with 0.5ml incubations would be twice the nmol of acetyl-CoA plus malonyl-CoA used, i.e. about 60μM at most. Hsu *et al.* (1965) have shown that 20μM-CoA inhibits pigeon liver fatty acid synthetase by 35%.

Increasing the amount of hydrolase relatively to fatty acid synthetase gradually changed the pattern of fatty acids synthesized from long- to medium-chain fatty acids without any change in the calculated rate of utilization of malonyl-CoA (Table 4). With 2mol of hydrolase/mol of fatty acid synthetase, which is the average ratio of these enzymes found in the cytosol, the pattern of fatty acids synthesized was very similar to that synthesized by lactating-rabbit mammary gland *in vivo* (Carey & Dils, 1972). No inhibitory effect of the hydrolase on the rate of fatty acid synthesis was seen.

The concentration-dependent effect observed on increasing the molar ratio of hydrolase to fatty acid synthetase could indicate a weak and non-specific interaction between the two enzymes. This was further investigated in the following experiment.

Specificity of the medium-chain acyl thioester hydrolase towards fatty acid synthetases from a number of different species

In these comparative experiments, fatty acid synthetases from lactating-rabbit, -cow, -goat and -sheep mammary gland, and from lactating-rabbit liver and -cow adipose tissue were used. In all cases the addition of the hydrolase changed the pattern of fatty acids synthesized from long- to medium-chain (Table 5). A comparison of the effect of the hydrolase on the different synthetases is difficult because of the difference in the amount of hydrolase added and the specific activities of the fatty acid synthetases used, but it seems that the lactating-rabbit mammary enzyme is the preferred substrate. However, the specificity of the hydrolase to terminate chain elongation is very broad with respect to fatty acid synthetases from different species and tissues.

Discussion

The concentration of medium-chain acyl thioester hydrolase and fatty acid synthetase in lactating-rabbit mammary gland calculated from the concentration in the particle-free supernatant fraction is 0.26–1.4mg and 2.8–8.9mg per g wet wt. of tissue respectively. The average molar ratio of the hydrolase to fatty acid synthetase was 1.99 ± 0.67 (mean ± S.D.).

An approximate value for lactating-rat mammary gland based on enzyme activity was 1–2 mol of hydrolase/mol of synthetase (Libertini & Smith, 1978). At rate-limiting concentrations of malonyl-CoA 2 mol of hydrolase/mol of fatty acid synthetase changed the pattern of fatty acids synthesized from long- to predominantly medium-chain fatty acids (Table 4). The pattern of fatty acids synthesized was greatly influenced by the molar ratio of hydrolase to fatty acid synthetase (Table 4) and by the rate of infusion of malonyl-CoA (Table 3).

It must be assumed from the results in Tables 1, 3 and 4 that malonyl-CoA has to be rate-limiting in lactating-rabbit mammary gland to ensure the predominant synthesis of medium-chain fatty acids as found in this tissue (Carey & Dils, 1972). Acetyl-CoA carboxylase is suggested to be rate-limiting in fatty acid synthesis *in vivo* (Numa, 1974). Furthermore the concentration of fatty acid synthetase in lactating-rabbit mammary gland [6–19 μmol/g wet wt. tissue (Table 1)] is of the same order as the concentration of malonyl-CoA found in rat liver, i.e. 4–38 μmol/g wet wt. of tissue (Cook *et al.*, 1977; Guynn *et al.*, 1972). Assuming similar concentrations of malonyl-CoA in lactating-rabbit mammary gland it is reasonable to expect that malonyl-CoA is rate-limiting and that the concentration of medium-chain acyl thioester hydrolase in lactating-rabbit mammary gland is sufficient to account for the predominant synthesis of medium-chain fatty acids in this tissue.

With the similar concentrations and molar ratios of enzymes as used in the present investigation, Libertini & Smith (1978) found only a slight effect of the lactating-rat mammary-gland medium-chain acyl thioester hydrolase (thioesterase II) on the pattern of fatty acids synthesized. These authors used non-rate-limiting concentrations of malonyl-CoA. Only if they increased the relative concentration of thioesterase II or of fatty acid synthetase were they able to get significant amounts of medium-chain fatty acids synthesized.

The effect of the relative concentration of hydrolase found by Libertini & Smith (1978) agrees with the results shown in Table 4. However, they found increased medium-chain fatty acid synthesis when they decreased the ratio of thioesterase II/fatty acid synthetase by increasing the concentration of fatty acid synthetase. This is not in accordance with the present result (Table 4), where a lower ratio of medium-chain acyl thioester hydrolase/fatty acid synthetase results in longer-chain fatty acids. This discrepancy is probably explained by decreased ratio of malonyl-CoA/fatty acid synthetase by increased additions of fatty acid synthetase in the experiments of Libertini & Smith (1978). A lowering of the ratio of malonyl-CoA/fatty acid synthetase will result in an increased synthesis of medium-chain fatty acids (Table 3).

Therefore qualitatively the medium-chain acyl thioesterases from lactating-rabbit and lactating-rat mammary gland seem to behave similarly.

The effect of low concentrations of albumin on peak height in the rocket immunoassay of the hydrolase (Figs. 1 and 2) is most likely caused by a decreased adsorption of the enzyme on glassware (Figs. 1 and 2). Similar conclusions were reached for the stimulatory effect of albumin on the hydrolysis of acyl-CoA by medium-chain acyl thioester hydrolase from lactating-rat mammary gland (Libertini & Smith, 1978).

The stimulatory effect of albumin on chain termination by the hydrolase can be explained in a similar way. By decreasing the adsorption on glassware, albumin would increase the true concentration of the hydrolase in the incubation medium and therefore increase the synthesis of medium-chain fatty acids (Table 4).

The increased rate of incorporation of malonyl-CoA caused by albumin in the presence, but not in the absence, of the hydrolase (Table 2) indicates that albumin might also increase the interaction between the hydrolase and fatty acid synthetase. An increased synthesis of medium-chain fatty acids upon adding 52 μg of albumin/ml in the presence of the hydrolase (Table 3) is most probably offset by the increased rate of incorporation of malonyl-CoA.

High concentrations of the hydrolase relative to fatty acid synthetase did not, as in previous experiments (Knudsen *et al.*, 1976), decrease the rate of fatty acid synthesis. The inhibitory effects seen in these previous experiments could therefore be due to inhibition of the acetyl-CoA carboxylase used to generate malonyl-CoA, though there is no direct evidence for this inhibition.

The gradual change from long- to medium-chain fatty acids synthesized with increasing concentration of the hydrolase relative to fatty acid synthetase (Table 4) without an optimum concentration suggests a weak interaction between the two enzymes. That the interaction between the two enzymes is weak and non-specific is further supported by the results from experiments with fatty acid synthetases from cow, goat and sheep lactating mammary gland, lactating-rabbit liver and lactating-cow adipose tissue (Table 5). Physiological concentrations of the hydrolase (1.4–6.1 mol/mol of fatty acid synthetase) changed the pattern from long- to medium-chain fatty acids.

The effect of increased relative concentrations of medium-chain acyl thioester hydrolase and malonyl-CoA on fatty acid chain length can be explained by the model for fatty acid termination and elongation proposed by Sumper *et al.* (1969) as follows. A higher concentration of hydrolase increases the probability for interaction with a given amount of the substrate, the proportion of acyl groups bound to acyl carrier protein that are medium-chain length at any instant.

This will increase the proportion of medium-chain fatty acids synthesized. The proportion of acyl groups bound to the acyl carrier protein of the synthetase are medium-chain length at any instant can be changed by either decreasing or increasing the rate of the competing reaction (i.e. the condensation reaction) by changing the concentration of malonyl-CoA.

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