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Isolated Rat Heart Mitochondria are able to Metabolize Pent-4-enoate to Tricarboxylic Acid-Cycle Intermediates

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The metabolism of four short-chain odd-number-carbon fatty acids, pentanoate, pent-4-enoate, propionate and acrylate, was studied in isolated rat heart mitochondria incubated in [^{14}C]bicarbonate buffer. Under these conditions pentanoate was metabolized with a concomitant accumulation of malate and incorporation of $^{14}CO_2$ into non-volatile compounds. The metabolism of propionate to tricarboxylic acid-cycle intermediates required the addition of ATP and oligomycin. After addition of a small amount of rotenone to the incubation medium, pent-4-enoate was metabolized with an increase in malate from less than 3 nmol/mg of protein to 34.0 ± 1.5 nmol/mg in 40 min, during which time the amount of $^{14}CO_2$ fixed in acid-stable compounds increased from 1.56 ± 0.30 to 41.1 ± 2.6 nmol/mg of protein. Acrylate was not metabolized under any of the conditions tested. The results show that cardiac mitochondria must have an enzyme system that is capable of reducing the double bond of either pent-4-enoate or its metabolites. That the metabolism of pent-4-enoate occurs through a reductive step and energy-dependent carboxylation is evident from the requirement for NAD^+ reduction by partial inhibition of the mitochondrial respiratory chain and the presence of ATP and CO_2. The results do not enable us to say whether the compound reduced is pent-4-enoyl-CoA or acryloyl-CoA.

Pent-4-enoate is the simplest hypoglycaemic analogue of hypoglycin (Glasgow & Chase, 1975; Billington *et al.*, 1978), which inhibits the β-oxidation of fatty acids in various systems (Senior *et al.*, 1968; Brendel *et al.*, 1969; Fukami & Williamson, 1971), and it is probably an intermediate formed during the β-oxidation of pent-4-enoate which is responsible for this inhibition (Holland *et al.*, 1973; Holland & Sherratt, 1973). The end-products of the β-oxidation of pent-4-enoate are acetyl-CoA and acryloyl-CoA (Holland *et al.*, 1973; Holland & Sherratt, 1973; Sherratt & Osmundsen, 1976). It has been suggested that CoA is liberated from acryloyl-CoA by unspecific deacylases, or else that acryloyl-CoA is hydrated to form hydroxypropionate, for example (Osmundsen & Sherratt, 1975; Williamson *et al.*, 1970). The metabolic fate of the acryloyl group is still uncertain. Perfused liver is able to oxidize pent-4-enoate continuously provided that its concentration in the perfusion medium is not too high (Williamson *et al.*, 1969, 1970), and we have suggested previously, on the basis of experiments with pent-4-enoate and perfused rat hearts, that cardiac muscle is able to metabolize it by a mechanism in which the carbon skeleton of the acryloyl part of pent-4-enoate is transformed to tricarboxylic acid-cycle intermediates via the propionate pathway (Hiltunen, 1978; Hiltunen *et al.*, 1978). The aim of the present work was to ascertain whether isolated heart mitochondria can metabolize pent-4-enoate. Of the three other short-chain fatty acids used as references, propionate, pentanoate and acrylate, the first two are saturated compounds rapidly metabolized by cardiac muscle. The metabolism of the C_3 fragments via the propionate pathway was judged by measuring changes in the size of the pool of tricarboxylic acid-cycle intermediates and the incorporation rate of $^{14}CO_2$ into non-volatile compounds.

We now present further data supporting the suggestion that pent-4-enoate is metabolized via the propionate pathway, and showing that isolated rat heart mitochondria are indeed able to metabolize pent-4-enoate into intermediates of the tricarboxylic

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acid cycle in the presence of ATP and low concentrations of rotenone by a mechanism involving CO_2 fixation.

Materials and Methods

Reagents

The enzymes, nucleotides, rotenone and oligomycin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and Boehringer Mannheim G.m.b.H., Mannheim, Germany, $NaH^{14}CO_3$ was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Pentanoic acid, pent-4-enoic acid, propionic acid and acrylic acid were obtained from Fluka A.G., Buchs SG, Switzerland.

Experimental procedure

Mitochondria were isolated from the hearts of Sprague–Dawley rats as described by Tyler & Gonze (1969). The experiments were conducted under liquid paraffin in 1.4ml of an incubation mixture containing final concentrations of 107mM-KCl, 17.9mM-Tris, 1.8mM-$MgCl_2$, 4.5mM-P_i, 22.3 mM-HCO_3^- (specific radioactivity 55000–60000 d.p.m./μmol), pH 7.2, and 2.0–2.7mg of mitochondrial protein. The final concentration of ATP, when present, was 2.5mM, that of oligomycin 2.5μg/ml, that of rotenone 3.5μM and that of the acid to be tested 2.86μM. The incubation time was 40min at room temperature (25°C), after which 0.15ml of 70% (v/v) $HClO_4$ was added into the incubation medium. The radioactive bicarbonate in the $HClO_4$ extract was eliminated by bubbling the acidified solution with unlabelled CO_2. When samples were prepared for chromatography on Dowex-1 (formate form), the volumes were doubled and the specific radioactivity of the bicarbonate was increased to 140000–190000d.p.m./μmol.

Metabolites

After neutralization of the $HClO_4$ extract with 3.75M-K_2CO_3/0.5M-triethanolamine hydrochloride, the metabolites were assayed by enzymic methods, measuring the appearance or disappearance of NADH in an Aminco DW-2 dual-wavelength spectrophotometer by using an $\varepsilon_{340}-\varepsilon_{385}$ value of 5.33×10^3 litre·mol^{-1}·cm^{-1} for NADH. Citrate was measured with citrate lyase (EC 4.1.3.6) (Gruber & Moellering, 1966), malate by the method of Williamson & Corkey (1969), 2-oxoglutarate as described by Narins & Passonneau (1970). In order to study the incorporation of radioactivity into tricarboxylic acid-cycle intermediates, 1μmol of each of the compounds studied was added to the $HClO_4$ extract as a carrier and the metabolites were isolated by ion-exchange chromatography on a Dowex-1 (formate form) column eluted with a linear gradient of formic acid followed by ammonium formate (LaNoue *et al.*, 1970). To determine the elution position of the relevant metabolites, succinate was determined as described by Williamson & Corkey (1969), glutamate with glutamate dehydrogenase (EC 1.4.1.3) (Bernt & Bergmeyer, 1970) and aspartate as described by Bergmeyer *et al.* (1970).

Protein

Protein was determined by the biuret procedure of Szarkowska & Klingenberg (1963).

Results and Discussion

The C_3 fragment, propionyl-CoA, remaining from the β-oxidation of saturated odd-numbered fatty acids in muscle tissue is metabolized via the propionate pathway (for references see Lowenstein, 1967), involving CO_2 fixation, to yield the C_4 compounds of the tricarboxylic acid cycle. In earlier experiments performed in our laboratory, pent-4-enoate caused a considerable increase in the concentration of tricarboxylic acid-cycle intermediates in perfused hearts, especially in that of malate, and pentanoate caused a similar metabolic change. These results suggested that acryloyl-CoA formed during the β-oxidation of pent-4-enoate could undergo reduction in cardiac muscle, after which the C_3 compound is metabolized to tricarboxylic acid-cycle intermediates (Hiltunen, 1978; Hiltunen *et al.*, 1978). The enzyme system which reduces acryloyl-CoA in mammalian tissue is not known, and the possibility that pent-4-enoyl-CoA is reduced to pentanoyl-CoA cannot be excluded.

The addition of pentanoate to mitochondria incubated in state 2 (Chance & Williams, 1955) increased the incorporation of $^{14}CO_2$ into non-volatile metabolites by 750% compared with the control experiments, i.e. far more than with the other fatty acids tested (Table 1). Propionyl-CoA, NADH, $FADH_2$ and acetyl-CoA are formed during the β-oxidation of pentanoate, and acetyl-CoA can be oxidized further by means of the tricarboxylic acid cycle. The low efficiency of propionate alone as an oxidizable fuel is exemplified by the ATP-dependence of $^{14}CO_2$ fixation during propionate metabolism (Table 1). Addition of ATP and oligomycin to the incubation medium increased the metabolic rate of propionate about 10-fold, as estimated from the incorporation of $^{14}CO_2$ and from the increase in the concentration of malate. Factors affecting the metabolism of pent-4-enoate under these conditions are the energy required for the activation of pent-4-enoate, fixation of CO_2 and 'reducing power' for the formation of propionyl-CoA. Therefore, without external manipulations providing energy and 'reducing power', isolated mitochondria metabolize pent-4-enoate at a very low

Table 1. *Metabolism of pentanoate, pent-4-enoate, propionate and acrylate in isolated rat heart mitochondria*
Mitochondria (2.0–2.7 mg of mitochondrial protein) were incubated in a reaction mixture containing 107 mM-KCl, 17.9 mM-Tris, 1.8 mM-$MgCl_2$, 4.5 mM-P_i and (unless otherwise stated) 22.3 mM-$H^{14}CO_3^-$ (specific radioactivity 55 000–67 000 d.p.m./μmol), pH 7.2, for 40 min at room temperature. The final volume was 1.4 ml. The concentration of metabolites and the rate of radioactivity incorporation into non-volatile compounds were determined from neutralized $HClO_4$ extracts as described in the Materials and Methods section. Values are means ± S.E.M. from the numbers of experiments given in parentheses.

C_3 or C_5 fatty acid (2.86 mM)	ATP (2.5 mM) + oligomycin (2.5 μg/ml)	Rotenone (3.5 μM)	Rate of metabolism (nmol/40 min per mg of protein)					
			$^{14}CO_2$ incorporation	Malate		Citrate		2-Oxoglutarate
— (t = 0 min control)	−	−	0	<3*	(11)	<5†	(6)	<3†† (8)
— (t = 40 min control)	−	−	1.56 ± 0.26 (14)	<3	(10)	<5	(6)	<3 (8)
Pentanoate	−	−	11.7 ± 2.3 (8)	10.8 ± 2.6	(8)	<5	(4)	<3 (6)
	+	−	24.9 ± 2.8 (8)	18.4 ± 2.3	(8)	<5	(4)	<3 (4)
	+	+	46.4 ± 4.7 (7)	41.3 ± 2.1	(8)	<5	(4)	<3 (6)
Pent-4-enoate	−	−	0.92 ± 0.28 (11)	<3	(12)	<5	(10)	<3 (8)
	+	−	2.57 ± 0.57 (11)	<3	(12)	<5	(10)	<3 (8)
	+	+	41.1 ± 2.6 (12)	34.0 ± 1.5	(12)	<5	(10)	<3 (8)
Propionate	−	−	4.90 ± 1.56 (6)	<3	(6)	<5	(4)	<3 (5)
	+	−	31.3 ± 5.6 (6)	7.58 ± 2.4	(4)	<5	(4)	<3 (6)
	+	+	95.7 ± 4.6 (5)	88.4 ± 9.0	(5)	<5	(4)	<3 (5)
Acrylate	−	−	1.20 ± 0.49 (4)	<3	(4)	<5	(4)	<3 (4)
	+	−	4.20 ± 0.43 (4)	<3	(4)	<5	(4)	<3 (4)
	+	+	4.27 ± 0.75 (4)	<3	(4)	<5	(4)	<3 (4)
HCO_3^--free buffer								
— (t = 40 min control)	−	−	−	<3	(4)	<5	(4)	<3 (4)
Pentanoate	−	−	−	<3	(4)	<5	(4)	<3 (4)
	+	+	−	<3	(4)	<5	(4)	<3 (4)
Pent-4-enoate	−	−	−	<3	(4)	<5	(4)	<3 (4)
	+	+	−	<3	(4)	<5	(4)	<3 (4)

* Detection limit of malate.
† Detection limit of citrate.
†† Detection limit of 2-oxoglutarate.

rate. The metabolism of pentanoate increased a little after the addition of ATP and oligomycin.

A further increase in the $^{14}CO_2$ incorporation and metabolite accumulation occurred when rotenone was added to the incubation medium in addition to ATP and oligomycin. This addition of rotenone increased the rate of CO_2 fixation by 86% in the presence of pentanoate, 16-fold in the presence of pent-4-enoate, and 3-fold in the presence of propionate, but did not alter the rate in the presence of acrylate. The most interesting finding was that the accumulation of malate and the incorporation of bicarbonate radioactivity into non-volatile compounds in the presence of ATP plus rotenone was almost the same with pent-4-enoate and pentanoate, being about one-half of that found with propionate. Intermediates of the tricarboxylic acid cycle were certainly synthesized under these conditions and not just re-arranged, as is evident from the low concentrations of all the metabolites measured in the control experiments (Table 1). No accumulation of tricarboxylic acid-cycle intermediates occurred when CO_2 was omitted from the incubation medium. Thus, in order to be metabolized in isolated heart mitochondria, pent-4-enoate needs ATP, CO_2 and reductive conditions, the last being obtained by means of rotenone in this case.

When the radioactive non-volatile intermediates of pent-4-enoate metabolism were identified by means of Dowex-1 chromatography, it was found that malate was the major intermediate, accounting for 63% of total incorporation. A minor peak of radioactivity (8% of the total) was eluted coincident with aspartate and glutamate. The [NADH]/[NAD^+] ratio is high in the presence of rotenone, and this shifts the equilibrium of the glutamate dehydrogenase reaction towards glutamate formation, and ammonia is always present in small amounts. Isolated mitochondria retain some amino acids, and label can be incorporated into these by transamination, resulting in an exchange of carbon stems between amino acids and the 2-oxo acids synthesized. In this connection it is noteworthy that the activity of glutamate dehydrogenase is relatively

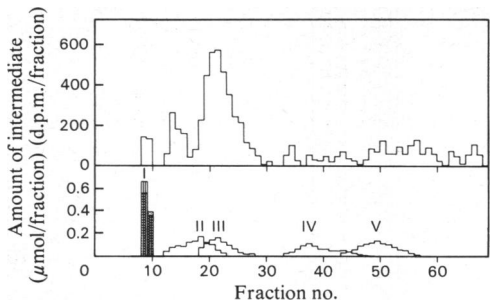

Fig. 1. *Isolation of radioactive non-volatile intermediates of pent-4-enoate metabolism by isolated rat heart mitochondria in a medium containing* [^{14}C]-*bicarbonate*

Means of results from six experiments are shown. Mitochondria were incubated in the complete mixture described in Table 1 in the presence of pent-4-enoate for 40min. A neutralized $HClO_4$ extract of the mixture was chromatographed on a Dowex-1 (formate form) column as described by LaNoue *et al.* (1970); 1μmol each of aspartate, glutamate, succinate, malate, citrate and α-oxoglutarate were added as carriers. The concentrations of carriers were measured from each fraction (volume 5.2ml) as described in the Materials and Methods section, and their radioactivity was determined. Elution positions were as follows: I, glutamate (vertical ruling) and aspartate (dotted area); II, succinate; III, malate; IV, citrate; V, 2-oxoglutarate.

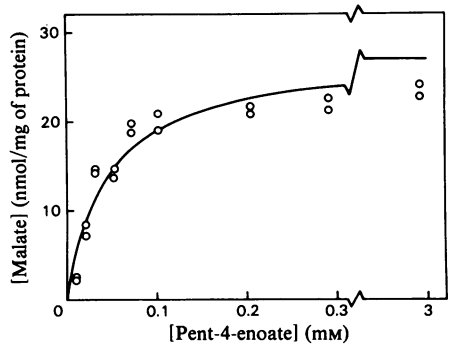

Fig. 2. *Dependence of malate accumulation on pent-4-enoate concentration in isolated rat heart mitochondria*

Mitochondria (1.23mg of mitochondrial protein/ml) were incubated in a medium consisting of 101mM-KCl, 16.9mM-Tris, 1.7mM-$MgCl_2$, 4.2mM-potassium phosphate, 24.7mM-$KHCO_3$, 2.6mM-ATP, 8.2mM-mannitol, 2.7mM-sucrose, 0.18μM-EDTA, 3μM-rotenone and oligomycin (2.9μg/ml). pH was 7.4 and incubation time 40min at 25°C. The curve was constructed by least-squares regression to give best fit to the experimental data in a 1/[pent-4-enoate] versus 1/[malate] plot.

high in cardiac muscle compared with skeletal muscle (T. Takala, J. K. Hiltunen & I. E. Hassinen, unpublished work). An unknown peak accounting for 19% of the total incorporation of radioactivity was eluted just before succinate (Fig. 1), and a small amount of radioactivity, about 10%, was distributed widely among different intermediates of the tricarboxylic acid cycle. The specific radioactivity of malate was $71 \pm 3\%$ ($n = 6$) of that of bicarbonate. The failure of the specific radioactivity of malate to reach that of bicarbonate was due to incomplete inhibition of the respiratory chain by rotenone. The metabolism of pent-4-enoate and pentanoate, however, was slow because of the blocking of the reoxidation of NADH produced in β-oxidation. Taking into account the 39% dilution observed in malate radioactivity, it can be estimated that the minimum rate of pent-4-enoate metabolism to malate must have been 1.2nmol$\cdot$min$^{-1}\cdot$(mg of protein)$^{-1}$ under the conditions used. The accumulation of tricarboxylic acid-cycle intermediates from pent-4-enoate metabolism occurs mostly in the form of malate, indicating that the citrate synthase reaction must be inhibited under these conditions. One reason for this could be that the equilibrium of the malate dehydrogenase reaction shifts strongly towards malate and the citrate synthase reaction becomes limited by oxaloacetate.

Under the conditions used, accumulation of malate in the presence of pent-4-enoate proceeded at a constant rate during the 40min observation period. The accumulation of malate in the presence of pent-4-enoate metabolism was concentration-dependent, as depicted in Fig. 2. The hyperbolic correlation between pent-4-enoate concentration and malate accumulation rate gave an apparent K_m of 45μM for pent-4-enoate. When pent-4-enoate concentration was 100μM, which is known to inhibit strongly palmitoylcarnitine oxidation by heart mitochondria (Fong & Schulz, 1978), the rate of malate accumulation was 70% of maximum.

Acrylic acid caused no clear incorporation of bicarbonate radioactivity into non-volatile compounds, nor any increase in the concentration of tricarboxylic acid-cycle intermediates. No decrease in the concentration of free CoA and no accumulation of tricarboxylic acid-cycle intermediates occurs when the heart is perfused with acrylate (J. K. Hiltunen, unpublished work; Hiltunen, 1978). These results, which agree with those of Holland & Sherratt (1973), who incubated liver mitochondria with 1mM-acrylate and did not find any short-chain acylation of CoA, demonstrate that the activation of acrylate to acryloyl-CoA in cardiac mitochondria is

slow compared with the deacylation reactions. The results can be reconciled with a tentative metabolic pathway for pent-4-enoate in cardiac muscle as follows:

$$\text{Pent-4-enoate} \xrightarrow{+\text{ATP, CoA}} \text{acetyl-CoA} + \text{acryloyl-CoA } (\beta\text{-oxidation})$$

$$\text{Acryloyl-CoA} \xrightarrow{+\text{NAD(P)H}} \text{propionyl-CoA} + \text{NAD(P)}^{+} \text{ (reduction enzyme?)}$$

$$\text{Propionyl-CoA} \xrightarrow{+\text{ATP, CO}_2} \text{malate} + \text{CoA} + \text{ADP (propionate pathway, tricarboxylic acid cycle)}$$

The rapidity of this pathway leading to physiological compounds explains the short half-life (20min) of blood pent-4-enoate (Sherratt, 1978; Osmundsen & Sherratt, 1978), the high concentrations (1mM) needed for inhibiting fatty acid oxidation and gluconeogenesis in bicarbonate-containing systems (Hiltunen *et al.*, 1978; Williamson *et al.*, 1970) and high doses (250mg/kg body wt.) needed for the hypoglycaemic effect of pent-4-enoate in animals (Corredor *et al.*, 1967).

The reason why the accumulation of tricarboxylic acid-cycle intermediates from pent-4-enoate has not been observed previously is probably that it is customary to conduct experiments with isolated mitochondria and tissue homogenates in bicarbonate-free buffers.

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