Metabolism of pent-4-enoate in rat heart

Reduction of the double bond

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(Received 24 June 1980/Accepted 18 September 1980)

1. Soluble extracts from rat heart and liver mitochondria were used to evaluate the early steps in the conversion of pent-4-enoyl-CoA into tricarboxylic acid-cycle intermediates. Hitherto the unresolved problem was the reduction of the double bond of pent-4-enoate. 2. Soluble extracts from heart mitochondria reduced pent-4-enoyl-CoA and penta-2,4-dienoyl-CoA in the presence of NADPH at rates (nmol/min per mg of protein) of 0.9 ± 0.1 and 132 ± 8 and from the liver mitochondria at the rates of 1.9 ± 0.2 and 52 ± 6 respectively. No reduction of acryloyl-CoA was found. 3. We show that primarily the double bond in position 4, not in position 2, of penta-2,4-dienoyl-CoA is reduced. 4. It is concluded that the principal metabolic pathway of penta-4-enoate is reduction of the double bond in position 4 after an initial oxidation to penta-2,4-dienoyl-CoA. The pent-2-enoyl-CoA thus formed can be further metabolized by the usual enzymes of β -oxidation, and by the further metabolism of propionyl-CoA to tricarboxylic acid-cycle intermediates.

The hypoglycaemic agent pent-4-enoate inhibits fatty acid oxidation under many different kinds of conditions (e.g. Senior *et al.*, 1968; Brendel *et al.*, 1969; Fukami & Williamson, 1971). Pent-4-enoate itself can be slowly metabolized through β -oxidation, forming acetyl-CoA and acryloyl-CoA as end products (Brendel *et al.*, 1969; Holland *et al.*, 1973), and the inhibition is apparently caused by some inhibitory intermediates during pent-4-enoate metabolism. We have shown, however, that in perfused rat hearts (Hiltunen, 1978) and in isolated heart mitochondria (Hiltunen *et al.*, 1980) pent-4-enoate causes a rapid increase of tricarboxylic acid-cycle intermediates, and that pent-4-enoate itself is the source of these extra carbon skeletons. The mechanism seems to be that the C_3 end products formed during the β -oxidation of pent-4-enoate are metabolized via the propionate pathway into the tricarboxylic acid cycle.

Reduction of the double bond is a prerequisite for the metabolism of pent-4-enoate by a mechanism suggested above. How and at which step of pent-4-enoate metabolism this takes place has remained unclear. We now show in experiments done with soluble extracts from heart and liver mitochondria and intermediates of pent-4-enoate metabolism that only the reduction of penta-2,4-dienoyl-CoA to pent-2-enoyl-CoA is rapid enough

to account for the rate of pent-4-enoate metabolism found in perfused heart and liver.

Materials and methods

Materials

Carnitine acetyltransferase (EC 2.3.1.7), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), CoA, oxalyl chloride, cytochrome c, 5,5'-dithio-bis-(2-nitrobenzoic acid) and nucleotides were purchased from Sigma, St. Louis, MO, U.S.A.; dichloromethane and phenazine methosulphate were from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; *trans*-pent-2-enoic acid and acryloyl chloride were from Fluka A.G., Buchs, Switzerland; pent-4-enoic acid was from Plaltz and Bauer, Stamford, CT, U.S.A.; enzyme-grade $(NH_4)_2SO_4$ was from Fisher Scientific Co., Fair Lawn, NY, U.S.A.; DEAE-Sephadex A-50 and Sephadex G-25 were from Pharmacia, Piscataway, NJ, U.S.A.; and preparative thin-layer cellulose plates (Avicel) were from Analtech, Newark, NJ, U.S.A. (–)-Carnitine was a gift from Otsuka Pharmaceutical, Tokushima, Japan.

Preparation of enzymes

Acyl-CoA dehydrogenase (octanoyl-CoA dehydrogenase, EC 1.3.99.3) was prepared from rabbit

livers essentially as described by Holland *et al.* (1973), but with omission of the final step. The enzyme was stored in 80%-satd. $(\text{NH}_4)_2\text{SO}_4$, and was used to prepare penta-2,4-dienoyl-CoA (see below). Enoyl-CoA hydratase (EC 4.2.1.17) was purified from ox liver as described by Stern (1955).

Determination of concentrations of acyl-CoA esters

Concentrations of pent-2-enoyl-CoA and penta-2,4-dienoyl-CoA were determined with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase at pH 9.5 as described by Decker (1963). Acryloyl-CoA and pent-4-enoyl-CoA were determined with 5,5'-dithiobis-(2-nitrobenzoic acid) and carnitine acetyltransferase in the presence of excess carnitine (Holland *et al.*, 1973; Chase & Tubbs, 1966).

Preparation of acyl-CoA esters

The acid chlorides of the corresponding acids were prepared by the method of Borrebaek *et al.* (1980). The acid chlorides were further purified by fractional distillation: the fraction of b.p. 115–128°C for pent-4-enoyl chloride (Holland & Sherratt, 1973) and that of b.p. 120–138°C for pent-2-enoyl chloride. The CoA esters were then prepared at 0°C as described by Seubert (1960). Thiol groups were protected by bubbling a stream of N_2 through the solutions during the reaction. Removal of thiol groups that had not reacted was followed as described by Holland *et al.* (1973). When all free thiol groups had disappeared the pH was adjusted to 2 with HCl, and free carboxy groups were removed by repeated extraction with ether. Recoveries were 40–60% for pent-4-enoyl-CoA and pent-2-enoyl-CoA, and 25–35% for acryloyl-CoA. Pent-2- and pent-4-enoyl-CoA were further purified on preparative cellulose thin-layer plates as described by Pullman (1973). Overall recoveries from the plates were about 60%. The identities and concentrations of these esters were further documented as follows: (i) both esters bleached permanganate, showing the presence of a double bond; (ii) the concentration of adenylates was determined after alkaline hydrolysis by measuring the A_{260} , by assuming a molar absorption coefficient of $16.4 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Stadtman, 1957); the ratio so estimated of adenylate to enzymically active acyl-CoA for both esters was 0.75–0.9; (iii) pent-4-enoyl-CoA reacted in a stoichiometric manner with carnitine acetyltransferase, demonstrating the presence of the thioester bond; (iv) pent-4-enoyl-CoA reacted with acyl-CoA dehydrogenase; and (v) pent-2-enoyl-CoA was measured with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase.

It was also possible to purify acryloyl-CoA on thin-layer plates, but it was unstable on brief storage at -20°C . Therefore acryloyl-CoA was used

directly after ether extraction and neutralization without further purification.

Penta-2,4-dienoyl-CoA was prepared from purified pent-4-enoyl-CoA by oxidation, with acyl-CoA dehydrogenase and phenazine methosulphate as electron acceptor, as follows. The pH of a freshly prepared mixture containing 0.2 mM-pent-4-enoyl-CoA and 5 mM-phenazine methosulphate in water was adjusted to pH 7.2 with 0.05 M-KOH. The reaction was started with purified acyl-CoA dehydrogenase (0.02 unit/ml of incubation mixture) and carried out at room temperature in the dark. Since 2,4-dienoyl compounds absorb light at 300 nm (Wakil & Hübscher, 1960), the progress of the reaction was followed at this wavelength (about 45 min). To remove phenazine methosulphate (it will oxidize NADPH immediately) the incubation medium was poured into a test tube containing Dowex-50 (0.1 ml/ml of incubation mixture) in 0.1 M-HCl. After vigorous mixing, Dowex-50 was removed by centrifugation (1000 g for 5 min) and the same treatment was repeated twice with the supernatant fluid. The pH was adjusted to 5–6 and the penta-2,4-dienoyl-CoA was partially dehydrated to a final concentration of 0.5–0.7 mM by freeze-drying. The yield of penta-2,4-dienoyl-CoA was virtually 100%, based on measurement with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase. In control experiments, pent-4-enoyl-CoA did not react in this coupled reaction. Attempts to purify penta-2,4-dienoyl-CoA on the thin-layer plates failed, because freeze-drying of penta-2,4-dienoyl-CoA solution to dryness resulted in a yellow oily drop which did not contain an acyl-CoA ester reacting with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase.

Other methods

Heart mitochondria from Wistar rats were prepared as described by Tyler & Gonze (1967), but with omission of proteinase, and liver mitochondria as described by Johnson & Lardy (1967). Particle-free supernatant fluid of sonicated mitochondria (Branson Sonifier W185; four 20 s bursts at 100 W on the mitochondrial suspension in 50 mM-potassium phosphate, pH 7.2) was used as source of fatty acid-metabolizing enzymes. Activities of enoyl-CoA reductases were determined as described by Kunau & Dommes (1978). Protein was determined by the biuret reaction (Gornall *et al.*, 1949) in the presence of 0.3% deoxycholate.

Results and discussion

On the basis of data published during the last few years, we know that the carbon skeleton of pent-4-enoate can be metabolized into intermediates of the tricarboxylic acid cycle (Hiltunen *et al.*, 1977;

Hiltunen, 1978; Hiltunen *et al.*, 1980). Thus the double bond in position 4 of pent-4-enoate must be reduced, but we do not know the sequences of those reactions. To get information about this, we have now studied the reduction of some intermediates of β -oxidation of pent-4-enoate with soluble protein from rat heart and liver mitochondria.

Table 1 summarizes the activities of soluble enoyl-CoA reductases of rat heart and liver mitochondria on pent-4-enoyl-CoA, pent-2-enoyl-CoA, penta-2,4-dienoyl-CoA and acryloyl-CoA in the presence of NADPH. In separate experiments no reduction of these compounds was found with NADH.

We (Hiltunen, 1978; Hiltunen *et al.*, 1980) have earlier suggested that acryloyl-CoA is the site of reduction in the pathway of pent-4-enoate metabolism. Furthermore, it has been reported that an acryloyl derivative (carnitine) is formed from pent-4-enoate by pigeon liver homogenates (Brendel *et al.*, 1969), or acryloyl-CoA is found slowly from penta-2,4-dienoyl-CoA (Holland *et al.*, 1973). However, our present data show no significant oxidation of NADPH by either heart or liver extracts in the presence of acryloyl-CoA (Table 1).

Pent-4-enoyl-CoA was reduced slowly by soluble extracts of both liver and heart (Table 1). If the rate of accumulation of tricarboxylic acid-cycle intermediates in K^+ -arrested heart perfused with pent-4-enoate (Hiltunen, 1978) is taken as a minimum rate of pent-4-enoate metabolism ($2.2 \mu\text{mol/min per g dry wt.}$), this is equivalent to about $50 \text{ nmol/min per mg}$ of solubilized mitochondrial protein. This estimate assumes 48 mg of mitochondrial protein/g wet wt. of heart (Farmer *et al.*, 1974), a fresh-weight/dry-weight ratio of 5 (Opie & Owen, 1975) and that proteins solubilized by ultrasonic treatment account for 22% of total mitochondrial protein (Lin & Davis, 1974). Thus the rate of reduction of pent-4-enoyl-CoA by the heart mitochondrial extract is about 50-fold less than a minimum estimate of the rate of pent-4-enoate metabolism by the perfused heart. Pent-4-enoate is also oxidized by perfused livers if

present in concentrations less than 1 mM (Williamson *et al.*, 1970). Also, the rate of reduction of pent-4-enoyl-CoA that we find cannot explain its rate of flux in intact livers.

Pent-4-enoyl-CoA has been shown previously to be a good substrate for oxidation by butyryl- and octanoyl-CoA dehydrogenase to penta-2,4-dienoyl-CoA (Holland *et al.*, 1973). Our data show (Table 1) that, with both heart and liver enzymes, penta-2,4-dienoyl-CoA is the only metabolite of pent-4-enoyl-CoA tested (including the latter) that undergoes reduction at a rate rapid enough to accommodate the rate of pent-4-enoyl-CoA metabolism in the intact organ. However, these data do not identify the position at which the reduction takes place, since it is theoretically possible that the double bond in position 2 is reduced, or both double bonds are reduced. To study these possibilities the following experiments were carried out: penta-2,4-dienoyl-CoA was incubated with soluble protein from heart mitochondria in the presence and absence of NADPH. When the rapid oxidation of NADPH had ceased, some products were identified. Control experiments were also run with pent-2-enoyl-CoA (Table 2). It should be pointed out that carnitine acetyltransferase is very active with pent-4-enoyl-CoA and n-pentanoyl-CoA, but reacts only very slowly with penta-2,4-dienoyl-CoA (Holland *et al.*, 1973). Thus significant amounts of pent-4-enoyl-CoA or n-pentanoyl-CoA were not present after incubation in the presence or absence of NADPH. This excludes the possibility that the double bond in position 2 or both the double bonds were reduced appreciably. This finding is in agreement with the low activity of 2-enoyl-CoA reductase found with pent-2-enoyl-CoA as substrate (Table 1).

Little or no 3-hydroxyacyl-CoA accumulated from penta-2,4-dienoyl-CoA if NADPH was not present, whereas pent-2-enoyl-CoA was recovered almost quantitatively as the 3-hydroxy derivative (Table 2). However, if NADPH was present, the 3-hydroxyacyl-CoA ester(s) was (were) formed from the former. These results, together with the obser-

Table 1. Activities of 4-enoyl-CoA and 2-enoyl-CoA reductases of rat heart and liver mitochondria

The activities of enzymes were determined as described in the Materials and methods section at 30°C . For measurement of the rate of penta-2,4-dienoyl-CoA reduction, the reaction was started by adding a reductase sample containing $40\text{--}80 \mu\text{g}$ of protein. Otherwise the reactions were started by adding the acyl-CoA ester to be tested into the cuvette containing reductase samples equivalent to $0.5\text{--}2.0 \text{ mg}$ of protein. The initial rates of NADPH oxidation were recorded and the results are expressed as nmol of NADPH oxidized/min per mg of soluble mitochondrial protein, and are means $\pm$ S.E.M. for the numbers of individual experiments given in parentheses.

NADPH oxidized (nmol/min per mg of soluble mitochondrial protein)

Substrate	Pent-4-enoyl-CoA	Penta-2,4-dienoyl-CoA	Pent-2-enoyl-CoA	Acryloyl-CoA
Heart	0.9 ± 0.1 (5)	132 ± 8 (6)	<0.6	<0.6
Liver	1.9 ± 0.2 (5)	52 ± 6 (5)	2.0 ± 0.3 (6)	<0.6

Table 2. Conversion of pent-2-enoyl-CoA and penta-2,4-dienoyl-CoA into some other intermediates of β -oxidation in the presence of NADPH and soluble mitochondrial protein of heart

The incubation mixtures contained, in 1.3 ml, 65 nmol of penta-2,4-dienoyl-CoA or pent-2-enoyl-CoA, 325 nmol of NADPH when present, and 64 μ g of soluble mitochondrial protein of heart, in 40 mM-potassium phosphate, pH 7.2. The decrease in A_{340} was recorded against a reference cuvette containing the same incubation mixture, but without acyl-CoA. After incubation at 30°C for 10 min, 2 M-HCl was added to lower the pH to 1.7–2.0. After neutralization and removal of protein the concentrations of metabolites were determined: 3-hydroxyacyl-CoA derivatives with 3-hydroxyacyl-CoA dehydrogenase, pent-2-enoyl-CoA and penta-2,4-dienoyl-CoA after addition of enoyl-CoA hydratase to the same cuvette, and acyl-CoA esters reacting with carnitine acetyltransferase as described in the Materials and methods section. The results, expressed as nmol, are means $\pm$ s.e.m. for triplicate experiments. Abbreviation: n.d., not detectable.

Substrate	Metabolites (nmol)			
	Penta-2,4-dienoyl-CoA		Pent-2-enoyl-CoA	
	+NADPH	–NADPH	+NADPH	–NADPH
NADPH oxidized	40 $\pm$ 1	—	n.d.	—
3-Hydroxyacyl-CoA esters found (reacting with 3-hydroxyacyl-CoA dehydrogenase)	29 $\pm$ 2	n.d.	63 $\pm$ 1	61 $\pm$ 2
Pent-2-enoyl-CoA or penta-2,4-dienoyl-CoA found (reacting with enoyl-CoA hydratase)	7 $\pm$ 1	61 $\pm$ 5	6 $\pm$ 1	6 $\pm$ 1
Acyl-CoA (reacting with carnitine acetyltransferase)	<5	<5	<5	<5

vation that the rate of reduction of pent-4-enoyl-CoA is negligible (Table 1), indicate that the hydration reaction takes place only after the double bond in position 4 has been reduced. To check this indication, we incubated penta-2,4-dienoyl-CoA at neutral pH with purified enoyl-CoA hydratase and then measured the products of the reaction (Fig. 1). The concentrations of 3-hydroxypent-4-enoyl-CoA and penta-2,4-dienoyl-CoA were determined by observing the appearance of NADH after the sequential additions of 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA reductase (Fig. 1a). This experiment shows that the latter enzyme carries out the reversible hydration of penta-2,4-dienoyl-CoA, but the equilibrium position of the reaction lies far to the side of dehydration. Essentially 100% was present as penta-2,4-dienoyl-CoA, but could be hydrated quantitatively at high pH and in the presence of 3-hydroxyacyl-CoA dehydrogenase. In control experiments carried out as described above (Fig. 1b), hydration of pent-2-enoyl-CoA was virtually complete at neutral pH. The equilibrium of enoyl-CoA hydratase with penta-2,4-dienoyl-CoA explains the results of Holland *et al.* (1973), who found no decrease in A_{300} if they incubated penta-2,4-dienoyl-CoA with purified enoyl-CoA hydratase. The activity of enoyl-CoA hydratase in heart is known to be manyfold higher than that of any other enzyme of β -oxidation (Kunau & Dommes, 1978; Fong & Schulz, 1978). Since the equilibrium position of the reaction catalysed by enoyl-CoA hydratase is very different with pent-2-enoyl-CoA and penta-2,4-dienoyl-CoA as substrates, we can

Fig. 1. Equilibrium of the reaction catalysed by enoyl-CoA hydratase with penta-2,4-dienoyl-CoA or pent-2-enoyl-CoA as substrate

For the experiments, 81 nmol of penta-2,4-dienoyl-CoA (a) or pent-2-enoyl-CoA (b) were incubated in 40 mM-potassium phosphate buffer, pH 7.2, in the presence of 3 units of purified enoyl-CoA hydratase at room temperature for 4 min (final volume 2.55 ml). The enzyme was denaturated by acidification with HCl, and then neutralized with KOH. The concentrations of metabolites were then determined in Tris/HCl buffer, pH 9.5, in the presence of 0.3 mM-NAD⁺ as follows: 3-hydroxypent-4-enoyl-CoA and 3-hydroxypentanoil-CoA with 3-hydroxyacyl-CoA dehydrogenase (1), and penta-2,4-dienoyl-CoA and pent-2-enoyl-CoA by adding enoyl-CoA hydratase to the same cuvette (2).

conclude that the 3-hydroxyacyl-CoA ester found when penta-2,4-dienoyl-CoA was incubated with soluble mitochondrial protein of heart and NADPH is 3-hydroxypentanoyl-CoA. As indicated above, the results show that heart mitochondrial extracts carry out the NADPH-dependent reduction of the double bond in position 4 of penta-2,4-dienoyl-CoA.

The lack of exact stoichiometry, both in the amount of pent-2,4-dienoyl-CoA added and in NADPH consumption (Table 2, second column), can be explained by the fact that 3-hydroxyacyl-CoA dehydrogenase activity in both heart and liver is high, and that NADP⁺ can replace NAD⁺ to some extent in this reaction (Wakil, 1963). Hence, it is likely that 3-oxopentanoyl-CoA was formed, resulting in a dismutation of NADPH, and thus an underestimation of its oxidation in the first reaction:

Two NADPH-dependent enoyl-CoA reductases are known, which carry out reduction of double bonds in the 2 and 4 positions respectively (Podack & Seubert, 1972; Kunau & Dommes, 1978). The substrates for the latter enzyme, however, possess a 2,4-diene structure rather than an isolated double bond in position 4. In our experiments, pent-2-enoyl-CoA was not reduced at a significant rate. Although we now have demonstrated that penta-2,4-dienoyl-CoA is reduced by heart enzymes in position 4, it is not certain that this compound is reduced by the enzyme studied by Kunau & Dommes (1978), since it differs from the substrates they used by having a double bond at the terminal methylene carbon.

General conclusions

Our previous observations have shown that (1) in perfused rat heart pent-4-enoate causes a rapid increase of tricarboxylic acid-cycle intermediates, (2) pent-4-enoate itself is the source of the extra carbon

skeletons, and (3) in isolated rat heart mitochondria the metabolism of pent-4-enoate occurs through a reductive step and energy-dependent carboxylation. With these data and the present results taken together, we can conclude that the principal metabolic pathway of pent-4-enoate in heart is as follows:

This is in agreement with Borrebaek *et al.* (1980), who came to the same conclusion indirectly when studying the effect of clofibrate treatment on pent-4-enoate metabolism in liver.

This work was supported by U.S. Public Health Service Grant AM 13939 and Grace M. Showalter Trust, U.S.A. Travel and expenses of J. K. H. were provided in part by the Magnus Ehrnrooth and Emil Aaltonen Foundations, Finland. We thank Dr. P. V. Blair for critical reading of this manuscript and for helpful suggestions.

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