Utilization of Methylmalonate for the Synthesis of Branched-Chain Fatty Acids by Preparations of Chicken Liver and Sheep Adipose Tissue

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1. The utilization of methyl[2-¹⁴C]malonyl-CoA for fatty acid synthesis was investigated using synthetase preparations from chicken liver and sheep adipose tissue. 2. The rate of fatty acid synthesis from acetyl-CoA and malonyl-CoA was greatly diminished in the presence of methylmalonyl-CoA. 3. In the absence of malonyl-CoA, methylmalonyl-CoA was utilized for fatty acid synthesis only very slowly by the synthetase from sheep adipose tissue and not at all by that from chicken liver. 4. Despite the inhibitory effect of methylmalonyl-CoA on fatty acid synthesis from malonyl-CoA, it was utilized by the synthetase preparations from both species to produce a complex mixture of methyl-branched fatty acids.

Abnormally high proportions of a variety of methyl-branched fatty acids and odd-numbered straight-chain fatty acids are present in the adipose-tissue triacylglycerols of young sheep and goats when they are fed on diets based on cereals such as barley, maize or wheat (Garton *et al.*, 1972; Duncan *et al.*, 1974, 1976). The occurrence of these fatty acids is associated with the assimilation of unusual amounts of propionate that are produced in the rumen as a result of bacterial fermentation of cereal starch (Ørskov *et al.*, 1974). Under such dietary circumstances, the capacity of the animals to metabolize propionate via methylmalonyl-CoA to succinyl-CoA is apparently exceeded, leading to the accumulation of propionyl-CoA and methylmalonyl-CoA.

Propionyl-CoA is the known primer unit for the biosynthesis of odd-numbered straight-chain fatty acids (Horning *et al.*, 1961), and it was postulated (Garton *et al.*, 1972; Duncan *et al.*, 1974) that incorporation of methylmalonyl-CoA (in place of malonyl-CoA) into fatty acids synthesized from acetyl-CoA or propionyl-CoA as primer unit could account for the formation of mono-, di- and trimethyl-branched fatty acids.

Studies by Feller (1958) suggested that methylmalonate could be incorporated into fatty acids by mouse adipose tissue, though the possibility that it is first converted to propionate was not excluded. Later it was reported by Cardinale *et al.* (1970) that methylmalonyl-CoA could apparently be utilized for fatty acid synthesis by rat liver preparations, and Buckner *et al.* (1978) have demonstrated that two polymethyl-substituted fatty acids were produced

from methylmalonyl-CoA by the fatty acid synthetase of goose uropygial gland.

In the present paper we describe the synthesis of branched-chain fatty acids from methylmalonyl-CoA by fatty acid synthetase from chicken liver and from adipose tissue of sheep. A preliminary account of some of the findings has been published (Scaife & Garton, 1975).

Materials and Methods

Materials

Chemicals. Methyl[2-¹⁴C]malonyl-CoA (specific radioactivity 8.6 Ci/mol) and [2-¹⁴C]malonyl-CoA (specific radioactivity 10.0 Ci/mol) were purchased from New England Nuclear, Dreieichenhain, West Germany, and [1-¹⁴C]palmitic acid and [1-¹⁴C]stearic acid from The Radiochemical Centre, Amersham, Bucks., U.K. Malonyl-CoA was synthesized by the method of Trams & Brady (1960), and acetyl-CoA was prepared as described by Simon & Shemin (1953). NADPH, avidin, bovine serum albumin (fraction V), glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Methyl 2-methyltridecanoate and methyl 2-methylheptadecanoate were kindly made available by our colleague, Dr A. Smith. All other fatty acids and reagents were of the purest grade obtainable commercially.

Animal tissues. Livers, obtained immediately *post mortem* from chickens killed at a commercial processing factory (Ross Poultry Ltd., Aberdeen, Scotland, U.K.), were put on crushed ice for transport to the laboratory. Pieces (each about 10 g) of inguinal

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adipose tissue were removed, at slaughter, from several 1-year-old female sheep which had been reared on a diet of grass or on one which contained 900 g/kg of rolled barley together with white-fish meal and appropriate amounts of minerals and vitamins; the tissue samples were taken to the laboratory in a polythene bag immersed in water at 37°C.

Methods

Preparation of crude fatty acid synthetase. About 50 g of liver or adipose tissue was taken, cut into small pieces and placed in 100 ml of 50 mM-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose; for liver, the buffer temperature was 0°C and, for adipose tissue, 37°C. Homogenates were prepared in an Ultra-Turrax high-speed homogenizer. The adipose-tissue homogenate was strained as quickly as possible through a double layer of surgical gauze to remove large particles and then it was centrifuged at 0°C for 10 min at 2000g, after which the fat at the tops of the centrifuge tubes was readily removed. The liver homogenate and the fat-free adipose-tissue homogenate were each centrifuged at 130 000g for 60 min at 0°C to yield particle-free supernatant solutions. $(\text{NH}_4)_2\text{SO}_4$ was then added to give 20% saturation of the supernatant, which was centrifuged at 18 000g for 20 min at 0°C. The precipitate was discarded and the supernatant again treated with $(\text{NH}_4)_2\text{SO}_4$ to give 40% saturation. After centrifuging at 18 000g for 20 min at 0°C, the particle-free supernatant was removed and the protein pellet was suspended in 5 ml of 0.1 M-potassium phosphate buffer, pH 7.4. This suspension constituted the crude partially purified fatty acid synthetase from each tissue.

Assay of fatty acid synthetase. The fatty acid synthetase activity in the particle-free supernatant and in the crude enzyme preparation was determined spectrophotometrically by following the oxidation of NADPH in an incubation system which contained potassium phosphate buffer, pH 7.4 (60 μmol), NADPH (0.5 μmol), dithioerythritol (10 μmol), acetyl CoA (0.07 μmol), malonyl-CoA (0.1 μmol) and approx. 1 mg of crude enzyme protein in a final volume of 1.5 ml; various concentrations (0.004–0.12 μmol) of unlabelled methylmalonyl-CoA were used in studies on its inhibitory effect on fatty acid synthesis. The spectrophotometer was a double-beam instrument (Pye–Unicam model SP. 1800) and the cell housing was heated to 36°C. The protein content of the crude enzyme preparations was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (fraction V) as standard.

Incubation of methylmalonyl-CoA with fatty acid synthetase. To each of five tubes containing crude fatty acid synthetase [0.5 ml of the suspension (about 13 mg of protein) in phosphate buffer] was added NADPH

(0.5 μmol), glucose 6-phosphate (10 μmol), glucose 6-phosphate dehydrogenase (5 units), malonyl-CoA (0.10 μmol) or $[2\text{-}^{14}\text{C}]\text{malonyl-CoA}$ (0.10 μmol , 1.0 μCi), acetyl-CoA (0.07 μmol), methyl $[2\text{-}^{14}\text{C}]\text{malonyl-CoA}$ (0.12 μmol , 1.0 μCi) and avidin (2 units). The total volume was adjusted to 1.5 ml by the addition of 0.1 M-potassium phosphate buffer, and the tubes were incubated for 1 h at 37°C in a shaking water bath.

Analysis of the products of synthesis. After the five tubes had been incubated for 1 h, lauric acid (2 mg in solution in ethanol) was added as carrier to each tube, followed by an excess of ethanolic 0.5 M-KOH. The lipids were allowed to saponify overnight at 40°C and then the contents of the tubes were pooled, diluted with 3 vol. of water and acidified dropwise with 5 M- H_2SO_4 . The fatty acids were extracted into n-hexane and the solutions evaporated to dryness. The acids were then converted into their methyl esters with an ethereal solution of diazomethane, the excess of which was removed by distillation. The methyl esters were subjected to radio-g.l.c. in a Panax radio-gas detector system in conjunction with a Pye–Unicam gas chromatograph [model 104, operated at 180°C with carrier gas (argon containing 5% of CO_2) flow rate of 55 ml/min]; the columns [215 cm (7 ft)] contained either 15% (w/w) Apiezon L grease or 15% (w/w) polymerized ethylene glycol adipate supported on Chromosorb W. A stream-splitter permitted 90% of the emergent gas to pass to the combustion unit of the radio-gas detector. To give increased mass and to act as indicators of retention times mixtures containing several methyl esters of even-numbered and odd-numbered straight-chain fatty acids in the range $\text{C}_{12:0}$ – $\text{C}_{19:0}$ were added to the products of synthesis before g.l.c. In addition, a mixture of methyl palmitate and methyl stearate containing methyl $[1\text{-}^{14}\text{C}]\text{palmitate}$ and methyl $[1\text{-}^{14}\text{C}]\text{stearate}$ was chromatographed to establish that the retention time of a given peak of radioactivity corresponded to that of its mass peak.

After the analysis by radio-g.l.c. of the methyl esters of the total fatty acids from each set of five incubation tubes, the branched-chain components in the mixture were separated from their straight-chain counterparts by the method of Ackman & Hooper (1968). To this end, 3 mg each of methyl 2-methyltridecanoate and methyl 2-methylheptadecanoate were added as carriers, and the mixture, in solution in methanol, was treated with urea, which removed most of the straight-chain esters as crystalline clathrates. The residual branched-chain components, derived from methyl $[2\text{-}^{14}\text{C}]\text{malonyl-CoA}$, contained more than 90% of the added ^{14}C radioactivity; in contrast, almost all of the ^{14}C radioactivity in the fatty acids derived from $[2\text{-}^{14}\text{C}]\text{malonyl-CoA}$ was present in the crystalline clathrates. Preparative t.l.c. on plates of Kieselgel G, with

diethyl ether/light petroleum (b.p. 40–60°C) (3:100, v/v) as developing solvent, was used to purify each concentrate of branched-chain esters. These concentrates were then analysed by radio-g.l.c. as described above for the methyl esters of the total fatty acids.

Results and Discussion

As determined spectrophotometrically, the fatty acid synthetase activity of the particle-free supernatant prepared from the adipose tissue of barley-fed sheep was about 10-fold greater than that of the corresponding preparation from the adipose tissue of grass-fed sheep (Fig. 1). This difference is illustrated by the examples given in Fig. 1. Enhanced synthetase activity in the particle-free supernatant from barley-fed sheep could be a response to the ready availability of glucose (as a result of gluconeogenesis from propionate), or it could be in part a compensatory phenomenon associated with inhibition of the fatty acid synthetase system by methylmalonyl-CoA (cf. Frenkel *et al.*, 1974). As the double-reciprocal plots

show (Fig. 1), the rate of fatty acid synthesis was markedly affected, regardless of whether the synthetase originated from the adipose tissue of a barley-fed sheep or a grass-fed sheep. Classical competitive inhibition occurred towards the synthetase in the particle-free supernatant from the adipose tissue of the grass-fed sheep, and a similar inhibitory effect was observed towards the synthetase in the particle-free supernatant from barley-fed sheep, except when the concentrations of methylmalonyl-CoA relative to that of malonyl-CoA were very high. In addition to inhibiting synthetase activity in chicken liver particle-free supernatant, methylmalonyl-CoA also inhibited fatty acid synthesis from malonyl-CoA by the crude synthetase preparations from both chicken liver and sheep adipose tissue.

Methylmalonyl-CoA has also been shown to be a competitive inhibitor of the fatty acid synthetase of rat brain and liver (Forward & Gompertz, 1970) and goose uropygial gland (Buckner *et al.*, 1978). Nevertheless it can be incorporated into newly synthesized fatty acids, thereby giving rise to branched chains. When the synthetase preparation from chicken liver

Fig. 1. Lineweaver-Burk plots showing the effect of methylmalonyl-CoA on fatty acid synthesis by particle-free supernatant from the adipose tissue of sheep

Conditions are detailed under 'Methods'. Particle-free supernatant was prepared from adipose tissue of (a) a barley-fed sheep and (b) a grass-fed sheep; v is expressed as nmol of NADPH oxidized/min per mg of protein; s is concentration (μM) of malonyl-CoA. The particle-free supernatant was prepared from adipose tissue of (a) a barley-fed sheep and (b) a grass-fed sheep: $\bullet$, without methylmalonyl-CoA; $\square$, with 20 μM -methylmalonyl-CoA; $\blacksquare$, with 40 μM -methylmalonyl-CoA.

or sheep adipose tissue was incubated with methyl-[2- 14 C]malonyl-CoA in the presence of unlabelled malonyl-CoA, 14 C radioactivity was found in fatty acids. The labelling pattern in the total fatty acids that resulted from the incubations is shown in the

radio-gas chromatograms that are reproduced in Figs. 2(a) and 2(b); for comparison the 14 C radioactivity in the fatty acids derived from [2- 14 C]malonyl-CoA is shown in Fig. 2(c). Whereas the products of synthesis from [2- 14 C]malonyl-CoA

Fig. 3. Radio-gas chromatograms of the methyl esters of the branched-chain components isolated from the total fatty acids after the incubation of methyl[2- 14 C]malonyl-CoA with crude fatty acid synthetase from (a) sheep adipose tissue and (b) chicken liver

For details see the legend to Fig. 2. The following carrier and/or indicator fatty acids were added, as methyl esters, to the samples before chromatography: C_{14:br} (methyl 2-methyltridecanoate), C_{15:0}, C_{17:0} and C_{18:br} (methyl 2-methylheptadecanoate).

Fig. 2. Radio-gas chromatograms of the methyl esters of the fatty acids produced when methyl[2- 14 C]malonyl-CoA was incubated with crude fatty acid synthetase from (a) sheep adipose tissue and (b) chicken liver and when (c) [2- 14 C]malonyl-CoA was incubated with the synthetase from sheep adipose tissue

The lower line of each chromatogram represents mass response and the upper (more jagged) line represents the simultaneous response to 14 C radioactivity. Changes made in the sensitivity to mass are shown by arrows and changes made in the number of 14 C c.p.s. are similarly indicated. The following carrier and/or indicator fatty acids were added, as methyl esters, to the samples before chromatography (a) C_{12:0}; (b) C_{12:0}, C_{15:0}, C_{17:0} and C_{19:0}; (c) C_{12:0}, C_{15:0} and C_{17:0}.

were exclusively even-numbered, straight-chain fatty acids (mostly $C_{14:0}$ and $C_{16:0}$), the fatty acids derived from methyl[2- ^{14}C]malonyl-CoA were a complex mixture, the peaks of radioactivity of which did not correspond to the mass peaks of the methyl esters of any straight-chain fatty acid having an even or an odd number of carbon atoms in its chain, irrespective of whether the phase used for g.l.c. was Apiezon L or ethylene glycol adipate. For illustrative purposes, the chromatograms reproduced in Fig. 2 and Fig. 3 all refer to separations effected on ethylene glycol adipate. It is apparent from Figs. 2(a) and 2(b) that the peaks of radioactivity probably represented branched-chain components, and it is noteworthy that, regardless of whether the synthetase was of avian or ovine origin, the products of biosynthesis were essentially similar.

Confirmation that the products of synthesis were indeed branched-chain fatty acids was forthcoming when the methyl esters of the fatty acids that did not form urea clathrates were resolved by radio-g.l.c. As is shown in Figs. 3(a) and 3(b), the patterns of peaks of radioactivity were similar to those given by the methyl esters of the corresponding total fatty acids (Figs. 2a and 2b), though some loss of short-chain components evidently occurred during the manipulations involved in the preparation of the concentrates. As expected, treatment with urea of the methyl esters of the total fatty acids that resulted from the incubation of [2- ^{14}C]malonyl-CoA with avian or ovine synthetase preparations removed all the ^{14}C -labelled fatty acids. The radio-gas chromatograms in Fig. 3 show that the methyl esters of the branched-chain acids can be considered as falling into three main groups: (1) short-chain components with retention times less than that of the carrier, methyl 2-methyltridecanoate; (2) components with retention times intermediate between those of the two carrier methyl esters; (3) another group of components with retention times greater than that of the second carrier methyl 2-methylheptadecanoate. The acids represented in group (2) probably consisted, for the most part, of components containing a total of 15 and 17 carbon atoms (i.e. isomeric methyl-substituted tetradecanoic and hexadecanoic acids respectively). In this context it is notable that such acids are among the major constituents of the branched-chain acids that occur in the adipose-tissue triacylglycerols of barley-fed sheep (Duncan *et al.*, 1974) and that were identified by capillary-column g.l.c. combined with mass spectrometry.

As mentioned in the introduction to this paper, Buckner *et al.* (1978) have reported that *in vivo* the

fatty acid synthetase of goose uropygial gland is responsible for the production of two polymethyl-substituted fatty acids. These acids, 2,4,6,8-tetramethyldecanoic acid and 2,4,6,8,10-pentamethyl-dodecanoic acid, were shown to arise as a result of repeated condensations of methylmalonyl-CoA starting with acetyl-CoA as primer unit; virtually no malonyl-CoA is available because the gland is unique in containing a highly active substrate-specific enzyme which decarboxylates malonyl-CoA. However, *in vitro*, the synthetase from goose uropygial gland could utilize malonyl-CoA for fatty acid synthesis and, in the presence of methylmalonyl-CoA, branched-chain fatty acids were formed; these acids were probably similar in structure to those that we have shown to be produced by the synthetase of chicken liver and sheep adipose tissue.

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