

Purification and some Properties of a Medium-Chain Acyl-Thioester Hydrolase from Lactating-Rabbit Mammary Gland which Terminates Chain Elongation in Fatty Acid Synthesis

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1. An acyl-thioester hydrolase was isolated from the cytosol of lactating-rabbit mammary gland. The purified enzyme terminates fatty acid synthesis at medium-chain ($C_{8:0}$ – $C_{12:0}$) acids when it is incubated with fatty acid synthetase and rate-limiting concentrations of malonyl-CoA. These acids are characteristic products of the lactating gland. 2. The mol.wt. of the enzyme is 29000 ± 500 (mean \pm s.d. of three independent preparations), as estimated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. 3. The enzyme also hydrolyses acyl-CoA esters of chain lengths $C_{10:0}$ – $C_{16:0}$ when these are used as model substrates. The greatest activity was towards dodecanoyl-CoA, and the three preparations had specific activities of 305, 1130 and 2010 nmol of dodecanoyl-CoA hydrolysed/min per mg of protein when $56 \mu M$ substrate was used. 4. The way in which this enzyme controls the synthesis of medium-chain fatty acids by fatty acid synthetase is briefly discussed.

The mechanism by which chain elongation is terminated during fatty acid synthesis *de novo* is not fully understood. Sumper *et al.* (1969) have put forward a model to explain chain termination by yeast fatty acid synthetase, which releases long-chain ($C_{16:0}$ – $C_{18:0}$) fatty acids as the acyl-CoA esters. They propose that the probability of any covalently bound acyl residue forming a product by being transferred to CoA is determined by the relative velocities of the elongating and of the terminating transfer reactions of the synthetase. The maximum chain length synthesized is governed by an increasing interaction of the growing alkane chain with the hydrophilic enzyme protein. This leads to termination of fatty acid synthesis at $C_{16:0}$ and $C_{18:0}$ acids. The model could also explain chain termination by fatty acid synthetases from mammalian and avian tissues and from *Escherichia coli*, which release unesterified long-chain ($C_{14:0}$ – $C_{18:0}$) fatty acids [see Vagelos (1974) for review]. With these latter synthetases, the acyltransferase transfers the acyl group to water (rather than to CoA) and so acts as an acyl-thioester hydrolase which is specific for long-chain acyl-thioesters.

Lactating-rabbit mammary gland terminates chain elongation at $C_{8:0}$ and $C_{10:0}$ fatty acids both *in vivo* (Carey & Dils, 1972) and *in vitro* (Strong & Dils, 1972). By contrast, purified fatty acid synthetase from

this tissue only synthesizes $C_{4:0}$ and $C_{16:0}$ fatty acids in significant proportions (Carey & Dils, 1970a). When acyl-CoA esters are used as model substrates, the acyl-thioester hydrolase activity of this synthetase is specific for $C_{14:0}$ and $C_{16:0}$ acyl-CoA esters (Knudsen *et al.*, 1975). The inability of this purified synthetase to release $C_{8:0}$ and $C_{10:0}$ acids can therefore be explained by the model of Sumper *et al.* (1969) by the greater velocity of the elongation reaction compared with that of the acyl-thioester hydrolase for covalently bound medium-chain acyl residues.

One explanation for the synthesis of medium-chain fatty acids by lactating-rabbit mammary gland is the presence in the tissue of an acyl-thioester hydrolase which could specifically release medium-chain acids from the acyl-carrier protein or from other leaving sites of the fatty acid synthetase. We have presented preliminary evidence for the presence of this enzyme in the cytosol of lactating-rabbit mammary gland and have shown that the enzyme terminates chain lengthening even when the synthesized fatty acids were not subsequently esterified as glycerides. The crude enzyme preparation also hydrolysed acyl-CoA esters of various chain lengths when these were used as model substrates to mimic fatty acids covalently bound to fatty acid synthetase (Knudsen & Dils, 1975; Knudsen *et al.*, 1975). The present paper describes the purification and some of the properties of the enzyme and compares the enzyme with fatty acyl-CoA hydrolases (EC 3.1.2.2) from other tissues.

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Materials and Methods

Materials

Malonyl-CoA, NADH, NADPH, ATP, 5,5'-dithiobis-(2-nitrobenzoic acid), dithiothreitol, bovine serum albumin (fraction V, fatty acid-poor), pepsin (EC 3.4.23.1), cytochrome *c*, trypsin (type III) (EC 3.4.21.4), ovalbumin, myoglobin and sodium dodecyl sulphate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylamide and bisacrylamide were obtained from Fluka A.G., Buchs, Switzerland, and CoA and peroxidase (EC 1.11.1.7) were from Boehringer, Mannheim, West Germany. Ultragel Aca 44 was supplied by LKB A.B., Bromma, Sweden, and Sephadex G-100 by Pharmacia Fine Chemicals A.B., Uppsala, Sweden. [1-¹⁴C]Acetic anhydride and 1-¹⁴C-labelled fatty acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Pepsin (EC 3.4.23.1) was also purchased from E. Merck, Darmstadt, West Germany. All the other reagents used were of analytical purity and were obtained from E. Merck.

Methods

Acetyl-CoA and [1-¹⁴C]acetyl-CoA were prepared from acetic anhydride and from [1-¹⁴C]acetic anhydride respectively as described by Stadtman (1957). Radioactive and non-radioactive long-chain acyl-CoA esters were synthesized by the method of Sánchez *et al.* (1973). To synthesize the CoA esters of butyric acid, hexanoic acid and octanoic acid, the method was modified as follows (D. N. Brindley, personal communication) by converting the sodium salts of these acids into the tetraethylammonium salts before the reaction with ethyl chloroformate. Tetraethylammonium hydroxide was prepared by shaking tetraethylammonium bromide (2.1g) with Ag₂O (3.5g) in 10ml of water for 10min. The AgBr was removed by centrifugation. A column (5cm × 0.5cm) of Dowex 50W (H⁺ form) was converted into the tetraethylammonium form by applying 3ml of the aqueous solution of tetraethylammonium hydroxide and then washing the column with water until the eluate was pH7.0. The sodium salts of butyric acid, hexanoic acid or octanoic acid (in each case 20 μmol in 0.4ml of water) were applied to the column. The eluate containing the tetraethylammonium salts was evaporated to dryness under N₂, and the salt was dissolved in methylene chloride.

All acyl-CoA esters were analysed and purified as described by Pullman (1973). [1-¹⁴C]Acyl-carnitine esters of chain lengths C_{4:0}-C_{16:0} were a gift from Dr. R. Breach, Department of Biochemistry, University of Liverpool, U.K. Their ester content was determined by the method of Stern & Shapiro (1956), and their rate of hydrolysis by purified medium-chain acyl-thioester hydrolase was measured at 3 and 10 μM

substrate concentrations under the assay conditions described below for the radiochemical assay of the hydrolysis of fatty acyl-CoA esters.

Acetyl-CoA carboxylase (EC 6.4.1.2) was purified from lactating-rabbit mammary gland by the method of Manning *et al.* (1976). Fatty acid synthetase was purified from this tissue by the method of Knudsen (1972).

Enzyme purification

Unless stated otherwise, all procedures were carried out at 4°C. Mammary tissue (approx. 100g wet wt.) from lactating rabbits (12–16 days *post partum*) was finely chopped with scissors. Excess of milk was removed by washing the mince with 0.25M-potassium phosphate buffer, pH7.0, containing 1mM-EDTA and 0.25mM-dithiothreitol (2ml/g wet wt. of tissue). The tissue was homogenized with this buffer (2ml/g wet wt. of tissue) in a Potter-Elvehjem homogenizer and the homogenate was centrifuged at 105000g_{av.} for 2h to yield the particulate-free supernatant fraction. Solid (NH₄)₂SO₄ was added continuously, with stirring, to this fraction. The protein precipitated between 245 and 390g of (NH₄)₂SO₄ per litre of initial volume was collected by centrifugation at 10000g_{av.} for 10min. The precipitate was dissolved in 5mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA and 0.25mM-dithiothreitol and was applied to a column (2.5cm × 80cm) of Ultragel Aca 44. The column was eluted with 0.1mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA and 0.25mM-dithiothreitol, and fractions (7ml) containing the enzyme were pooled. The enzyme was concentrated by adding 470g of (NH₄)₂SO₄/litre of the pooled fractions. The precipitate was dissolved in the minimum volume (about 5ml) of 5mM-potassium phosphate buffer, pH7.8, containing 1mM-EDTA and 1mM-dithiothreitol and was then dialysed against 2 litres of the same buffer for 4h with one change of buffer.

A portion of the dialysed sample (20–30mg of protein) was applied to a preparative electrophoresis column (Shandon Scientific Co. Ltd., Willesden, London N.W.10, U.K.) of 18.8% (w/v) polyacrylamide (2.7% cross-linked; 4cm long) which had been polymerized in 8mM-Tris/HCl buffer, pH7.8, by riboflavin. The upper and lower buffer compartments contained 5mM-Tris/glycine buffer, pH8.5. The column was run for about 2h before the enzyme was applied. Electrophoresis was carried out for 18–20h at 2°C with a constant current of 10mA at 400V. The protein bands were then continuously eluted from the column with 14mM-Tris/HCl buffer, pH7.8, containing 1mM-EDTA and 0.25mM-dithiothreitol, at a flow rate of 4.8ml/h. Fractions (1.2ml) containing medium-chain acyl-thioester hydrolase were pooled and stored at -70°C.

Assay of medium-chain acyl-thioester hydrolase activity by chain-length termination in fatty acid synthesis

The purified enzyme was assayed by its ability to terminate fatty acid synthesis at medium-chain fatty acids. The incubation system (final volume 0.5 ml) contained 0.1 M-potassium phosphate buffer, pH 7.0, 1 mM-EDTA, 100 μ M-[1- 14 C]acetyl-CoA (5.75 μ Ci/ μ mol), 10 mM-KHCO₃, 5 mM-ATP, 8 mM-MgCl₂, 5 mM-tripotassium citrate, 0.24 mM-NADPH, fatty acid synthetase purified from lactating-rabbit mammary gland (218 μ g, of specific activity 1.16 μ mol of NADPH oxidized/min per mg of protein) and purified medium-chain acyl-thioester hydrolase as shown. [1- 14 C]Malonyl-CoA was generated *in situ* by adding rate-limiting amounts of acetyl-CoA carboxylase purified from lactating-rabbit mammary gland (10.5 μ g, of specific activity 520 nmol of malonyl-CoA formed/min per mg of protein). The mixture was incubated at 37°C for 10 min and the reaction was stopped by adding aqueous NaOH to a final concentration of 2.5 M. The radioactive fatty acids were extracted and were analysed by radio-g.l.c. as described by Knudsen (1976). In the absence of medium-chain acyl-thioester hydrolase this system synthesized negligible proportions of medium-chain fatty acids. All assays were performed in duplicate.

Assay of medium-chain acyl-thioester hydrolase activity with acyl-CoA esters as model substrates

(1) *Spectrophotometric assay.* For convenience throughout the purification procedure, the enzyme activity was assayed by measuring the release of thiol groups from dodecanoyl-CoA as model substrate. The assay mixture contained 0.4 M-Tris/HCl buffer, pH 7.4, 1 mM-EDTA, 0.2 mM-5,5'-dithiobis-(2-nitrobenzoic acid) and enzyme protein. It was preincubated for 3 min at 37°C. The reaction was started by adding dodecanoyl-CoA (final concentration 56 μ M; see Fig. 2) and was followed spectrophotometrically at 412 nm. The amount of thiol released was calculated from the molar extinction coefficient $\epsilon = 1.36 \times 10^4$ litre \cdot mol⁻¹ \cdot cm⁻¹ (Means & Feeney, 1971).

(2) *Radiochemical assay.* The specificity of the purified enzyme towards acyl-CoA esters of various chain lengths when these are used as model substrates was determined by measuring the release of [1- 14 C]-fatty acids from the radioactive acyl-CoA esters. The assay mixture contained 0.1 M-potassium phosphate buffer, pH 7.5, 1 mM-EDTA, 1 mM-dithiothreitol, 3 or 8 μ M-[1- 14 C]acyl-CoA esters (0.4–1.6 nCi/nmol) and albumin (as indicated). It was incubated at 37°C for 5 min. The reaction was started by adding 1–4 μ g of enzyme protein which had been preincubated at 37°C for 2 min in 14 mM-Tris/HCl buffer, pH 7.8. The final volume of the reaction mixture was 0.5 ml. The reaction was stopped after 0.5–3.0 min with 2.0 ml of

Dole's (1956) reagent. Unesterified 14 C-labelled fatty acids of chain lengths C_{8:0}–C_{16:0} were extracted by the method of Bar-Tana *et al.* (1971). The top phase, which contained the unesterified 14 C-labelled fatty acids, was mixed with 10 ml of xylene containing 4.0 g of 2,5-diphenyloxazole/litre and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre, and the radioactivity determined by liquid scintillation. Portions (0.5–1.0 ml) of the bottom phase were mixed with 10 ml of Triton X-100/xylene (1:2, v/v) containing 5.5 g of 2,5-diphenyloxazole/litre and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre. Water (0.1–0.5 ml) was added, and the radioactivity determined by liquid scintillation. When butyryl-CoA or hexanoyl-CoA was used as substrate, the reaction was stopped by adding 0.5 ml of 0.1 M-HCl. The unesterified fatty acids were extracted four times with 1.0 ml portions of diethyl ether and their radioactivity was determined by liquid scintillation. The recovery of [14 C]butyric acid was 93%. Control incubations without added enzyme were always used. All assays were performed in triplicate.

Molecular weight

The molecular weight of partly purified medium-chain acyl-thioester hydrolase was determined by gel filtration (Andrews, 1970). The molecular weight and the homogeneity of the purified enzyme was established by polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). A 10% (w/v) polyacrylamide gel was used.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Enzyme purification

Details of the purification of the enzyme are shown in Table 1. The only effective step in the final stage of the purification was found to be preparative polyacrylamide-gel electrophoresis. This increased the specific activity about 12-fold with relatively little loss of enzyme activity. Attempts to use cellulose ion-exchange chromatography or hydroxyapatite treatment at this stage of the purification were unsuccessful.

Three independent preparations of the enzyme purified by the procedures given in Table 1 had specific activities of 305, 1130 and 2010 nmol of dodecanoyl-CoA hydrolysed/min per mg of protein as determined by the spectrophotometric assay at a substrate concentration of 56 μ M. The three preparations were homogeneous by the criterion of analytical

Table 1. Purification of medium-chain acyl-thioester hydrolase from mammary gland of lactating rabbits

Details of the purification are given in the Materials and Methods section. One unit of enzyme activity is defined as 1 nmol of dodecanoyl-CoA hydrolysed/min at a substrate concentration of 56 μ M in the spectrophotometric assay.

| | Protein (mg) | Activity (units) | Specific activity (units/mg of protein) | Purification | Yield (%) |
|--|-----------------|---------------------|--|--------------|--------------|
| 1. Particle-free supernatant | 2160 | 15600 | 7.2 | 1.0 | 100 |
| 2. $(\text{NH}_4)_2\text{SO}_4$ fraction (245–390 g/litre) | 280 | 5040 | 21.6 | 3.0 | 39 |
| 3. Ultragel Aca 44 eluate [concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and dialysed] | 46.2 | 4350 | 94.2 | 13.1 | 26 |
| 4. Preparative-electrophoresis eluate | 2.1 | 2380 | 1130 | 157 | 15 |

polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate on 10% (w/v) gels (Plate 1).

Molecular weight

The approximate mol.wt. of the enzyme as the material precipitated by 245–290 g of $(\text{NH}_4)_2\text{SO}_4$ /litre from the particle-free supernatant was estimated by Sephadex G-100 gel filtration to be about 28000. Albumin (mol.wt. 68000), peroxidase (mol.wt. 40000), pepsin (mol.wt. 35000) and cytochrome *c* (mol.wt. 11700) were used as reference proteins. The mol.wt. of the three preparations of the purified enzyme, as established by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, was 29000 ± 500 (mean \pm S.D.). Albumin, ovalbumin (mol.wt. 43000), pepsin, trypsin (mol.wt. 23300), myoglobin (mol.wt. 17200) and cytochrome *c* were used as reference proteins.

Effects of the enzyme on chain termination in fatty acid synthesis

The incubation system used to test the effects of the purified enzyme on chain termination contained purified fatty acid synthetase and [^{14}C]acetyl-CoA plus purified acetyl-CoA carboxylase so as to generate rate-limiting amounts of malonyl-CoA. This system synthesized predominantly $\text{C}_{4:0}$ and $\text{C}_{14:0}$ acids plus a varying proportion of $\text{C}_{16:0}$ (Table 2, Expt. *a*); when malonyl-CoA is not rate-limiting, $\text{C}_{16:0}$ is the major product (Carey & Dils, 1970*b*).

When albumin (260 μ g) was added as a control, it increased the rate of fatty acid synthesis and the proportion of $\text{C}_{14:0}$ synthesized, but did not increase the proportions of medium-chain fatty acids formed (Table 2, Expt. *b*). This increased rate may be due to a direct effect of albumin on the activity of acetyl-CoA carboxylase or to the removal of unesterified fatty acids synthesized which may inhibit this enzyme.

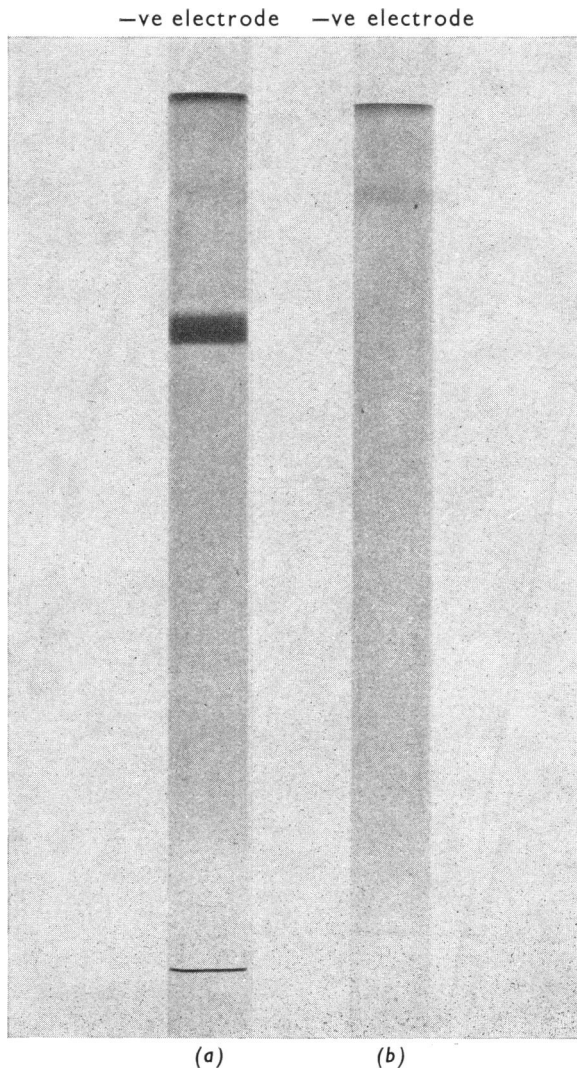
When 15 μ g of the three preparations of purified hydrolase (specific activities 305, 1130 and 2010 nmol/min per mg of protein respectively) was added in the

absence of albumin, there was a consistent increase in the proportion of $\text{C}_{4:0}$ acid synthesized (Table 2, Expts. *a*, *c*, *e* and *k*). With 30 and 45 μ g of the purified hydrolase of specific activity 1130 nmol/min per mg of protein, the proportion of $\text{C}_{4:0}$ acid formed increased further until it was the predominant product (Table 2, Expts. *a*, *e*, *g* and *i*). These effects probably reflect the decreased rates of fatty acid synthesis observed.

In the presence of 15 μ g of the three preparations of the hydrolase with no added albumin, there was a decrease in the proportion of long-chain ($\text{C}_{14:0}$ and $\text{C}_{16:0}$) fatty acids synthesized and an increase in the proportion of medium-chain ($\text{C}_{8:0}$ and $\text{C}_{10:0}$) acids formed (Table 2, Expts. *a*, *c*, *e* and *k*). These changes were related to the units of hydrolase activity added.

The most striking changes were observed when albumin was present in the incubation mixture. When 15 μ g of the three preparations of purified hydrolase was added, the increase in the proportion of $\text{C}_{4:0}$ acid synthesized was much less marked (Table 2, Expts. *b*, *d*, *h* and *l*). This reflected the much higher rates of fatty acid synthesis in the presence of albumin compared with those in its absence. The proportions of medium-chain fatty acids synthesized increased with the units of hydrolase activity added and there was a corresponding decrease in the proportions of long-chain fatty acids formed (Table 2, Expts. *b*, *d*, *f* and *l*). Chain termination at $\text{C}_{8:0}$ and $\text{C}_{10:0}$ acids also occurred when 30 μ g of the purified hydrolase of specific activity 1130 nmol/min per mg of protein was added (Expt. *h*). However, when the amount of enzyme preparation was increased to 45 μ g, there was a decrease in the proportion of medium-chain acids formed. $\text{C}_{4:0}$ acid again became the major product, even though the rate of fatty acid synthesis did not decrease substantially (Expt. *j*).

The mol.wt. of fatty acid synthetase purified from lactating-rabbit mammary gland is 9.1×10^5 (Carey & Dils, 1970*a*), whereas that of the purified hydrolase is 29000. These values have been used to gain some insight into the relative proportions (on a protein basis) of the two enzymes required for chain termination at $\text{C}_{8:0}$ and $\text{C}_{10:0}$ acids in the presence of albumin. When



EXPLANATION OF PLATE I

Purity of medium-chain acyl-thioester hydrolase by the criterion of analytical polyacrylamide-gel electrophoresis

(a) Samples of the enzyme (6 μ g of protein) were analysed by polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate on 10% (w/v) gels (Weber & Osborn, 1969). Three independent preparations of the enzyme showed the same degree of homogeneity. (b) Control experiments carried out in the absence of enzyme showed a minor band which migrated at about half the rate of that shown by the enzyme.

Table 2. Effects of purified medium-chain acyl-thioester hydrolase on the proportions of fatty acids synthesized by fatty acid synthetase

The incubation system is described in the Materials and Methods section (under 'Assay of medium-chain acyl-thioester hydrolase activity by chain-length termination in fatty acid synthesis'). The incubations contained fatty acid synthetase purified from lactating-rabbit mammary gland (218 μg of specific activity 1.16 μmol of NADPH oxidized/min per mg of protein) and acetyl-CoA carboxylase purified from lactating-rabbit mammary gland (10.5 μg of specific activity 520 nmol of malonyl-CoA formed/min per mg of protein). The three preparations of purified medium-chain acyl-thioester hydrolase (abbreviated to 'hydrolase' in the Table) and albumin (260 μg) were added as shown. Their specific activities are given as nmol of dodecanoyl-CoA hydrolysed/min per mg of protein at a substrate concentration of 56 μM in the spectrophotometric assay. The rates of incorporation are mean values \pm half the difference between duplicate incubations.

| Expt. | Addition(s) | Percentage distribution (mol/100mol) of radio-activity in fatty acids | | | | | | | Total acetate incorporated from [^{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} | |
| (a) | None | 38 | 12 | 5 | 3 | 6 | 30 | 6 | 27.7 \pm 1.6 |
| (b) | Albumin | 18 | 6 | 0 | 2 | 6 | 55 | 13 | 44.0 \pm 0.8 |
| (c) | Hydrolase (15 μg ; specific activity 305) | 64 | 11 | 6 | 8 | 5 | 6 | 0 | 16.4 \pm 1.6 |
| (d) | Hydrolase (15 μg ; specific activity 305) plus albumin | 33 | 7 | 7 | 16 | 13 | 21 | 3 | 35.9 \pm 0.9 |
| (e) | Hydrolase (15 μg ; specific activity 1130) | 62 | 11 | 9 | 12 | 4 | 2 | 0 | 18.9 \pm 0.1 |
| (f) | Hydrolase (15 μg ; specific activity 1130) plus albumin | 16 | 4 | 17 | 36 | 17 | 8 | 2 | 43.0 \pm 1.3 |
| (g) | Hydrolase (30 μg ; specific activity 1130) | 78 | 12 | 6 | 4 | 0 | 0 | 0 | 11.0 \pm 0.3 |
| (h) | Hydrolase (30 μg ; specific activity 1130) plus albumin | 29 | 9 | 26 | 28 | 6 | 2 | 0 | 37.8 \pm 3.4 |
| (i) | Hydrolase (45 μg ; specific activity 1130) | 89 | 9 | 1 | 1 | 0 | 0 | 0 | 6.4 \pm 0.1 |
| (j) | Hydrolase (45 μg ; specific activity 1130) plus albumin | 53 | 12 | 23 | 11 | 1 | 0 | 0 | 29.5 \pm 0.3 |
| (k) | Hydrolase (15 μg ; specific activity 2010) | 59 | 18 | 10 | 11 | 2 | 0 | 0 | 9.8 \pm 2.0 |
| (l) | Hydrolase (15 μg ; specific activity 2010) plus albumin | 26 | 10 | 20 | 30 | 10 | 3 | 1 | 32.2 \pm 4.4 |

the molar ratio of synthetase to hydrolase was maintained at 1:0.5, chain termination was greatest when the two hydrolase preparations with the highest specific activities were used (Table 2, Expts. *d*, *f* and *l*). When increasing amounts of hydrolase of specific activity 1130 nmol/min per mg of protein were added, the maximum proportion (54 mol%; Expt. *h*) of medium-chain fatty acids synthesized was observed at a molar ratio of synthetase to hydrolase of 1:1 (Expts. *f*, *h* and *j*).

The effects of albumin and the chain termination by the hydrolase shown in Table 2 have been reproduced in other experiments with these and other preparations of the hydrolase.

Properties of the purified enzyme with acyl-CoA esters as model substrates

Acyl-CoA esters were then used as model substrates for the purified enzyme so as to compare its properties with those of long-chain acyl-CoA hydrolases in other tissues.

Substrate specificity. The critical micellar concentrations of acyl-CoA esters of different chain

lengths are not known for the incubation conditions used to assay the purified enzyme. Hence meaningful values for K_m and V_{max} could not be obtained in these experiments. To avoid this problem of critical micellar concentrations, the specificity of the purified enzyme in the absence of albumin towards acyl-CoA esters of different chain lengths was measured at low substrate concentrations, i.e. 8 μM for acyl-CoA esters of chain lengths C_{4:0}–C_{12:0}, and 3 μM for acyl-CoA esters of chain lengths C_{8:0}–C_{16:0} (Fig. 1).

At both 3 and 8 μM substrate, the rate of hydrolysis was greatest with dodecanoyl-CoA. The enzyme did not hydrolyse butyryl-CoA or hexanoyl-CoA at measurable rates. The rates of hydrolysis of octanoyl-CoA and of decanoyl-CoA were about 5 and 50% respectively of that of dodecanoyl-CoA with all three enzyme preparations. Tetradecanoyl-CoA and hexadecanoyl-CoA were hydrolysed at rates about 60% and 55% respectively of that of dodecanoyl-CoA when the enzyme with the highest specific activity was used (preparation c, Fig. 1*a*). There was little difference in the rate of hydrolysis of fatty acyl-CoA esters of chain lengths C_{12:0}–C_{16:0} when the enzyme with the lowest specific activity was used (preparation b,

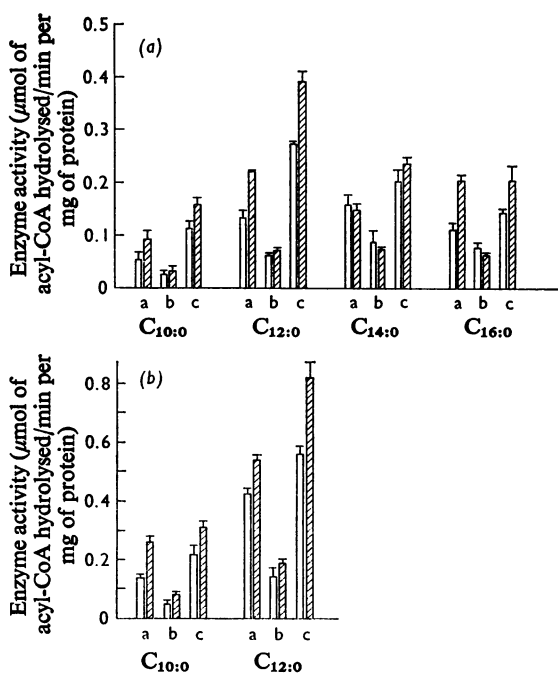


Fig. 1. Specificity of purified medium-chain acyl-thioester hydrolase towards acyl-CoA esters as model substrates

The radiochemical assay used to determine the substrate specificity is described in the Materials and Methods section. In (a) and (b) the substrate concentrations were 3 and 8 μM respectively. Three purified enzyme preparations were used. Preparations a, b and c had, respectively, specific activities of 1130, 305 and 2010 nmol of dodecanoyl-CoA hydrolysed/min per mg of protein as determined by the spectrophotometric assay with 56 μM substrate (see the Materials and Methods section). The shaded columns are enzyme activity in the presence of 30 μg of albumin/ml of incubation mixture. The open columns are enzyme activity in the absence of albumin. The error bars are the standard deviations of triplicate incubations.

Fig. 1a). The enzyme was unable to hydrolyse acyl-carnitine esters of chain lengths $\text{C}_{4:0}$ – $\text{C}_{16:0}$ at concentrations of 3 and 10 μM .

Properties of the enzyme with dodecanoyl-CoA as model substrate

Since the purified enzyme showed the highest rate of hydrolysis with dodecanoyl-CoA, this was used as the substrate in the following experiments.

pH-activity curve. The enzyme showed the highest activity at pH 7.0–7.5 towards 8 μM -[1- ^{14}C]dodecanoyl-CoA in 0.1 M-potassium phosphate buffer containing 1 mM-EDTA and 1 mM-dithiothreitol.

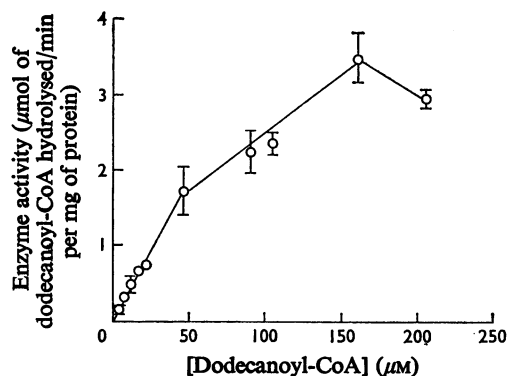


Fig. 2. Effect of substrate concentration on the hydrolysis of dodecanoyl-CoA by medium-chain acyl-thioester hydrolase

The radiochemical assay used is described in the Materials and Methods section. The substrate concentration was varied as shown. The error bars represent the standard deviation of triplicate incubations. Similar results were obtained with all three preparations of the purified enzyme.

Substrate concentration. The rate of hydrolysis of dodecanoyl-CoA increased linearly with substrate concentration up to 45 μM (Fig. 2). The rate decreased at higher concentrations, and a slight inhibition was observed at 212 μM substrate, though it was difficult to obtain reproducible results at high substrate concentrations. This may be due to the differing extent of micelle formation even though the substrate was preincubated with buffer for 5 min before the enzyme was added.

Effects of albumin. Fatty acid-poor albumin increased the rate of hydrolysis of dodecanoyl-CoA by the two preparations of the purified enzyme of high specific activity (Fig. 3). The maximum increase occurred with 30 μg of albumin/ml of incubation mixture; higher concentrations decreased the rate, which may be due to the binding of substrate. This optimum concentration of albumin was found to increase the rate of hydrolysis of all fatty acyl-CoA esters of chain lengths $\text{C}_{8:0}$ – $\text{C}_{16:0}$ (except for the $\text{C}_{14:0}$ ester) when the two enzyme preparations of highest specific activity were used (Fig. 1, preparations a and c). However, it did not affect the overall pattern of chain-length specificity.

This stimulatory effect of albumin could have been due to the removal of inhibitory products. Increasing concentrations of unesterified dodecanoic acid were therefore added to the incubation system described in the legend to Fig. 3, except that albumin was omitted from the system containing 8 μM -dodecanoyl-CoA.

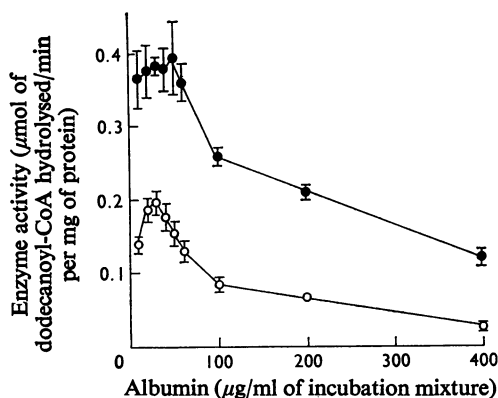


Fig. 3. Effect of albumin on the activity of purified medium-chain acyl-thioester hydrolase towards dodecanoyl-CoA

The radiochemical assay used is described in the Materials and Methods section. Fatty acid-poor albumin was added as shown. The substrate was $3\ \mu\text{M}$ -dodecanoyl-CoA (\circ) or $8\ \mu\text{M}$ -dodecanoyl-CoA (\bullet). The errors bars represent the standard deviation of triplicate incubations. The purified enzyme preparation had a specific activity of $1130\ \text{nmol}$ of dodecanoyl-CoA hydrolysed/min per mg of protein as determined by the spectrophotometric assay with $56\ \mu\text{M}$ substrate (see the Materials and Methods section). Similar results were obtained by using the purified enzyme preparation of specific activity $2010\ \text{nmol}$ of dodecanoyl-CoA hydrolysed/min per mg of protein.

No decrease in the rate of hydrolysis of dodecanoyl-CoA was observed even when $3\ \text{mM}$ -dodecanoic acid was added. This indicates that the effect of albumin is unlikely to be due to the removal of inhibitory products.

Discussion

Purified fatty acid synthetase from lactating-rabbit mammary gland synthesizes $\text{C}_{4:0}$ and $\text{C}_{16:0}$ fatty acids in the presence of optimum concentrations of acetyl-CoA and malonyl-CoA (Carey & Dils, 1970b). With the assay system described in Table 2, there is a constant and rate-limiting generation of malonyl-CoA. This will limit the probability of chain elongation according to the model of Sumper *et al.* (1969). If the rate of elongation is decreased sufficiently, $\text{C}_{14:0}$ should be the predominant fatty acid synthesized according to this model. This is because the rate of hydrolysis of shorter-chain fatty acids by the acyl-thioester hydrolase of the synthetase is low compared with the rate of elongation. The results in Table 2 show that purified fatty acid synthetase from rabbit mammary gland behaves according to this model. It can also be predicted from the model that medium-chain fatty acids would be synthesized if medium-chain acyl-thioester hydrolase activity was added,

which could specifically release these acids from the synthetase.

Preliminary evidence for the occurrence of this chain-terminating enzyme in the cytosol of lactating-rabbit mammary gland (Knudsen *et al.*, 1975) has now been confirmed and the enzyme has been purified. When added to a system which is synthesizing short- and long-chain fatty acids from rate-limiting amounts of malonyl-CoA the behaviour predicted from the model of Sumper *et al.* (1969) is observed. That the decrease in the chain length of the synthesized fatty acids is related to the amount of medium-chain acyl-thioester hydrolase activity added can be seen from the results given in Table 2. The purified medium-chain acyl-thioester hydrolase must therefore be able to use as substrates medium-chain acyl residues which are bound to synthetase, probably as acyl-carrier protein derivatives.

It is not known whether the effects of albumin on chain termination (Table 2) are due to changes in protein concentration or to the removal of unesterified fatty acids synthesized, which are likely to inhibit acetyl-CoA carboxylase.

The results presented here may well explain the synthesis of $\text{C}_{8:0}$ and $\text{C}_{10:0}$ fatty acids by lactating-rabbit mammary gland *in vivo* (Carey & Dils, 1972) and *in vitro* (Strong & Dils, 1972). The conditions required for the purified hydrolase to function in chain termination are the generation of low and rate-limiting amounts of malonyl-CoA. Acetyl-CoA carboxylase is thought to be the rate-limiting enzyme of fatty acid biosynthesis *de novo* in a number of tissues [see Numa (1974) for review]. Smith & Abraham (1970) have found the activity of fatty acid synthetase to be 2.5 times that of acetyl-CoA carboxylase in extracts of lactating-rat mammary gland when the enzymes were assayed under optimum conditions. This ratio is 4.35 in the microsomal plus particle-free supernatant fraction of homogenates of lactating-rabbit mammary gland (Smith *et al.*, 1966). Conditions favourable for the medium-chain acyl-thioester hydrolase to function in chain termination could therefore occur in lactating mammary gland *in vivo*.

Comparison with long-chain acyl-CoA hydrolases

Molecular weight. The mol.wt. of long-chain acyl-CoA hydrolase isolated from seven tissues from the rat was 50000 as determined by sucrose-density-gradient centrifugation. However, the mol.wt. of the enzyme from rat heart, spleen and blood was about 100000 as estimated by Sephadex G-100 gel filtration (Kurooka *et al.*, 1972). Long-chain acyl-CoA hydrolase from bovine brain showed two peaks of activity when chromatographed on Sephadex G-200 at 2°C . This is due to the dissociation of a dimer of mol.wt. 96000 into monomers of mol.wt. 46000

(Anderson & Erwin, 1971). This formation of a dimer could explain the mol.wt. of 100000 found for the enzyme in rat heart, spleen and blood by gel filtration.

The mol.wt. of purified medium-chain acyl-thioester hydrolase from lactating-rabbit mammary gland (29000) is similar to that of the subunits (30000) of the higher-mol.wt. form (90000–117000) of long-chain acyl-CoA hydrolase from *E. coli* (Barnes *et al.*, 1970; Bonner & Bloch, 1972). This latter enzyme hydrolyses palmitoyl-acyl-carrier protein and might therefore be involved in the synthesis of long-chain fatty acids. The mol.wt. of the medium-chain acyl-thioester hydrolase is somewhat higher than that of a second long-chain acyl-CoA hydrolase from *E. coli* (22000). It has been shown that this second hydrolase from *E. coli* participates in chain-length termination leading to the synthesis of long-chain unesterified fatty acids (Barnes & Wakil, 1968). The most interesting similarity is between the mol.wt. of the medium-chain acyl-thioester hydrolase and the long-chain acyl-thioester hydrolase (32000), which can be cleaved from fatty acid synthetase purified from lactating-rat mammary gland by proteolytic hydrolysis (Smith *et al.*, 1976; Agradi *et al.*, 1976).

Substrate specificity

The substrate specificity of purified medium-chain acyl-thioester hydrolase from rabbit mammary gland towards acyl-CoA esters as model substrates determined at low substrate concentrations showed that the enzyme has the highest activity towards dodecanoyl-CoA (Fig. 1), but substantial activity towards decanoyl-CoA. This specificity differs from that of the low-molecular-weight enzyme from *E. coli*, which shows maximum activity towards tetradecanoyl-CoA and hexadecanoyl-CoA but very low activity towards dodecanoyl-CoA and shorter-chain acyl-CoA esters (Barnes & Wakil, 1968). The high-molecular-weight enzyme from *E. coli* shows increasing hydrolytic activity with increasing chain length of acyl-CoA esters, with maximum activity towards hexadecanoyl-CoA and octadecanoyl-CoA (Barnes *et al.*, 1970). The mammalian enzymes show a wide specificity towards medium- and long-chain acyl-CoA esters, which varies with the tissue from which they were isolated (Anderson & Erwin, 1971; Kurooka *et al.*, 1972). However, comparison with mammalian enzymes is difficult, since their substrate specificities have been determined by estimating K_m and V_{max} values which are doubtful value, owing to micellar formation by the substrates.

Effects of albumin

The effect of albumin in increasing the rate of hydrolysis of acyl-CoA esters by the high-specific-activity preparations of medium-chain acyl-thioester

hydrolase (Fig. 1) is unlikely to be due to the removal of inhibitory unesterified fatty acids formed during hydrolysis. High concentrations of dodecanoic acid did not affect the activity of the enzyme when albumin was omitted from the incubation system. Though Barnes & Wakil (1968) found that albumin increased the activity of the low-molecular-weight enzyme from *E. coli*, they used a high concentration (14.4 μM) of hexadecanoyl-CoA as substrate. The albumin could therefore have prevented the inhibition of the enzyme by substrate in the micellar form. This is unlikely to be the explanation here, since the increase in activity was more pronounced at the lower substrate concentration (Fig. 3). The lack of effect of albumin when the purified enzyme of low specific activity was used could be due to the high concentration (4 $\mu\text{g}/\text{ml}$) of enzyme protein used compared with that when the high-specific-activity enzyme was used (1 $\mu\text{g}/\text{ml}$). If so, this would indicate a protein-concentration effect.

It is not clear whether the effect of albumin in the chain-termination assay (Table 2) is related to its effects on hydrolase activity when acyl-CoA esters are used as model substrates (Figs. 1 and 3).

Summary

In summary, the presence of medium-chain acyl-thioester hydrolase activity in extracts of lactating-rabbit mammary gland explains, at least in part, previous reports (Carey & Dils, 1973*a,b*; Strong *et al.*, 1973; Knudsen, 1976) which indicated an unidentified factor(s) in these extracts which controlled chain termination.

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