A study of the metabolism of [U-¹⁴C]3-methyl-2-oxopentanoate by rat liver mitochondria using h.p.l.c. with continuous on-line monitoring of radioactive intact acyl-coenzyme A intermediates

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1. A reverse-phase h.p.l.c. system for the resolution of the acyl-CoA intermediates of the degradation of 3-methyl-2-oxopentanoate is described. 2. The validation of a method for the measurement of radioactive intermediates produced by the incubation of [U-¹⁴C]3-methyl-2-oxopentanoate with rat liver mitochondrial fractions is described. 3. The absence of bicarbonate caused the accumulation of [¹⁴C]propionyl-CoA. 4. The accumulation of [¹⁴C]2-methylbutyryl-CoA was observed in incubations with mitochondrial fractions derived from riboflavin-deficient animals. 5. Studies of the accumulation of labelled intermediates with time suggest that there is regulation of the pathway of isoleucine degradation at methylmalonyl-CoA mutase, as suggested for valine [Corkey, Martin-Requero, Walajtys-Rode, Williams & Williamson (1982) *J. Biol. Chem.* **257**, 9668–9676]. 6. These studies demonstrate that h.p.l.c. with on-line continuous radiochemical detection is a powerful method for the investigation of the control of intermediary metabolism.

INTRODUCTION

The regulation of the metabolism of 4-methyl-2-oxopentanoate (2-ketoisocaproate), 3-methyl-2-oxopentanoate (2-keto-3-methylvalerate) and 3-methyl-2-oxobutyrate (2-ketoisovalerate), the transamination products of leucine, isoleucine and valine respectively, has been the object of much study. Most attention has been directed at the first step of these pathways, the oxidative decarboxylation to the respective saturated acyl-CoA esters, isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA respectively, which is catalysed by the branched-chain oxo acid dehydrogenase complex (Wohlhueter & Harper, 1970; Pettit *et al.*, 1978; Parker & Randle, 1978a; Danner *et al.*, 1979). The complex is regulated by end-product inhibition by NADH and acyl-CoA (Pettit *et al.*, 1978; Parker & Randle, 1978a) and in addition by phosphorylation/dephosphorylation (Parker & Randle, 1978b, 1980; Fatania *et al.*, 1981; Odessey, 1982; Randle *et al.*, 1984). Phosphorylation, which causes inactivation of the complex, is catalysed by a co-purifying kinase (Fatania *et al.*, 1981; Odessey, 1982; Paxton & Harris, 1982; Lawson *et al.*, 1983), which is itself subject to regulation and is inhibited by octanoate [I_{40} (concn. giving 40% inhibition) = 0.5mM], NADP⁺ (I_{40} = 1.5mM), methylmalonyl-CoA (I_{40} = 0.2mM) and most potently by acetoacetyl-CoA (I_{40} = 0.01 mM) (Paxton & Harris, 1984).

There have been few studies of the regulation of the degradation of the branched-chain oxo acids at sites other than at the dehydrogenase complex. However, investigations of the degradation of 3-methyl-2-oxobutyrate by isolated rat hepatocytes and rat liver mitochondria suggested an additional site of regulation at methylmalonyl-CoA mutase (L-methylmalonyl-CoA CoA-carboxylmutase, EC 5.4.99.2; Williamson *et al.*, 1979; Corkey *et al.*, 1982). These experiments were made possible by the development of a reverse-phase h.p.l.c. system

Fig. 1. Pathway of isoleucine degradation

Abbreviation: TPP, thiamin pyrophosphate.

for the determination of intact acyl-CoA intermediates (Corkey *et al.*, 1981). The metabolism of 3-methyl-2-oxopentanoate has, to our knowledge, not been studied. We

report here a reverse-phase h.p.l.c. system for the resolution of all of the acyl-CoA intermediates of 3-methyl-2-oxopentanoate degradation (Fig. 1). Preliminary experiments suggested that simple u.v. detection of the intermediates was insufficiently sensitive, and therefore we have developed a method for the on-line continuous radiochemical detection of labelled acyl-CoA esters derived from [U-¹⁴C]3-methyl-2-oxopentanoate. We describe here our validation of this technique and its application to the study of the regulation of 3-methyl-2-oxopentanoate degradation. Some of the data described here have appeared in a preliminary form (Causey *et al.*, 1985).

EXPERIMENTAL

Materials

CoA, methylmalonyl-CoA, butyryl-CoA, hexanoyl-CoA, 2-methylbut-2-enoyl-CoA, succinyl-CoA (3-carboxypropionyl-CoA) and crotonase (enoyl-CoA hydratase; EC 4.2.1.17) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Methanol (h.p.l.c.-grade) was purchased from Rathburn Chemicals, Walkerburn, Scotland, U.K. All other reagents were of highest available purity. L-[U-¹⁴C]isoleucine (sp. radioactivity 12.5 GBq/mmol) and [U-¹⁴C]acetyl-CoA (sp. radioactivity 2.13 GBq/mmol) were obtained from Amersham International, Amersham, Bucks., U.K.

Preparation of [U-¹⁴C]3-methyl-2-oxopentanoate

[U-¹⁴C]3-Methyl-2-oxopentanoate was prepared from L-[U-¹⁴C]isoleucine (diluted to 0.757 GBq/mmol) and purified as described by Rudiger *et al.* (1972). The purity of the preparation was monitored by radio-h.p.l.c. using a Bio-Rad HPX-87H organic acid column (see below). The preparation was stored at -40 °C and the purity checked before each experiment.

Synthesis of acyl-CoA esters

Acetyl-CoA and propionyl-CoA were prepared from their respective anhydrides by the method of Simon & Shemin (1953). Yields of 75–80% were obtained as judged by assay with 5,5'-dithiobis-(2-nitrobenzoic acid) (Bartlett & Gompertz, 1974). The purity of the preparations was assessed by h.p.l.c. (see below). 2-Methylbutyryl-CoA was synthesized from the 1-acylimidazole (Kawaguchi *et al.*, 1981) and purified by h.p.l.c. 2-Methylacetoacetyl-CoA was synthesized and purified as previously described (Middleton & Bartlett, 1983). 3-Hydroxy-2-methylbutyryl-CoA was synthesized from 2-methylbut-2-enoyl-CoA by the action of crotonase. The incubation (1.0 ml) contained 20 μmol of KH₂PO₄, 0.5 μmol of 2-methylbut-2-enoyl-CoA and 0.4 unit of crotonase. The pH was 5.3. The reaction (2 min, 37 °C) was stopped by freezing at -70 °C. The mixture was freeze-dried, and 3-hydroxy-2-methylbutyryl-CoA was purified by h.p.l.c. Although the reaction goes to completion, the equilibrium greatly favours 2-methylbut-2-enoyl-CoA, so that the yield is about 10%.

Preparation of rat liver mitochondrial fractions

Mitochondrial fractions were prepared from the livers of 18 h-starved male Wistar rats (approx. 250 g). The isolation medium contained 300 mM-mannitol, 10 mM-Hepes buffer (pH 7.2) and 1 mM-EGTA. All manipulations were done at 0–4 °C. Rats were killed by cervical dislocation. Finely chopped liver (5–8 g) was homo-

genized in 40 ml of isolation medium in a loose-fitting Potter-Elvehjem homogenizer. The nuclear fraction was sedimented by centrifugation (Sorvall RC-5B refrigerated centrifuge; temperature = 4 °C; 7×10^6 g_{av.}-min) and the supernatant was centrifuged as above (2.95×10^8 g_{av.}-min). The mitochondrial pellet was resuspended in 40 ml of isolation medium and re-centrifuged. Finally, the mitochondrial fraction was resuspended in isolation medium at a concentration of 30–40 mg/ml. The respiratory control ratio (Chance & Williams, 1956) was routinely 4–6, as measured with 10 mM-glutamate and 1 mM-malate (Turnbull *et al.*, 1983). Mitochondrial fractions were also prepared from animals made riboflavin-deficient by the method of Gregersen & Kolvraa (1982). Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Mitochondrial incubations and sample preparation

Incubations were made at 30 °C in a shaking water bath (120 strokes/min) in 1 ml of freshly prepared medium containing 110 mM-KCl, 5 mM-KH₂PO₄, 9.5 mM-EDTA, 2 mM-MgCl₂, 25 mM-KHCO₃, 20 mM-Hepes buffer and 1 mM-[U-¹⁴C]3-methyl-2-oxopentanoate (diluted to 64.4 MBq/mmol) in stoppered tubes. The tubes were gassed with O₂/CO₂ (19:1) for 1 min and temperature-equilibrated for 5 min before mitochondrial suspension (5–6 mg of protein) was added to start the reactions. The reactions were stopped at appropriate times by the addition of 100 μl of 5 M-HClO₄. Internal standard (20 nmol of hexanoyl-CoA) was added, and precipitated protein and KClO₄ were removed by centrifugation (5×10^4 g_{av.}-min). The supernatant was extracted with 3 × 5 vol. of diethyl ether to remove unused substrate, adjusted with 1 M-KHCO₃ to pH 7.0 and freeze-dried. The residue was extracted with 400 μl of h.p.l.c.-grade water, and a 200 μl sample analysed by radio-h.p.l.c.

Radio-h.p.l.c. analysis of acyl-CoA esters

H.p.l.c. was carried out with a Spectra-Physics SP8700 system with a LiChrosorb 10RP18 column (250 mm × 4.6 mm) at 45 °C. Samples were introduced with a manual Rheodyne loop injector (model 7125) fitted with a guard column (75 mm × 2.1 mm, packed with pellicular reverse-phase material; Chrompack, Middleburg, Holland). All solvents were continuously sparged with helium to de-gas them. Acyl-CoA esters were resolved by a gradient of methanol in water containing 50 mM-KH₂PO₄ (pH 5.3) and 4 mM-2, 2-thiodiethanol: isocratic 5% (v/v) (5 min), then isocratic 10% (v/v) (10 min), then linear to 17% (v/v) (18 min), then linear to 45% (v/v) (25 min) and finally isocratic 45% (v/v) (10 min). The flow rate was 1 ml/min. In order to maintain column performance, it is essential that the column is carefully re-equilibrated at the end of each working day, as described by Corkey *et al.* (1981). The solvent system resolved all of the intermediates of 3-methyl-2-oxopentanoate oxidation (Fig. 1), although methylmalonyl-CoA was poorly separated from free CoA (results not shown). Resolved compounds were detected by a Pye-Unicam variable-wavelength u.v. detector (model LC3; 8 μl flow cell; path-length 10 mm) at 260 nm. The output was displayed on a Kipp and Zonen dual-channel strip-chart recorder (model BD9) and also a Spectra-Physics SP4270 integrator.

Radioactivity associated with eluted peaks was detected on-line with a Reeve Analytical radiochemical detector fitted with a 900 μ l flow cell. A homogeneous radioactivity-counting system was used. The effluent from the u.v. detector (1 ml/min) was mixed with scintillation fluid (1.4 ml/min; 10 g of 2,5-diphenyl-oxazole, 330 ml of Triton X-100, 670 ml of xylene, 150 ml of methanol) by a Reeve Analytical precision splitter/mixer and then passed to the radiochemical detector. The radiochemical-detector output was displayed by using a Hewlett-Packard 85B personal computer and the second channel of the strip-chart recorder. The pens were offset to compensate for the dead volume between the u.v. and radiochemical detectors, to generate superimposable dual traces. The integrators were used to determine peak areas and accurate retention times.

The radioactivity monitor is constructed around two photomultipliers sited on either side of the flow cell. The major contribution to background noise is chemiluminescence derived from flow phenomena, which is decreased by coincidence circuitry with a response time of 10 ns to distinguish multiphoton events from chemiluminescence. The signal is averaged by an algorithm which uses a variable rolling average. Thus, at low count rates a maximum time constant is used, whereas at high count rates a minimum time constant is used. This method improves the signal-to-noise ratio but produces peak-skewing. This problem is circumvented by averaging the data stack from the centre, working alternatively around the centre-point. For a full discussion of the theory of operation of this algorithm, see Reeve & Crozier (1977) and the manufacturers' literature (Reeve Analytical, Glasgow, Scotland, U.K.).

The precision of the radiochemical detector was determined with [1- 14 C]acetyl-CoA. Integration of the radioactive peak gives the total number of radioactive counts recorded per transit time (flow cell volume/rate of flow through cell): counts per minute are calculated by dividing the integrated counts by the transit time. Counting efficiency is then calculated in the usual way. It is not always appreciated that, in order to determine counting efficiency, account must be taken of the transit time. This becomes obvious if one considers that the probability that a disintegration will be registered by the detector is dependent on the length of time a given molecule resides in the flow cell. In this particular instance the total flow rate through the cell (column effluent plus scintillant) is 4.0 ml/min, the transit time is therefore 0.23 min, and thus the observed integrated counts must be multiplied by 4.35 to obtain c.p.m. Thus the accuracy of the determination of c.p.m. is heavily dependent on the accuracy of measurement of the flow rate. Although the flow rate did not vary significantly either within or between runs on any given day, it was necessary to determine the flow rate at the start of each working day.

Radio-h.p.l.c. detection of organic acids

In order to confirm the identity of the acyl-CoA esters, some samples were divided in two. One half was analysed for intact acyl-CoA esters as described above, and the other half was incubated with 100 μ l of 0.1 M-KOH for 30 min at 40 °C. The resultant liberated organic acids were analysed on a Bio-Rad HPX-87H organic acid column (300 mm $\times$ 7.8 mm; particle size 9 μ m) after the addition of standard carrier organic acids. The column

was run at 25 °C with 2.5 mM- H_2SO_4 at 0.8 ml/min. The u.v. detection was at 210 nm. The scintillation-fluid flow rate was 2.4 ml/min.

Fig. 2. H.p.l.c. chromatograms of: (a) a standard mixture of the acyl-CoA intermediates of isoleucine degradation (u.v. detection); (b) extract of mitochondria incubated with unlabelled 3-methyl-2-oxopentanoate (u.v. detection); (c) extract of mitochondria incubated with [U- 14 C]3-methyl-2-oxopentanoate (radiochemical detection); (d) extract of mitochondria from a riboflavin-deficient rat incubated with [U- 14 C]3-methyl-2-oxopentanoate (radiochemical detection)

The incubation conditions were as described in the Experimental section; incubation times were 5 min. The peaks are as follows: 1, 3-methyl-2-oxopentanoate; 2, methylmalonyl-CoA; 3, succinyl-CoA; 4, acetyl-CoA; 5, 2-methylacetoacetyl-CoA; 6, propionyl-CoA; 7, 3-hydroxy-2-methylbutyryl-CoA; 8, 2-methylbut-2-enoyl-CoA; 9, 2-methylbutyryl-CoA; 10, hexanoyl-CoA (internal standard); 11, Phenol Red.

Fig. 3. Radio-h.p.l.c. chromatogram of an extract of mitochondria incubated with $[U-^{14}C]$ 3-methyl-2-oxopentanoate and hydrolysed to liberate the free organic acids

The incubation conditions were as described in the Experimental section; incubation times were 5 min. The peaks are as follows: 1, citrate, isocitrate, oxaloacetate, 2-oxoglutarate; 2, 3-methyl-2-oxopentanoate; 3, methylmalonate; 4, succinate; 5, acetate; 6, propionate; 7, 2-methylbutyrate; 8, 2-methylbut-2-enoate.

RESULTS

The resolution of the acyl-CoA intermediates of 3-methyl-2-oxopentanoate degradation is shown in Fig. 2(a). Hexanoyl-CoA was used as internal standard. Preliminary experiments showed that detection by u.v. absorption alone was neither sufficiently sensitive nor sufficiently discriminatory. A typical chromatogram with u.v. detection of the products of reaction derived from the incubation of mitochondria with 3-methyl-2-oxopentanoate is shown in Fig. 2(b). A large number of analytes other than acyl-CoA esters are detected by this procedure. The complex of peaks eluted in the first 10 min were nucleotides. Furthermore two large late-running u.v.-absorbing peaks were observed which were stable to alkaline hydrolysis (retention times 15.0 and 39.0 min) and which were therefore not acyl-CoA esters. We therefore concluded that the use of radioactive substrate was necessary and furthermore that fraction collection and subsequent liquid-scintillation counting was impracticable, since 200–300 fractions per chromatogram would be required to obtain radiochemical resolution. Initial experiments with a heterogeneous radiochemical detector proved unsuccessful because of adsorption of analytes by the solid scintillant, and we therefore used a homogeneous detector throughout these studies as described in the Experimental section. A typical radiochemical chromatogram is shown in Fig. 2(c).

In order to prove the identities of the radio-labelled acyl-CoA esters, a sample of the incubation mixture was subjected to alkaline hydrolysis and analysed for free organic acids. The results are shown in Fig. 3. Radioactive peaks were observed which co-chromatographed with acetate, propionate, methylmalonate, succinate, 2-methylbutyrate and 2-methylbut-2-enoate. Furthermore the corresponding acyl-CoA esters, that is

those peaks which co-chromatogrammed with the standards and which were both u.v.-absorbing at 260 nm and radioactive, had disappeared after saponification (results not shown).

Integration of the radio-chromatogram peak area yields an estimate of c.p.m. directly. Counting efficiencies of 68–70% were routinely obtained which were independent of the composition of the h.p.l.c. eluent and did not vary over the course of a gradient. Methanol concentrations in the range 10–50% (v/v) gave the same counting efficiency. The precision of the estimates of c.p.m. was examined by analysis of standard $[U-^{14}C]$ acetyl-CoA. Replicate analyses ($n = 5$) of 1028, 8447 and 25 330 d.p.m. yielded coefficients of variation of 4.2%, 2.9% and 2.1% respectively.

The reproducibility of the mitochondrial incubations was examined with five replicates (Table 1). The estimates of recovery of the acyl-CoA esters were based on the integrated peak area of the u.v. detection of hexanoyl-CoA and was in the range 85.4–99.2%, and demonstrates the necessity for internal standardization.

Incubations with various concentrations of mitochondrial protein between 1.9 and 9.4 mg/ml were made. There was a linear relationship between the amount of mitochondrial protein and the radioactivity associated with each acyl-CoA ester (Fig. 4). All subsequent experiments were made within this range of protein concentrations.

The appearance of radioactive acyl-CoA esters with respect to time was examined. Duplicate incubations were quenched at intervals over the range 15 s to 5 min. The results are shown in Fig. 5. 2-Methylbutyryl-CoA increased to a maximum concentration over the first 1 min and thereafter declined to about half-maximal after 5 min. Similar time courses were observed for 2-methylbut-2-enoyl-CoA and propionyl-CoA, although

Table 1. Precision of the radiochemical determination of acyl-CoA esters from replicate mitochondrial incubations

Values shown are means $\pm$ S.E.M.; the numbers in parentheses are the numbers of independent observations used to calculate the means. The incubation conditions were as described in the Experimental section; incubation times were 5 min.

Acyl-CoA ester	Retention time (min)	Radioactivity (d.p.m./mg of protein)
2-Methylbutyryl-CoA	49.59 $\pm$ 0.09 (10)	466 $\pm$ 21 (5)
2-Methylbut-2-enoyl-CoA	47.65 $\pm$ 0.06 (10)	178 $\pm$ 10 (5)
Propionyl-CoA	32.62 $\pm$ 0.14 (10)	426 $\pm$ 7 (5)
Acetyl-CoA	20.67 $\pm$ 0.11 (10)	342 $\pm$ 17 (5)
Methylmalonyl-CoA	12.58 $\pm$ 0.04 (8)	774 $\pm$ 37 (5)
Succinyl-CoA	13.96 $\pm$ 0.06 (8)	468 $\pm$ 12 (5)

Fig. 4. Effects of increasing mitochondrial protein concentration on the production of radioactive acyl-CoA esters

The incubation conditions were as described in the text; incubation times were 5 min.

the maximal radioactivities were lower than for 2-methylbutyryl-CoA. No radioactivity associated with either 3-hydroxy-2-methylbutyryl-CoA or 2-methyl-acetoacetyl-CoA was observed at any time point. However, the maximum methylmalonyl-CoA concentration was at 2 min, and thereafter fell to 67% of maximum

after 5 min. The time course of radioactivity associated with succinyl-CoA was markedly different from that of the other acyl-CoA esters and increased over the 5 min period of study. Radioactivity associated with acetyl-CoA reached a maximum after 2 min and then fell, a profile very similar to that for methylmalonyl-CoA.

We have studied the effects of riboflavin deficiency or the omission of bicarbonate from the incubation mixture (Table 2 and Fig. 2*d*). In both instances the system responded as expected. Incubations with mitochondria from riboflavin-deficient animals resulted in the accumulation of 2-methylbutyryl-CoA and a diminution of the other acyl-CoA esters compared with controls. The omission of bicarbonate from the incubations resulted in the accumulation of propionyl-CoA and a diminution in methylmalonyl-CoA.

DISCUSSION

There have been many studies of the catabolism of the branched-chain amino acids and their corresponding oxo acids, in isolated mitochondria, isolated cells, tissues or perfused organs. To our knowledge there have been no studies of the acyl-CoA esters of isoleucine degradation, presumably because of the lack of suitable analytical methods and some of the acyl-CoA intermediates.

CoA has traditionally been assayed by enzyme-linked spectrophotometric (Tubbs & Garland, 1969), fluorimetric (Michal & Bergmeyer, 1974) or radiometric methods (Skrede & Bremer, 1970; Smith, 1978). All these methods are indirect, laborious and limited in their application. Similarly, separations of acyl-CoA esters by t.l.c. (Pullman, 1973) and ion-exchange chromatography (Gregolin *et al.*, 1968) are time-consuming and give poor resolution. More recently several h.p.l.c. methods have been developed for the separation of acyl-CoA esters. Acyl-CoA esters are polar and charged compounds which do not readily chromatograph with conventional reversed-phase systems. Even if a weak solvent (100% water) and a C_{18} stationary phase are used, acyl-CoA esters are eluted in the solvent front. Baker & Schooley (1979) were the first to develop an ion-pairing reverse-phase system for the analysis of a homologous series of straight-chain saturated acyl-CoA esters (C_2 – C_{18}) by using tetrabutylammonium phosphate. However, this method has not proved very popular and, with the exception of the detection of valproyl-CoA derived from the incubation of valproic (2-propylpentanoic) acid with mitochondria (Turnbull *et al.*, 1983), there have been no

Fig. 5. Time course of the production of radioactive acyl-CoA esters by mitochondria

Each point represents a separate incubation. The incubation conditions were as described in the Experimental section. The incubations were stopped at the times indicated.

Table 2. Effects of riboflavin deficiency and the omission of bicarbonate from the incubations on the accumulation of radioactive acyl-CoA esters

Results shown are means $\pm$ S.E.M. (ND, not detected; *data from two individual animals). Values in parentheses represent the numbers of independent determinations used to calculate the mean. The incubation conditions were as described in the Experimental section; incubation times were 5 min. $\dagger P < 0.05$ (Mann-Whitney U test on unpaired samples) compared with controls.

Acyl-CoA ester	Radioactivity (d.p.m./mg of mitochondrial protein)		
	Controls	Riboflavin-deficient animals*	Minus-bicarbonate incubations
2-Methylbutyryl-CoA	668 $\pm$ 85 (9)	1197, 1744	354 $\pm$ 14 (3) $\dagger$
2-Methylbut-2-enoyl-CoA	165 $\pm$ 38 (9)	11, ND	111 $\pm$ 41 (3)
Propionyl-CoA	723 $\pm$ 165 (8)	243, 88	1638 $\pm$ 545 (3) $\dagger$
Acetyl-CoA	377 $\pm$ 78 (8)	187, 51	243 $\pm$ 66 (3)
Methylmalonyl-CoA	994 $\pm$ 170 (8)	338, 163	492 $\pm$ 39 (3) $\dagger$
Succinyl-CoA	398 $\pm$ 115 (5)	51, 24	291 $\pm$ 83 (3)

reports, to our knowledge, of the application of this method to the analysis of biological samples.

Ingebretsen *et al.* (1979) described an assay for CoA using an isocratic phosphate-buffer methanol system, which was subsequently modified to allow analysis of CoA in neutralized HClO_4 extracts of biological samples (Ingebretsen & Farstad, 1980). Subsequent developments of this system have extended the method to the analysis of short-chain acyl-CoA esters from a variety of substrates and biological sources (Corkey *et al.*, 1981; Ingebretsen *et al.*, 1982; Debuysere & Olson, 1983). The

present study required the resolution of the intermediates of 3-methyl-2-oxopentanoate oxidation, and this was achieved with a methanol/phosphate-buffer gradient. We have examined the effects of buffer concentration and pH on the retention times of a standard mixture of acyl-CoA esters (CoA, acetyl-CoA, propionyl-CoA, butyryl-CoA, crotonyl-CoA). The retention times increased with increasing buffer concentration and decreasing pH (results not shown). We have used a lower concentration of phosphate buffer (50 mM) than in previously reported methods to extend the range of acyl-CoA esters

analysed to C_6 . This lower phosphate concentration retains resolution of the lower- M_r esters, but avoids prolonged analysis times. Unsaturation decreased retention time (crotonyl-CoA < butyryl-CoA, 2-methylbut-2-enoyl-CoA < 2-methylbutyryl-CoA), as did the presence of an oxygenated functional group (2-methyl-3-oxobutyryl-CoA < 2-methylbutyryl-CoA).

Integration of the radiochemical detector signal provided an estimate of c.p.m. per chromatographic peak which was highly reproducible. The coefficients of variation obtained were very similar to those obtained by replicate counts of the same amounts of radioactivity by a conventional scintillation counter when the counting time was equal to the flow-cell transit time. The precision of the estimates of acyl-CoA radioactivity from replicate incubations is shown in Table 1. The coefficients of variation were in the range 3.7–12.6%, depending on the analyte. These values are of course higher than those obtained from replicate analyses of standard [$U-^{14}C$]acetyl-CoA, since there are errors associated with the incubations themselves. The analytical rigour of the method was confirmed by the results shown in Fig. 4. A linear relationship between radioactivity and mitochondrial protein was observed.

We have validated our experimental system by measuring the effects of riboflavin deficiency and of omission of bicarbonate (Table 2 and Fig. 2d). The effect of riboflavin deficiency was striking. In particular the ratio 2-methylbutyryl-CoA/2-methylbut-2-enoyl-CoA was elevated (control, mean = 4.9, S.E.M. = 0.8, $n = 9$; riboflavin-deficient animals, > 100). This result is consistent with a loss of branched-chain acyl-CoA dehydrogenase activity arising from riboflavin deficiency. An analysis of the urinary organic acids from these animals revealed the presence of large amounts of 2-methylbutyrylglycine and isovalerylglycine (results not shown), which confirms the loss of acyl-CoA dehydrogenase activity (Sherratt *et al.*, 1985). However, it is not clear whether this is due to loss of acyl-CoA dehydrogenase, electron-transferring flavoprotein (ETF), or ETF dehydrogenase, all of which are flavoproteins. The omission of bicarbonate resulted in an increase in propionyl-CoA and a decrease in succinyl-CoA and methylmalonyl-CoA. The K_m with respect to bicarbonate of propionyl-CoA carboxylase is 2 mM (Hegre & Lane, 1966) and presumably bicarbonate derived from the oxidation of endogenous fuels is insufficient to maintain V_{max} conditions. Thus the perturbation of isoleucine metabolism resulting from either riboflavin deficiency or omission of bicarbonate resulted in the expected changes in the pattern of acyl-CoA esters.

The concentration of the acyl-CoA esters in the mitochondrial matrix can be calculated from the specific radioactivity of 3-methyl-2-oxopentanoate; this involves a number of assumptions. Estimates of the matrix space depend on the nature of the extramitochondrial marker used; however, it has been argued that a value of 0.5 μ l/mg of protein obtained with mannitol as an impermeant marker provides the most reliable estimate (Halestrap & Quinlan, 1983). Using this value, and further assuming no dilution by 3-methyl-2-oxopentanoate or other endogenous intermediates, we calculate that the matrix concentration of methylmalonyl-CoA is 1.0 mM under our standard incubation conditions. This is in reasonable agreement with the value of 2.6 mM obtained by Corkey *et al.* (1982) in a study of valine catabolism by intact rat

hepatocytes. However, it is possible that there is dilution of labelled methylmalonyl-CoA or its precursors by endogenous compounds. These workers assumed a matrix volume of 0.2 ml/g dry wt. of liver (Tischler *et al.*, 1977). It is presumed that the metabolism observed is primarily in the mitochondrial matrix, although some small contribution from peroxisomes, which are an inevitable contaminant of the mitochondrial fraction, cannot be ruled out.

The results of the time-course experiment have several interesting features. The increase and subsequent decrease in 2-methylbutyryl-CoA is consistent with the known product inhibition of the branched-chain oxo acid dehydrogenase, which has been demonstrated with the chain isomer isovaleryl-CoA (Danner *et al.*, 1978; Parker & Randle, 1978a,b, 1980; Pettit *et al.*, 1978) and with a rise in the [NADH]/[NAD⁺] ratio. The accumulation of methylmalonyl-CoA, which was also noted by Corkey *et al.* (1982), confirms that methylmalonyl-CoA mutase may be a site of regulation and suggests that isoleucine as well as valine catabolism may be subject to multi-site regulation. Our estimate of the matrix concentration of methylmalonyl-CoA, although lower than that of Corkey *et al.* (1982), is still 4 times the K_m for methylmalonyl-CoA mutase (0.25 mM; Cannata *et al.*, 1966). The accumulation of methylmalonyl-CoA has another important implication. Methylmalonyl-CoA inhibits the branched-chain oxo acid dehydrogenase kinase and therefore causes the activation of the dehydrogenase (Paxton & Harris, 1984), which would result in positive feedback of the pathway. The slow accumulation of succinyl-CoA that we observed is presumably the consequence of our use of mitochondrial fractions rather than intact hepatocytes, in which succinyl-CoA would be partly utilized for gluconeogenesis. The pathway of isoleucine degradation is not closed, since it is possible that acyl-CoA esters could undergo hydrolysis, formation of glycine conjugates or formation of carnitine esters. Furthermore, acetyl-CoA is utilized for ketogenesis. The effects of carnitine and glycine on the flux through the pathway require examination. Although we have developed a radio-h.p.l.c. method because of the increased sensitivity and specificity which result from the use of labelled substrates, it was possible to detect some metabolites by u.v. absorption. Preliminary results suggest that it should be possible by dual detection to obtain directly an estimate of the specific radioactivity of some of the intermediates. This would allow the direct measurement of flux across each step of the pathway and thus an estimate of control strength.

We thank Action Research for the Crippled Child for the h.p.l.c. equipment. The University of Newcastle-upon-Tyne is thanked for the award of the John William Luccock Research Studentship to A.G.C. Dr. K. Veitch and Dr. H. S. A. Sherratt are thanked for the provision of riboflavin-deficient animals and for helpful discussion.

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