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Skeletal muscle mitochondrial β-oxidation

A study of the products of oxidation of [U-^{14}C]hexadecanoate by h.p.l.c. using continuous on-line radiochemical detection

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Well-coupled mitochondrial fractions were prepared from rat skeletal muscle without the use of proteolytic enzymes. The products of [U-^{14}C]hexadecanoate oxidation by rat skeletal muscle mitochondrial fractions were analysed by h.p.l.c. with on-line radiochemical detection. In the presence of 1 mM-carnitine, 70 % of the products is acetylcarnitine. In agreement with Veerkamp *et al.* [Veerkamp, van Moerkerk, Glatz, Zuurveld, Jacobs & Wagenmakers (1986) Biochem. Med. Metab. Biol. **35**, 248–259] $^{14}CO_2$ release is shown to be an unreliable estimate of flux through β-oxidation in skeletal muscle mitochondrial fractions. The flux through β-oxidation is recorded unambiguously polarographically in the presence of 1 mM-carnitine and the absence of citrate cycle intermediates.

INTRODUCTION

It has been known for some years that there is near stoichiometric production of ketone bodies from the β-oxidation of long-chain fatty acids by liver mitochondrial fractions, isolated hepatocytes and perfused liver preparations (Garland, 1968; Krebs & Hems, 1970). Investigation of the control of flux through β-oxidation has centred on studies of the liver, particularly with regard to the role of carnitine palmitoyltransferase and malonyl-CoA (McGarry & Foster, 1977, 1980). Relatively little attention has been paid to other, non-ketogenic, tissues. Since about 70 % of the energy requirements of resting muscle is met by fatty acid oxidation (Felig & Wahren, 1975), it is surprising that the control of β-oxidation in this tissue has not been studied more intensively.

In order to study the control of fatty acid β-oxidation in muscle it was essential to define the immediate products of β-oxidation by muscle mitochondrial fractions under different conditions. Many authors have measured the rate of $^{14}CO_2$ production from [^{14}C]fatty acids to determine the rate of fatty acid oxidation. [^{14}C]Acetyl units derived from [^{14}C]fatty acids, yield $^{14}CO_2$ from their oxidation by the citrate cycle at the isocitrate and 2-oxoglutarate steps only after the carbon atoms have completely traversed the cycle once (Greville, 1968). $^{14}CO_2$-release may seriously underestimate the rate of oxidation of [U-^{14}C]hexadecanoate by cultured cells, tissue homogenates or mitochondrial fractions. The measurement of radioactive acid-soluble metabolites in perchloric acid-quenched incubations is a better estimate of flux (Sherratt & Osmundsen, 1976; Veerkamp *et al.*, 1986). These acid-soluble metabolites may include citrate cycle intermediates and short-chain acylcarnitines (van Hinsbergh *et al.*, 1978), although they have not been rigorously identified. Our approach to this problem entails the incubation of rat skeletal muscle mitochondrial fractions with [U-^{14}C]hexadecanoate and measurement of $^{14}CO_2$ and [^{14}C]acid-soluble products which were further analysed by quantitative radio-h.p.l.c.

Inherited defects of mitochondrial β-oxidation are an important newly-discovered group of genetic diseases (Turnbull *et al.*, 1987). The diagnosis of these diseases is frequently attempted by measuring the release of $^{14}CO_2$ from [^{14}C]fatty acids of different chain lengths by cultured skin fibroblasts or blood leucocytes (Chalmers & English, 1987). This method often produces equivocal results, even from cell lines derived from patients with proven defects (Bennett *et al.*, 1987). CO_2 is a minor end-product of β-oxidation in preparations of non-ketogenic tissues. Measurement of other specific products may lead to the development of more satisfactory screening methods.

Part of this work has appeared in a preliminary form (Watmough *et al.*, 1987).

METHODS

Materials

Standard acyl-L-carnitine esters were prepared from their respective acyl chlorides (Bhuiyan *et al.*, 1987) and their purity was established by h.p.l.c. as described below. [U-^{14}C]Hexadecanoic acid (sp. radioactivity 34.3 GBq/mmol), [1-^{14}C]sodium acetate (sp. radioactivity 2.2 GBq/mmol), [2-^{14}C]sodium proprionate (sp. radioactivity 2.2 GBq/mmol), [1-^{14}C]acetyl-CoA (sp. radioactivity 2.2 GBq/mmol) and [^{14}C]sodium bicarbonate (sp. radioactivity 2.2 GBq/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). L-Carnitine and acetyl-L-carnitine were kindly donated by Sigma-tau s.p.a. (Rome, Italy). All h.p.l.c.

Abbreviations used: $S_{0.5}$, substrate concentration giving half maximal rate; BSA, bovine serum albumin.
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solvents were purchased from Rathbone Chemicals (Walkerburn, Scotland, U.K.). All other chemicals were of highest available purity unless otherwise specified.

Preparation of [1-^{14}C]acetylcarnitine

[1-^{14}C]Acetylcarnitine was synthesized from [1-^{14}C]-acetyl-CoA as follows. [1-^{14}C]Acetyl-CoA (5 μmol; sp. radioactivity 37 MBq/mmol) was incubated with 5 μmol of l-carnitine, 10 μmol of *N*-ethylmaleimide, 200 μunits of carnitine acetyltransferase, in a total volume of 7 ml for 1 h at 25 °C. The reaction mixture was applied to a Dowex 1 8X (chloride form) column, eluted with water and 4 ml was collected and freeze-dried. The residue was extracted with methanol and dried under N_2. The product was pure as judged by radio-h.p.l.c. (see below). The yield was 89%.

Preparation of rat skeletal muscle mitochondrial fractions

Mitochondrial fractions were prepared from hind-leg muscle of overnight-fasted male Wistar rats by a modification of the method of Makinen & Lee (1968). Hind-leg muscle was dissected out from rats killed by cervical dislocation, excess fat and connective tissue trimmed, weighed and placed in medium A [120 mM-KCl/20 mM-Hepes/5 mM-$MgCl_2$/1 mM-EGTA/BSA (5 mg/ml) pH 7.4]. The muscle (about 10 g) was chopped finely with scissors, rinsed twice with about 30 ml of medium A, passed through a hand mincer in 5 g batches, homogenized (Ystral Y/20, setting 9 for 5 s) and made up to 20 vol. with respect to the original wet wt. of tissue with medium A. Cell debris was removed by centrifugation (Sorvall RC5B centrifuge fitted with a ω^2dt integrator; rotor SS34) at 600 $g_{av.}$ for 10 min (4.08×10^7 rad$^2 \cdot$s). The pellet was resuspended in 8 vol. of medium A and recentrifuged. The supernatants were combined, filtered through four layers of cheesecloth to remove fat, and centrifuged at 17000 $g_{av.}$ for 10 min (1.09×10^9 rad$^2 \cdot$s). The pellet was resuspended in 10 vol. of medium A and centrifuged at 7000 $g_{av.}$ for 10 min (5.03×10^8 rad$^2 \cdot$s). The pellet was then resuspended in 5 vol. of medium B (300 mM-sucrose/2 mM-Hepes/0.1 mM-EGTA, pH 7.4) and centrifuged at 3500 $g_{av.}$ for 10 min (2.51×10^8 rad$^2 \cdot$s). This final pellet was resuspended in 0.5–1.5 ml of medium B to give a final protein concentration of 20–30 mg/ml.

Spectrophotometric and polarographic determination of oxidation rates

The mitochondrial oxidation of acetylcarnitine was measured by following the reduction of ferricyanide in a Hitachi 557 spectrophotometer at 420–475 nm (Turnbull *et al.*, 1982). O_2 uptake by mitochondrial fractions was measured polarographically at 30 °C and pH 7.4 in a final volume of 0.74 ml containing 1–3 mg of protein, 120 mM-KCl/10 mM-Hepes/10 mM-phosphate/1 mM-EDTA supplemented with BSA (2 mg/ml) and cytochrome *c* (0.2 mg/ml) (Sherratt *et al.*, 1988). Coupling conditions were obtained by adding 0.5 mM-ADP and uncoupling conditions by adding 20 μM-2,4-dinitrophenol. Respiratory control ratios were determined as described by Chance & Williams (1956) using 10 mM-pyruvate plus 1 mM-malate and ADP:O ratios were determined as described by Nicholls (1982).

Radiochemical incubation conditions

Incubations were made at 30 °C in a final volume of 1.00 ml containing 110 mM-KCl/10 mM-Hepes/5 mM-$MgCl_2$/10 mM-phosphate/1 mM-EGTA/cytochrome *c* (0.2 mg/ml)/5 mM-ATP/1 mM-carnitine/100 μM-CoASH, pH 7.4, and mitochondrial suspension (1–3 mg protein) contained in 20 ml glass scintillation vials fitted with silicone rubber seals. Each vial also contained a 0.4 ml microcentrifuge tube placed inside a 1.5 ml microcentrifuge tube. The vials were preincubated for 5 min with shaking (120 strokes/min) before the addition of 120 nmol of [U-^{14}C]hexadecanoate (sp. radioactivity 185 MBq/mmol) complexed to fat-free BSA (Boehringer Mannheim GmbH) in a molar ratio of 5:1. Duplicate incubations were quenched by the addition of 100 μl of 5 M-$HClO_4$ at various times (30–300 s), followed by 100 μl of 0.5 M-$KHCO_3$. Hyamine hydroxide [300 μl of 11% (w/v) in methanol] was added to the central microcentrifuge tube, and the vials were left at 20 °C for 2 h to allow complete trapping of $^{14}CO_2$. Radioactivity was determined in the hyamine solution by scintillation counting using the external-standard-channels ratio method. Preliminary experiments using $KH^{14}CO_3$ showed that this method gave quantitative recovery of $^{14}CO_2$ (results not shown).

Simple preparation

To the acid-quenched reaction mixture was added 98.2 nmol of [2-^{14}C]propionate (sp. radioactivity 3.7 MBq/mmol) and 250 nmol of propionylcarnitine as internal standards. Acid-insoluble material was removed by centrifugation (9000 g for 10 min). Total acid-soluble radioactivity was determined in 50 μl of the supernatant. Organic acids were determined in 600 μl of the supernatant by radio-h.p.l.c. (Causey *et al.*, 1986). Correction for dilution was made by reference to the [2-^{14}C]-propionate internal standard. Preliminary experiments showed that the recovery of standard [1-^{14}C]acetate from quenched mitochondrial incubations by this procedure was 98%. The remainder of the acid-soluble fraction (approx. 650 μl) was analysed for acylcarnitines by radio-h.p.l.c. as described below.

Radio-h.p.l.c. analysis of acylcarnitines

The perchloric acid supernatant was neutralized and applied to a 30 mm × 5 mm column of Dowex 50 × 8W (200–400 mesh) in the pyridinium form which was washed with 2 ml of 10 mM-HCl and 2 ml of water. The acylcarnitines were eluted with 3.5 ml of 0.5 M-pyridinium acetate, pH 4.5, in 50% (v/v) aqueous ethanol and recovered by freeze-drying which also removed the pyridinium acetate. The resultant acylcarnitines were derivatized as previously described (Bhuiyan *et al.*, 1987).

A Waters 600 solvent-delivery system with a Lichrosorb 10RP8 column (250 mm × 4.6 mm) was used for h.p.l.c. The column temperature was maintained at 30 °C. Samples were introduced with a Waters U6K injector. All solvents were de-aereated by continuous He sparging. Derivatized acylcarnitines were resolved by elution with a gradient of acetonitrile in water containing 4.5 mM-triethylamine phosphate (pH 5.6). The gradient was developed linearly from 70% (v/v) acetonitrile to a final concentration of 85% (v/v) acetonitrile over 20 min. This was preceded by 5 min isocratic elution at 70%

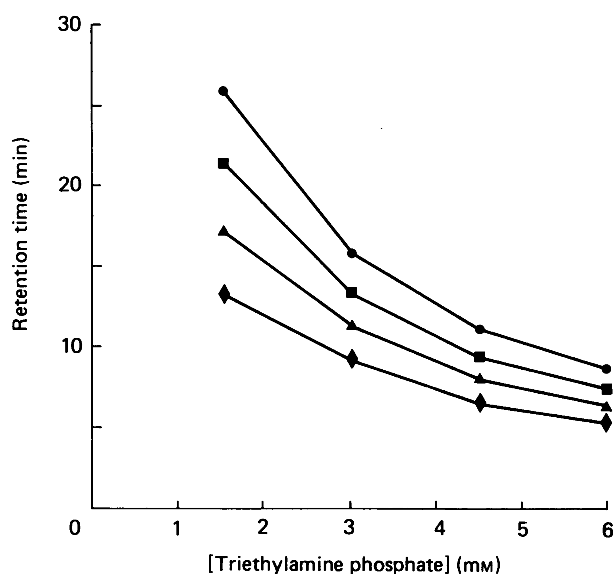

Fig. 1. Chromatographic behaviour of acylcarnitines

A standard mixture of the bromophenacyl derivatives of carnitine (◆), acetylcarnitine (▲), propionylcarnitine (■) and butyrylcarnitine (●) was analysed by h.p.l.c. with a mobile phase containing 70% (v/v) acetonitrile and varying concentrations of triethylamine phosphate. Other chromatographic conditions are described in the Methods section.

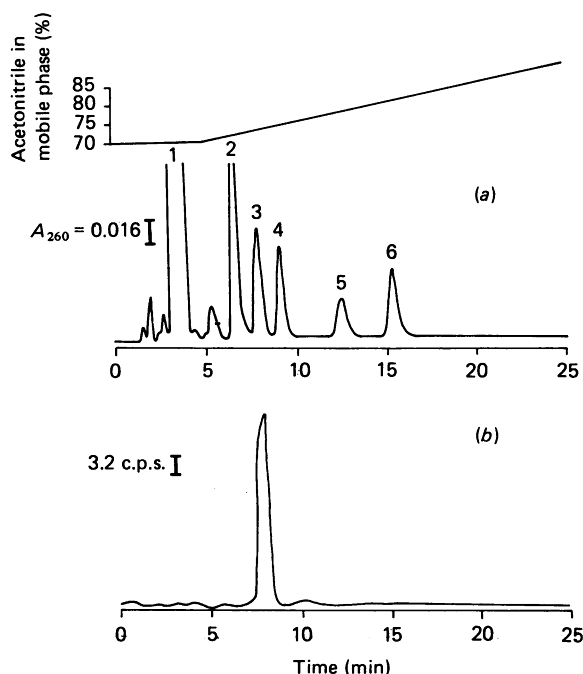

Fig. 2. H.p.l.c. of the *p*-bromophenacyl derivatives of acylcarnitine esters

(*a*) U.v. detection of a standard mixture: peak 1, excess derivatizing reagent; peak 2, carnitine; peak 3, acetylcarnitine; peak 4, propionylcarnitine (internal standard); peak 5, butyrylcarnitine; peak 6, 2-methylbutyrylcarnitine. (*b*) Radiochromatogram of the *p*-bromophenacyl derivatives of acylcarnitines derived from the incubation of a skeletal muscle mitochondrial fraction with [U-^{14}C]hexadecanoate.

(v/v) acetonitrile to elute excess derivatizing reagent ahead of the analytes of interest. The flow rate was 1.5 ml/min. This improves on the method of Tracey *et al.* (1986) in that it avoids excess derivatizing reagent chromatographing in the middle of the gradient. The concentration of triethylamine phosphate has a marked effect on retention times, peak shape and resolution. At high concentrations (> 5 mM) resolution is lost (Fig. 1), whereas at lower concentrations (< 4 mM) there is poor symmetry, peak broadening and prolonged retention times. This concentration (4.5 mM) is a compromise between these effects. There was little effect on chromatography by pH in the range 3–7 (results not shown). Nevertheless this system allowed baseline resolution of the compounds of interest (Fig. 2*a*).

Analytes were detected by a Pye-Unicam variable-wavelength u.v. detector (model LC3; 8 μl of flow cell; path length 10 mm). Radioactivity associated with eluted compounds was determined on-line with an LKB Betacord as described previously (Causey *et al.*, 1986), except that the scintillant flow-rate was 4.9 ml/min. The efficiency of counting was 77% and was independent of acetonitrile concentration as assessed by isocratic chromatography of known amounts of derivatized [1-^{14}C]acetylcarnitine at different acetonitrile concentrations. The theory of operation of continuous on-line radioactivity detectors has been fully discussed elsewhere (Reeve & Crozier, 1977; Causey *et al.*, 1986; Bartlett & Causey, 1988).

The u.v.-detector and radioactivity-detector flow cells were connected in series and the signals were acquired by a Waters chromatography data station (model 840). This allowed the generation of superimposable u.v. and radioactivity traces. The time lag between the two detectors was determined by chromatography of standard [1-^{14}C]acetylcarnitine.

Measurement of carnitine and acylcarnitines in tissues

Carnitine was determined by the radio-enyzmic method of McGarry & Foster (1976) with some modifications. Freeze-clamped tissue (approx. 100 mg) was homogenized with a Polytron homogenizer in 600 μl of 1 M-$HClO_4$ to which was added hexadecanoyl-[CH_3-^{3}H]carnitine (8000 d.p.m., 50 Ci/mol, 1.85 × 10^3 GBq) as internal standard (A. K. M. J. Bhuiyan & K. Bartlett, unpublished work). The homogenate was centrifuged and free and short-chain acylcarnitine esters were determined in the neutralized supernatant. For determination of long-chain acylcarnitine esters, the pellet was dissolved in 200 μl of 1 M-KOH and incubated at 50 °C for 2 h. Protein was precipitated by 300 μl of 1 M-$HClO_4$ and removed by centrifugation. The supernatant (400 μl) was neutralized with 95 μl of 1 M-KOH and 200 μl of 1 M-Hepes, pH 7.2, and precipitated $KClO_3$ was removed by centrifugation. Free carnitine formed by hydrolysis was determined and corrected for incomplete recovery by reference to the internal standard. Plasma samples were analysed in the same way except that the homogenization step was omitted.

RESULTS AND DISCUSSION

Preparation of mitochondria

The first requirement of this study was the reliable preparation in good yield of skeletal muscle mitochondrial fractions. Tissues were disrupted by mincing

Table 1. Recovery and respiratory characteristics of rat skeletal muscle mitochondrial fractions

Respiratory rates of six separate preparations of rat skeletal muscle mitochondrial fractions were determined as described in the Methods section using 10 mM-malate plus 1 mM-pyruvate as substrates. The results are expressed as means ± S.E.M. ($n = 6$).

Yield (mg of mitochondrial protein/g wet wt. of tissue)	Respiratory rate: State 4 (ngatoms of O $min^{-1}\cdot$mg of $protein^{-1}$)	Respiratory rate: State 3 (ngatoms of O $min^{-1}\cdot$mg of $protein^{-1}$)	Respiratory control ratio	ADP/O ratio
4.5 ± 0.3	66.5 ± 3.5	257.2 ± 15.5	3.9 ± 0.2	2.35 ± 0.03

followed by homogenization with a Ystral Y/20 tissue disintegrator, since the use of some proteolytic enzymes to enhance mitochondrial yield (Makinen & Lee, 1968), may inactivate the long-chain acyl-CoA synthetase (Pande & Blanchaer, 1970). BSA is included in the homogenization medium to prevent uncoupling of mitochondria by long-chain fatty acids and lysolecithins liberated during homogenization (van den Bergh, 1967; Scarpa & Lindsay, 1972). The recoveries of mitochondria in the fractions were about 20% of theoretical [based on citrate synthase and succinate ferricyanide reductase activities (results not shown), see Sherratt *et al.*, 1988]. Acceptable respiratory control ratios and ADP/O ratios were found indicating that the mitochondria were intact and well coupled although vigorous homogenization was needed to release them (Table 1). By contrast, rat liver mitochondria are uncoupled by vigorous homogenization (Aldridge, 1957). The exclusion of Mg^{2+} and the presence of 1 mM-EDTA to chelate Mg^{2+} in the polarographic incubation medium is also important. Mg^{2+} ions activate the myofibrillar ATPase which is an unavoidable contaminant of the mitochondrial fraction. Thus in the presence of Mg^{2+} ions, ATP, which is formed by oxidative phosphorylation, will be hydrolysed to ADP and inorganic phosphate and will maintain state 3 respiration with consequent apparent uncoupling (see Sherratt *et al.*, 1988).

Identification of the products of β-oxidation

Preliminary experiments showed that little free [^{14}C]acetate resulted from the incubation of a rat skeletal muscle mitochondrial fraction with [U-^{14}C]hexadecanoate. However following alkaline hydrolysis [^{14}C]acetate accounted for about 90% of the total acid-soluble products. Initial analysis of the ^{14}C-labelled products by radio-h.p.t.l.c. by the method of Bhuiyan *et al.* (1987) suggested that the major product was [^{14}C]acetylcarnitine. This contrasts with liver mitochondrial β-oxidation where the products are ketone bodies. We therefore devized radio-h.p.l.c. methods for the quantitative analysis of the labelled products of muscle mitochondrial β-oxidation described in the Methods section. The identity of [^{14}C]acetylcarnitine was confirmed by h.p.l.c. and Fig. 2(*b*) shows a radiochromatogram of the derivatized acylcarnitines formed during a 5 min incubation in the absence of malate.

Effect of carnitine concentration

Fig. 3 illustrates the effects of varying the concentration of carnitine in the absence of malate. The relative proportions of $^{14}CO_2$ and [^{14}C]acetylcarnitine depend on the concentration of carnitine. Thus below a concentration of 0.05 mM-carnitine, more $^{14}CO_2$ than [^{14}C]acetylcarnitine is formed, whereas at higher concentrations the reverse is true. This has important implications for the diagnosis of inherited defects of β-oxidation. It is common practice to measure only $^{14}CO_2$ generated by incubation of ^{14}C-labelled substrates with cultured human fibroblasts or tissue fractions to determine the flux through β-oxidation. Our results indicate that small variations in the carnitine concentration are likely to have a marked effect on the result in non-ketogenic tissues.

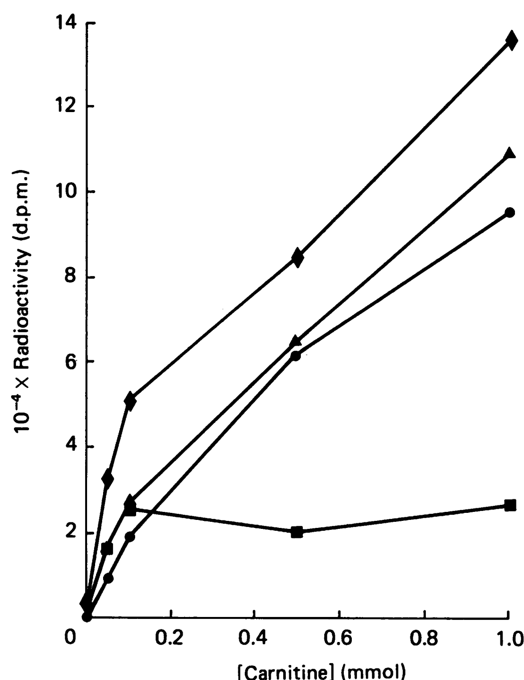

Fig. 3. Effect of carnitine on the distribution of the products of β-oxidation of [^{14}C]hexadecanoate by skeletal muscle mitochondrial fractions

Total ^{14}C-labelled products (◆), $^{14}CO_2$ (■), [^{14}C]acetylcarnitine (●), [^{14}C]acid-soluble products (▲). The conditions used are described in the Methods section.

Above a concentration of 0.1 mM-carnitine, the acid-soluble metabolites increased approximately linearly with respect to carnitine concentration. Thus at a concentration of 1.0 mM-carnitine, [^{14}C]acetylcarnitine (8×10^5 d.p.m.) represented 70% of the total products, and $^{14}CO_2$ represented 20% (2.7×10^5 d.p.m.), whereas at 0.05 mM-carnitine, [^{14}C]acetylcarnitine represented

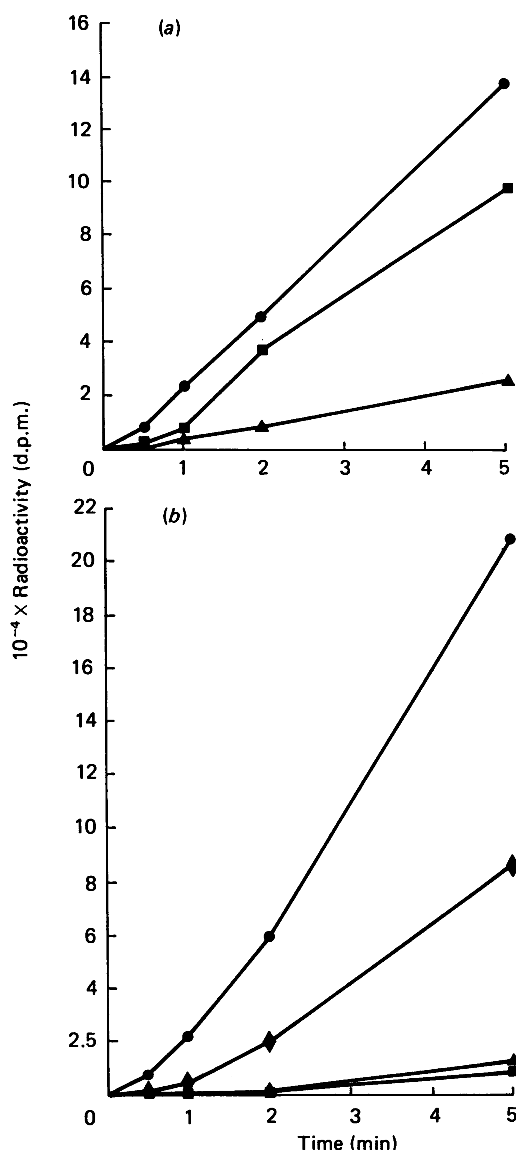

Fig. 4. Formation of the products of [U-^{14}C]hexadecanoate by skeletal muscle mitochondrial fractions with time in the absence (*a*) and presence (*b*) of 1 mM-malate

Conditions are described in the Methods section. Total ^{14}C-labelled products (●), $^{14}CO_2$ (▲), [^{14}C]acetylcarnitine (■), [^{14}C]succinate (◆).

27% of the total products (0.9×10^5 d.p.m.) and $^{14}CO_2$, 49% (1.7×10^5 d.p.m.) (Fig. 3). Under these conditions no labelling of acetate and tricarboxylic acid cycle intermediates was detected by radio-h.p.l.c. of organic acids (results not shown).

Effect of malate

The time courses of incubations were determined in the absence (Fig. 4*a*) and presence (Fig. 4*b*) of 1 mM-malate. In the absence of malate the major product was always [^{14}C]acetylcarnitine. In the presence of malate the major product was [^{14}C]succinate and the production of both [^{14}C]acetylcarnitine and $^{14}CO_2$ was decreased. A number of other citrate cycle intermediates (citrate, isocitrate and 2-oxoglutarate) were detected but poorly resolved on h.p.l.c.

The rate of β-oxidation, as estimated by the rate of production of $^{14}CO_2$ plus ^{14}C-labelled acid-soluble metabolites, was stimulated by malate. Moreover, the addition of malate increases the pool size of citrate cycle intermediates with consequent dilution of the label. This is shown by a decrease in $^{14}CO_2$ production relative to the total output of ^{14}C-labelled products (Fig. 4).

Conclusions

We interpret these findings as follows. In the absence of malate, acetylcarnitine is formed from acetyl-CoA by carnitine acetyltransferase, exported from the mitochondrial matrix and diluted into the bulk phase. However, in the presence of malate there is an adequate supply of oxaloacetate to combine with acetyl-CoA to form citrate which can enter the citrate cycle. Succinate, formed by the partial oxidation of citrate, is then exported in exchange for malate or phosphate. This is because muscle mitochondria lack a citrate transporter (Chappell & Robinson, 1968; England & Robinson, 1969).

Osmundsen & Sherratt (1975) determined the stoichiometry of O_2 uptake and the amount of substrate added, to investigate the completeness of β-oxidation in control and hypoglycin-poisoned rat liver mitochondria. This is possible because, if the citrate cycle is inhibited by either 5 mM-malonate or 20 μM-fluorcitrate, there is quantitative conversion of palmitate to ketone bodies by rat liver mitochondria and O_2 consumption is therefore an unambiguous measure of β-oxidation (see Garland, 1968).

Table 2. Tissue and plasma concentrations of carnitine, short-chain acylcarnitine and long-chain acylcarnitine in the rat

The results are expressed as means ± S.E.M. The numbers in parentheses indicate the numbers of observations.

Tissue	Concentration (nmol/g wet wt. of tissue or *nmol/ml) of: Carnitine	Short-chain acylcarnitine	Long-chain acylcarnitine	Proportion acylated (%)
Muscle (6)	573 ± 17	239 ± 18	78 ± 10	36 ± 2
Heart (6)	510 ± 21	230 ± 7	26 ± 1	34 ± 1
Liver (9)	167 ± 7	40 ± 4	22 ± 2	27 ± 2
Kidney (6)	293 ± 10	104 ± 8	20 ± 1	30 ± 2
Brain (6)	62 ± 4	11 ± 3	21 ± 2	34 ± 3
Plasma* (9)	28 ± 1	5 ± 0.5	1.1 ± 0.2	33 ± 2

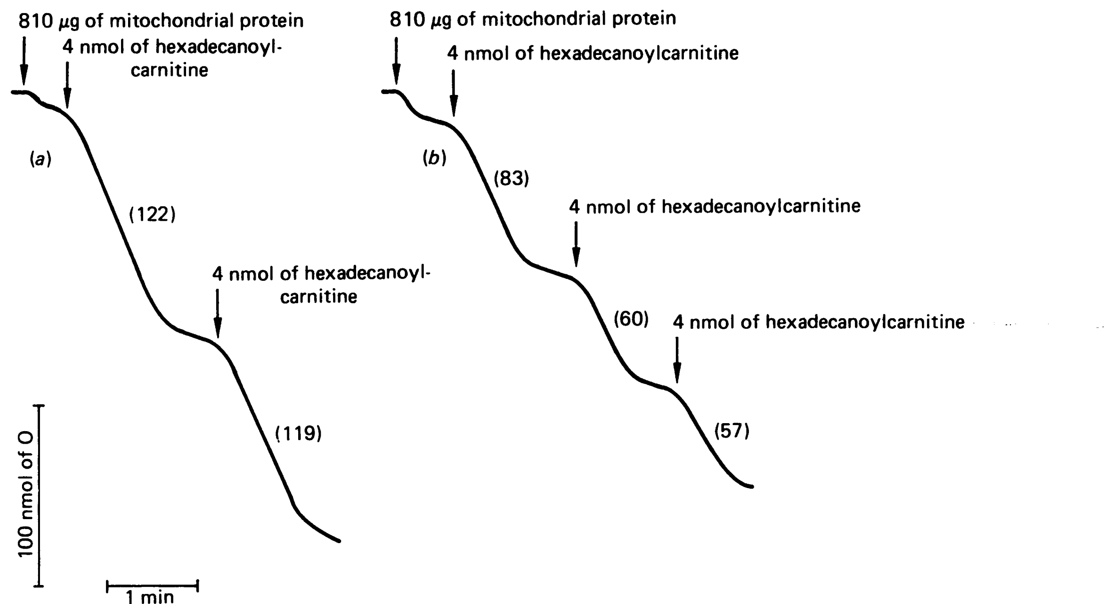

Fig. 5. Polarographic estimation of β-oxidation of hexadecanoylcarnitine by rat skeletal muscle mitochondrial fractions

Mitochondria were added to the medium containing 100 μM-2,4-dinitrophenol supplemented with either 1 mM-malate (*a*) or 1 mM-carnitine (*b*). Other conditions are described in the Methods section. The numbers in parentheses represent the extra oxygen (ngatoms) consumed after the addition of a limiting amount of hexadecanoylcarnitine. The larger first pulse in (*b*) can be explained by traces of endogenous citrate cycle intermediates causing some oxidation of acetyl-CoA.

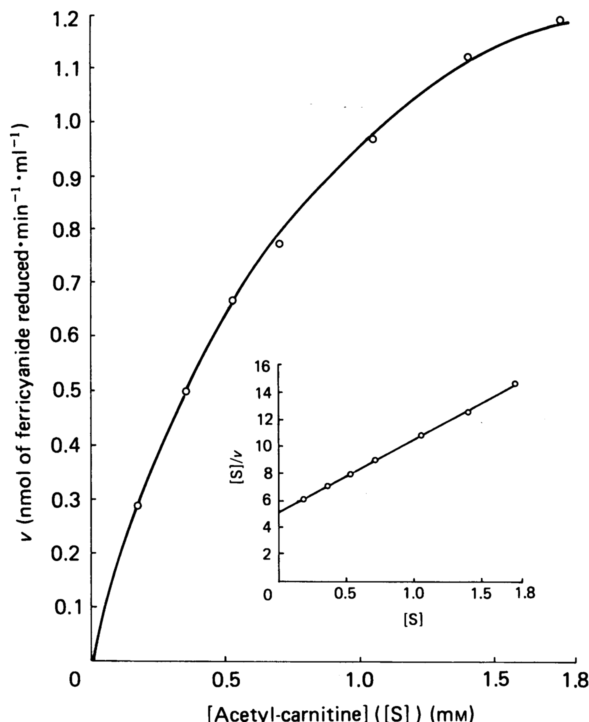

Fig. 6. Dependence on concentration of the rate of acetylcarnitine oxidation by rat skeletal muscle mitochondrial fractions

The rate of oxidation of acetylcarnitine at 30 °C was determined spectrophotometrically as described by Turnbull *et al.* (1982) in a final volume of 2.85 ml using 780 μg of mitochondrial protein in the presence of 1 mM-malate. The line of best-fit to the plot of [acetylcarnitine]/v versus [acetylcarnitine] (inset) and $S_{0.5}$ were determined to be 0.97 mM using the computerized direct linear plot method of Eisenthal & Cornish-Bowden (1974). The conditions are described in the Methods section. [S], [acetylcarnitine].

In the presence of 1 mM-carnitine and the absence of malate, > 70% of the products of hexadecanoate oxidized by rat skeletal muscle mitochondrial fractions is converted to acetylcarnitine (Fig. 4). It is reasonable to assume that, if a limiting amount of substrate is added to skeletal muscle mitochondria under identical conditions, the oxygen uptake will be a measure only of the flux through β-oxidation. This is illustrated in Fig. 5(*b*). The stoichiometry (14 ngatoms of O/nmol of hexadecanoylcarnitine) is consistent with the oxidation of hexadecanoylcarnitine as far as acetyl-CoA and its subsequent export as acetylcarnitine. If the carnitine is omitted and replaced with 1 mM-malate, both the rate and the amount of O_2 consumption is increased (Fig. 5*a*). However, the increased O_2 consumption is only about two thirds the theoretical maximum of 42 ngatom/nmol for the complete oxidation of hexadecanoylcarnitine, which indicates incomplete oxidation of the acetyl-CoA.

It has been established for some years that inhibition of the citrate cycle by either fluoroacetylcarnitine, a precursor of fluorcitrate (Bremer & Davis, 1973), or malonate (Pande, 1971) results in inhibition of oxidation of hexadecanoylcarnitine by skeletal muscle mitochondria. This inhibition can be overcome by the addition of carnitine and, under such conditions, O_2 uptake is due only to β-oxidation (Pande, 1971). Thus, in the presence of 1 mM-carnitine and the absence of malate, hexadecanoate-dependent O_2 consumption by skeletal muscle mitochondrial fractions is due solely to β-oxidation, and there is very little oxidation of the acetyl-CoA generated which is instead exported from the mitochondrion as acetylcarnitine.

The question that remains is whether such an acetyl buffer operates in skeletal muscle *in vivo*. There are several indications that it does. Firstly, heart and skeletal muscle contain high concentrations of short-chain

acylcarnitine compared with kidney, liver and brain (Table 2). Secondly, skeletal muscle mitochondrial fractions oxidize exogenous acetylcarnitine (Fig. 6). The rate has a hyperbolic relation to acetylcarnitine concentration and the $S_{0.5}$ is 0.97 mM. This is consistent with the K_m of carnitine acetyltransferase for acetylcarnitine (0.38 mM), and contrasts with the K_m for acetyl-CoA in the direction of acetylcarnitine formation which is lower (35 μM; Chase, 1967). Thirdly, acetylcarnitine is also generated from other sources of acetyl-CoA, such as pyruvate generated by heart mitochondrial fractions (Łisiack *et al.*, 1986). Finally, acetylcarnitine concentrations increase in rat skeletal muscle during high-intensity short-duration exercise (Carter *et al.*, 1981).

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