The Synthesis of Medium-Chain Fatty Acids by Lactating-Rabbit Mammary Gland Studied *in vitro*

By CHRISTOPHER R. STRONG, ERIC M. CAREY* and RAYMOND DILS
Department of Biochemistry, Medical School, University of Nottingham,
Nottingham NG7 2RD, U.K.

(Received 31 October 1972)

The proportion of C_{8:0} and C_{10:0} fatty acids synthesized by the microsomal plus particle-free supernatant fraction from lactating rabbit mammary gland is enhanced at high protein concentrations. This fraction appears to contain a soluble high-molecular-weight factor that modifies the specificity of the fatty acid synthetase complex for termination of the growing acyl chain.

The fatty acid synthetase complex isolated from lactating-rabbit mammary gland synthesizes predominantly short-chain (C_{4:0} and C_{6:0}) and long-chain (C_{14:0} and C_{16:0}) fatty acids (Carey & Dils, 1970b). The proportions of these fatty acids synthesized can be modified by altering the availability of malonyl-CoA. However, medium-chain fatty acids (C_{8:0} and C_{10:0}), which are prominent in rabbit milk triglycerides (Smith *et al.*, 1968), are only synthesized in amounts approximately equimolar to the fatty acid synthetase. By contrast, studies on fatty acid synthesis *in vivo* (Carey & Dils, 1972) and in tissue slices (Strong & Dils, 1972) show the predominant synthesis of C_{8:0} and C_{10:0} fatty acids.

Although subcellular fractions of this tissue synthesize fatty acids of chain lengths C_{4:0}–C_{18:0}, the proportion of C_{8:0} and C_{10:0} fatty acids varied considerably and seemed to be related to the overall rate of fatty acid synthesis (Smith & Dils, 1964, 1966). We present here the results of experiments that show that the proportions of fatty acids synthesized from ¹⁴C-labelled sodium acetate by subcellular fractions of lactating-rabbit mammary gland are influenced by the concentration (measured as protein) of the subcellular fractions in the incubation medium.

Materials and methods

Animals. Lactating rabbits of mixed strain were used at 4–10 days *post partum*.

Chemicals. Sodium [1-¹⁴C]acetate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Biochemical reagents were obtained from Boehringer Corp. (London) Ltd., London W5 2T2, U.K.

Preparation of subcellular fractions. The method of Smith & Dils (1964) was used to prepare the microsomal plus particle-free supernatant and the particle-free supernatant fractions of lactating-rabbit mammary gland. The homogenization medium was 0.3M-

sucrose containing 50mM-potassium phosphate buffer, pH7.0, and 1mM-dithiothreitol. The concentration of the subcellular fractions was 30–35mg of protein/ml. Protein precipitated from the particle-free supernatant fraction between 0 and 40% saturation with respect to (NH₄)₂SO₄ was removed by centrifugation. The resulting supernatant was dialysed twice each for 6h against 10vol. of 100mM-potassium phosphate buffer, pH7.0, containing 1mM-dithiothreitol. The non-diffusible material was concentrated to about 30mg of protein/ml by ultrafiltration with a Millipore Pellicon membrane with an exclusion limit of mol.wt. 25000, and stored at –15°C (the F₄₀ fraction). The F₄₀ fraction was prepared from the particle-free supernatant fraction of rabbit liver in an identical manner. All the operations described were at 0–4°C.

The F₄₀ fraction was stored under N₂ at –15°C until required. In some experiments the microsomal plus particle-free supernatant fraction was stored under these conditions for 2–3 weeks.

Incubation conditions. Duplicate incubation mixtures contained, in a final volume of 1.1ml, 40mM-potassium phosphate buffer, pH7.3, 10mM-ATP, 0.20mM-CoA, 10mM-MgCl₂, 25mM-KHCO₃, 7mM-NADP⁺, 1mM-NADH, 5mM-*rac*-glycerol 3-phosphate, 1mM-dithiothreitol and 1mM-sodium [1-¹⁴C]-acetate (2μCi). The subcellular fraction (alone or in the presence of the F₄₀ fraction) was incubated at room temperature for 5min and the reaction was started by addition of the incubation mixture. Incubations were at 37°C for 15min. The reaction was stopped with 0.1ml of 10M-KOH. After saponification, the ¹⁴C-labelled fatty acids synthesized were extracted and analysed by radio-g.l.c. (Strong *et al.*, 1972).

Results

The effect of the concentration of the microsomal plus particle-free supernatant fraction on the proportion of fatty acids synthesized is shown in Table 1.

* Present address: Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, U.K.

Table 1. *Effect of concentration of the microsomal plus supernatant fraction of lactating-rabbit mammary gland on the pattern of fatty acids synthesized*

All incubation mixtures contained the same microsomal plus particle-free supernatant fraction. The rates of fatty acid synthesis are mean values from duplicate incubations $\pm$ half the range between the two values. The deviation from the mean value of duplicate results for the distribution of radioactivity in fatty acids was less than 2% for major components (i.e. those containing more than 5% of the radioactivity incorporated). The results were used in the statistical analysis referred to in the text.

Concn. of microsomal + supernatant protein in the incubation mixture (mg/ml)	Rate of fatty acid synthesis (nmol of acetate incorporated/ 15 min per mg of microsomal + particle-free supernatant protein)	Percentage distribution of radioactivity in fatty acids						
		C _{4:0}	C _{6:0}	C _{8:0}	C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}
23.0	8.5 $\pm$ 0.5	22	18	41	17	1	1	0
11.6	19.6 $\pm$ 0.5	17	15	32	31	4	1	0
7.0	30.9 $\pm$ 0.8	23	11	21	35	8	2	0
2.3	59.0 $\pm$ 0.5	22	10	7	21	16	17	7
1.3	74.1 $\pm$ 3.8	23	7	4	10	11	25	20
2.3*	64.8 $\pm$ 1.2	25	12	20	30	10	2	1
2.3†	45.5 $\pm$ 0.4	23	10	10	26	18	10	3

* These also contained 11.0mg of F₄₀ protein obtained from the particle-free supernatant fraction of homogenates of lactating-rabbit mammary gland.

† These also contained 17.0mg of F₄₀ protein obtained from the particle-free supernatant fraction of homogenates of lactating-rabbit liver.

The same fraction was used throughout. At high concentrations there was a considerable proportion of C_{8:0} and C_{10:0} fatty acids synthesized. As the concentration decreased there was a progressive increase in the proportion of longer-chain fatty acids formed. In all incubations there was a substantial proportion of short-chain fatty acids (C_{4:0} and C_{6:0}) formed, as there is with purified fatty acid synthetase (Carey & Dils, 1970*a,b*). There was a curvilinear relationship between protein concentration and medium-chain fatty acid synthesis. However, the proportion of C_{8:0} fatty acid synthesized increased linearly with increasing protein concentration over the range 1.1–12.8mg of protein/ml. From the results of five experiments (40 incubations) the correlation coefficient for the linear regression was 0.97, $P < 0.001$. In preliminary studies we were also able to show an effect of protein concentration on medium-chain fatty acid synthesis with three microsomal plus particle-free supernatant fractions that were incubated under slightly different conditions.

The rates of fatty acid synthesis in these experiments were not proportional to the protein added (Table 1). Since the rate of fatty acid synthesis can also influence the pattern of fatty acids synthesized in these experiments (Smith & Dils, 1964, 1966), it was necessary to distinguish between the effects of concentration and of the rate of synthesis on the pattern of synthesized fatty acids. In a separate experiment with 2.7mg of protein of the microsomal plus particle-free supernatant fraction the rate

of fatty acid synthesis was varied over the range 8.5–45.5nmol of acetate incorporated/15min per mg of microsomal plus particle-free supernatant protein (i.e. the range shown in Table 1). This was done by altering the concentrations of the cofactors used and the pH of the incubation mixture. There was no significant change in the proportion of C_{8:0} plus C_{10:0} fatty acids synthesized. Hence the changes in the proportions of fatty acids synthesized shown in Table 1 do not merely reflect altered rates of fatty acid synthesis per unit of microsomal plus particle-free supernatant protein.

The F₄₀ fraction from lactating-rabbit mammary gland contains less than 2% of the fatty acid synthetase activity of the particle-free supernatant fraction. When the F₄₀ fraction was added to incubation mixtures containing 2.3mg of microsomal plus particle-free supernatant protein/ml there was an increase in the proportion of medium-chain fatty acids synthesized but no significant change in the overall rate of fatty acid synthesis. Addition of the F₄₀ fraction from lactating-rabbit liver had little effect on the pattern of fatty acids synthesized.

The increased synthesis of medium-chain fatty acids on the addition of F₄₀ fraction from mammary gland has been observed with three such fractions each added to a fresh microsomal plus particle-free supernatant fraction. In another experiment the F₄₀ fraction enhanced medium-chain fatty acid synthesis with a stored microsomal plus particle-free supernatant fraction. Analysis of the results from the four

experiments where 3.2–18.1 mg of F_{40} protein was added in a total of 25 incubations showed that the synthesis of $C_{8:0}$ plus $C_{10:0}$ fatty acids was significantly increased ($P < 0.001$) compared with 13 control incubations containing no added F_{40} protein.

The F_{40} fraction was also found to be effective in altering the pattern of fatty acids synthesized by purified fatty acid synthetase from lactating-rabbit mammary gland. With optimum concentrations of [$1-^{14}C$]acetyl-CoA, malonyl-CoA and NADPH the purified enzyme at 0.27 mg of protein/ml of incubation mixture synthesizes mainly $C_{4:0}$ and $C_{16:0}$ fatty acids (Carey & Dils, 1970b). In the presence of 9 mg of the F_{40} fraction from homogenates of lactating-rabbit mammary gland the proportion of $C_{8:0}$ plus $C_{10:0}$ fatty acids synthesized was increased from 1% to 24%.

The particle-free supernatant fraction of homogenates of lactating-rabbit mammary gland also synthesized a high proportion of medium-chain fatty acids at high (above 5 mg of protein/ml) but not at low (less than 1 mg of protein/ml) concentrations. Since the products were unesterified fatty acids, the concentration effect is not necessarily related to esterification of the synthesized fatty acids.

Discussion

The results presented suggest that the F_{40} fraction of lactating-rabbit mammary gland contains a factor (or factors) that alters the specificity of fatty acid synthetase for chain termination so as to favour the release of medium-chain fatty acids. Sumper *et al.*

(1969) have suggested that termination of chain-lengthening at $C_{16:0}$ and $C_{18:0}$ fatty acids by yeast fatty acid synthetase is due to the chain-termination site on the enzyme being more hydrophobic than the site of chain elongation. The probability of hydrophobic long-chain acyl groups being transferred to the elongation site is therefore decreased, while the probability of their release from the enzyme is increased. It will be interesting to discover whether the altered specificity of fatty acid synthetase for chain termination reported here is due to an induced conformational change in the enzyme. If so, this could affect the relative hydrophobicity of the chain-termination and -elongation sites.

We thank the Medical Research Council for financial support. Miss B. Dixon provided excellent technical assistance.

- Carey, E. M. & Dils, R. (1970a) *Biochim. Biophys. Acta* **210**, 371–387
Carey, E. M. & Dils, R. (1970b) *Biochim. Biophys. Acta* **210**, 388–399
Carey, E. M. & Dils, R. (1972) *Biochem. J.* **126**, 1005–1007
Smith, S. & Dils, R. (1964) *Biochim. Biophys. Acta* **84**, 776–778
Smith, S. & Dils, R. (1966) *Biochim. Biophys. Acta* **116**, 23–40
Smith, S., Watts, R. & Dils, R. (1968) *J. Lipid Res.* **9**, 52–57
Strong, C. R. & Dils, R. (1972) *Comp. Biochem. Physiol.* **43**, 643–652
Strong, C. R., Forsyth, I. & Dils, R. (1972) *Biochem. J.* **128**, 509–519
Sumper, M., Oesterhelt, D., Riepertinger, C. & Lynen, F. (1969) *Eur. J. Biochem.* **10**, 377–387