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Measurement of the acyl-CoA intermediates of β-oxidation by h.p.l.c. with on-line radiochemical and photodiode-array detection

Application to the study of [U-^{14}C]hexadecanoate oxidation by intact rat liver mitochondria

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The quantitative isolation of acyl-CoA esters of chain length C_2–C_{17} from mitochondrial incubations and their analysis by reverse-phase radio-h.p.l.c. is described. Photodiode-array detection was used to characterize 2-enoyl-CoA esters. The chromatographic behaviour of all 27 intermediates of the β-oxidation of hexadecanoyl-CoA is documented. Only C_{16}, C_{14} and C_{12} intermediates were detected in uncoupled mitochondria oxidizing [U-^{14}C]hexadecanoyl-CoA in the presence of fluorocitrate and carnitine, providing evidence for some organization of the enzymes of β-oxidation [Garland, Shepherd & Yates (1965) Biochem. J. **97**, 587–594; Sumegi & Srere (1984) J. Biol. Chem. **259**, 8748–8752]. Rotenone increased concentrations of 3-hydroxyacyl-CoA and 2-enoyl-CoA esters and inhibited flux. These experiments provide the first direct unambiguous measurements of acyl-CoA esters in intact respiring rat liver mitochondrial fractions.

INTRODUCTION

Mitochondrial β-oxidation of saturated acyl-CoA esters proceeds by a repeated cycle of four concerted reactions: flavoprotein-linked dehydrogenation, hydration, NAD^+-linked dehydrogenation and thiolysis. The three chain-length-specific acyl-CoA dehydrogenases which catalyse the first dehydrogenation step are linked to the respiratory chain by the electron-transferring flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QO). The second dehydrogenation step is catalysed by two chain-length-specific NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenases (El Fakhari & Middleton, 1982). The control of β-oxidation in the mitochondrial matrix occurs at several steps and depends on the redox state and the rate of recycling of CoA (Lopes-Cardozo & Van den Bergh, 1972, 1974*a*,*b*; Schultz, 1985). The rate is lowered with reduced states, since high NAD^+/NADH ratios impair the activity of the hydroxyacyl-CoA dehydrogenase (Bremer & Wojtczak, 1972; Latipää *et al.*, 1986) and increase the formation of ETF semiquinone (ETF_{sq}), which is a potent inhibitor of the acyl-CoA dehydrogenases (Beckmann *et al.*, 1981). These changes affect the steady-state concentrations of acyl-CoA intermediates, which in turn may change the control strength of other enzymes of the pathway.

In liver mitochondria, acetyl-CoA produced by each cycle of β-oxidation has four major routes of disposal: ketogenesis, oxidation by the citrate cycle, conversion into acetylcarnitine or hydrolysis to acetate; each of these reactions generates free CoA. During maximum flux through β-oxidation, up to 95% of the mitochondrial CoA pool is acylated (Garland *et al.*, 1965), and thus the rate of recycling of CoA may partly control β-oxidation (Billington *et al.*, 1979). Increased steady-state concentrations of some acyl-CoA esters may also occur when one or more of the enzymes of β-oxidation is inhibited, as in hypoglycin poisoning (Sherratt, 1986), or where one or more of the enzymes of the pathway is absent. Such inborn errors of β-oxidation are being increasingly recognized as important causes of disease, especially in children (Vianey-Liaud *et al.*, 1987; Turnbull *et al.*, 1988), and deficiencies of long-chain-acyl-CoA dehydrogenase (Hale *et al.*, 1985), medium-chain-acyl-CoA dehydrogenase (Stanley *et al.*, 1983), short-chain-acyl-CoA dehydrogenase (Turnbull *et al.*, 1984; Amendt *et al.*, 1987), ETF, ETF:QO (Frerman & Goodman, 1985) and acetoacetyl-CoA thiolase (Middleton & Bartlett, 1983) have been described.

In order to study the control of the enzymes of β-oxidation, their organization within the mitochondrial matrix, and their perturbation by disease and toxins, we developed a method to measure directly the 27 acyl-CoA intermediates of hexadecanoyl-CoA oxidation. Early methods for the analysis of CoA and its esters relied on fractionation by acid precipitation followed by measurement of non-esterified and esterified CoA by a variety of spectrophotometric, fluorimetric or luminometric methods (Michal & Bergmeyer, 1974). Synthetic acyl-CoA esters have been analysed by t.l.c. (Pullman, 1973) and ion-exchange chromatography (Gregolin *et al.*, 1968). More recently h.p.l.c. methods for the analysis of intact acyl-CoA esters using ion-pair reverse-phase chromatography have been described. Isocratic elution of a C_8 column with methanol/water containing tetrabutylammonium phosphate as an ion-pair reagent, resolved a mixture of nucleotides and short-chain acyl-CoA esters. A gradient of methanol in water separated a homologous series of acyl-CoA esters (C_2–C_{20}) and free

Abbreviations used: ETF, electron-transferring flavoprotein; ETF_{sq}, ETF semiquinone, ETF:QO, electron-transferring flavoprotein:ubiquinone oxidoreductase; CPT, carnitine palmitoyltransferase (EC 2.3.1.21); CAT, carnitine acetyltransferase (EC 2.3.1.19); PPO, 2,5-diphenyloxazole.

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CoA. Resolution was improved using a concave gradient of acetonitrile in water (Baker & Schooley, 1979, 1981). This system was used to characterize synthetic 2-methylacetoacetyl-CoA (Middleton & Bartlett, 1983) and to demonstrate the synthesis of valproyl-CoA from valproate by rat liver mitochondria (Turnbull *et al.*, 1983). However, the ion-pair reagent used is corrosive, and column performance rapidly deteriorates.

CoA, acetyl-CoA and dephospho-CoA was measured in neutralized $HClO_4$ tissue extracts using a C_{18} column with a phosphate buffer/methanol mobile phase (Ingebretsen & Farstad, 1980; Ingebretsen *et al.*, 1979, 1982). This method was modified by eluting a C_{18} column stepwise with increasing concentrations of methanol. This separated the acyl-CoA ester intermediates of leucine and valine catabolism and was used to study the regulation of these pathways in rat hepatocytes (Corkey *et al.*, 1981, 1982). Similar results were obtained using a complex phosphate/methanol gradient (De Buysere & Olson, 1983). The addition of chloroform to the mobile phase resulted in improved resolution of 3-hydroxy-3-methylglutaryl-CoA, succinyl-CoA, acetoacetyl-CoA and acetyl-CoA. Replacing methanol with acetonitrile enables a wider range of acyl-CoA esters, particularly with respect to chain length, to be separated (Woldegiorgis *et al.*, 1985; Causey & Bartlett, 1986).

Measurement of acyl-CoA ester concentrations by h.p.l.c. in hepatocytes, isolated mitochondrial fractions and freeze-clamped tissues have relied entirely on u.v. detection at 260 nm and co-chromatography with standard compounds (Corkey *et al.*, 1981, 1982; King & Reiss, 1985; Woldegiorgis *et al.*, 1985; Hosokawa *et al.*, 1986; Malaparast *et al.*, 1988). This is not only rather poor proof of identity, but is insensitive. An alternative approach using continuous on-line radiochemical detection of acyl-CoA esters has been developed (Bartlett & Causey, 1988; Bartlett *et al.*, 1988). This has previously been used to study 3-methyl-2-oxopentanoate catabolism by rat liver mitochondrial fractions (Causey *et al.*, 1985, 1986). However, reliance on radiochemical detection alone can lead to errors. For example acylcarnitine esters as well as acyl-CoA esters are extracted by chloroform/methanol (Bhuiyan & Bartlett, 1988). We therefore have devised a sample extraction procedure which removes acyl-carnitines. The combination of sample preparation, radiochemical detection and photodiode-array detection results in unambiguous identification and measurement of acyl-CoA esters generated from the oxidation of [U-^{14}C]hexadecanoate by isolated mitochondria.

EXPERIMENTAL

Materials

Acyl-CoA oxidase (EC 6.2.1.3), bovine serum albumin (Fraction V; fatty-acid-free), CoA (grade II; trilithium salt), cytochrome *c*, ADP and ATP were supplied by Boehringer Corp., Mannheim, Germany. Acetyl-CoA, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA and dodecanoyl-CoA were supplied by Sigma Chemical Co. Poole, Dorset, U.K. L-Carnitine was a gift from Sigma-Tau Spa, Rome, Italy. DEAE-Sephacel was supplied by Pharmacia. Acetonitrile (S grade) was purchased from Rathburn Chemicals, Walkerburn, Peebleshire, Scotland, U.K. H.p.l.c.-grade water, Analar-grade chloroform, methanol, xylene, Scintran-grade Triton X-100 and Scintran-grade 2,5-diphenyloxazole (PPO) were supplied by BDH, Poole, Dorset, U.K. Soluble bovine liver carnitine palmitoyltransferase (CPT) (Ramsay *et al.*, 1987) was a gift from Mr. J. P. Derrick, Department of Biochemistry, University of Cambridge, Cambridge, U.K.

[U-^{14}C]Hexadecanoate (34.3 GBq/mmol) was obtained from Amersham International, Amersham, Bucks., U.K., and 1.2 mM-[U-^{14}C]hexadecanoate (155.4 MBq/mmol) complexed to albumin in a 5:1 molar ratio was prepared (Sherratt *et al.*, 1988).

Synthesis of acyl-CoA esters

Tetradecanoyl-CA, hexadec-2-enoyl-CoA, hexadecanoyl-CoA, [U-^{14}C]hexadecanoyl-CoA and heptadecanoyl-CoA were prepared from their respective *N*-hydroxysuccinimide esters by the method of Al-Ahrif & Blecher (1969), modified as described by Osmundsen *et al.* (1987). Hex-2-enoyl-CoA, oct-2-enoyl-CoA and dec-2-enoyl-CoA were prepared from the corresponding mixed anhydrides by the method of Benert & Sprecher (1977). Hexadec-2-ynoic acid, synthesized by the method of Wood & Lee (1981), was used to prepare the corresponding CoA ester from the mixed anhydride (Benert & Sprecher, 1977). This was converted to 3-oxohexadecanoyl-CoA with crotonase (Thorpe, 1986).

Standard mixtures of some acyl-CoA, 2-enoyl-CoA, 3-hydroxyacyl-CoA and 3-oxoacyl-CoA esters were prepared enzymically from saturated acyl-CoA esters using acyl-CoA oxidase, crotonase and 3-hydroxyacyl-CoA dehydrogenase.

Preparation of rat liver mitochondrial fractions

Mitochondrial fractions were prepared from the livers of 18 h-starved male Wistar rats (180–220 g) in 250 mM-sucrose/2 mM-Hepes/0.1 mM-EGTA, pH 7.5 at 0–4 °C, and resuspended in isolation medium at a concentration of 25–40 mg·ml^{-1} (Causey *et al.*, 1986). Respiratory-control ratios, measured with 10 mM-glutamate plus 1 mM-malate as substrate (Watmough *et al.*, 1988), were routinely 5.5–7.5. Mitochondrial protein was determined by the method of Peterson (1978), with bovine serum albumin as standard.

Incubations with [U-^{14}C]hexadecanoate

Incubations were made in 1.0 ml of medium containing 110 mM-KCl, 2.5 mM-phosphate, 1 mM-EDTA, 5 mM-$MgCl_2$, cytochrome *c* (0.2 mg/ml), 50 μM-sodium fluorocitrate, 0.5 mM-L-carnitine, excess ATP (5 mM), 0.1 mM-CoA and 40 μM-dinitrophenol, pH 7.5, using 3.2–6 mg of mitochondrial protein at 30 °C in a shaking water bath (160 strokes/min). After 5 min preincubation the reactions were started by addition of 36 nmol of [U-^{14}C]-hexadecanoate and terminated as appropriate by addition of 200 μl of acetic acid followed by 20 nmol of heptadecanoyl-CoA internal standard. A 50 μl sample was withdrawn and added to 150 μl of 5 M-$HClO_4$. Precipitated protein and remaining substrate were removed by centrifugation (90000 $g_{av.}$·min) and radioactivity measured in 150 μl of the supernatant by scintillation counting. The remainder of each sample was used to prepare an acyl-CoA fraction for analysis as described below.

Preparation of fraction containing acyl-CoA esters

The sample preparation is summarized in Scheme 1.

Incubation mixture (1 ml)
↓
Quench with 200 μl of acetic acid and 20 nmol of heptadecanoyl-CoA internal standard
↓
Portion (50 μl) removed for determination of acid-soluble radioactivity
↓
Satd. $(NH_4)_2SO_4$ (100 μl) added to remainder and mixture heated at 100 °C for 2 min
↓
Extracted twice with 12 ml of diethyl ether
↓
Aqueous phase extracted with 8 ml of methanol/chloroform (2:1, v/v) for 1 h
↓
Centrifuge (40000 $g_{av.}$·min); pellet re-extracted with 3 ml of methanol/chloroform
↓
Solvent removed from combined extracts under N_2 at 60 °C
↓
Add 2 ml of 1 M-ammonium acetate and freeze-dry
↓
Residue extracted with 2.4 ml of methanol and centrifuged (90000 $g_{av.}$·min)
↓
Pellet re-extracted with 0.8 ml of methanol
↓
H.p.l.c.-grade water (0.8 ml) added to combined extracts and applied to DEAE-Sephacel
↓
Acylcarnitines eluted with 5 ml of methanol/water (4:1; v/v) and discarded
↓
Acyl-CoA esters eluted with 6 ml of methanol/water (4:1; v/v) containing 0.5 M-ammonium acetate and 10 mM-acetic acid
↓
Methanol removed under N_2 at 60 °C and 2–3 ml of h.p.l.c.-grade water added and the mixture freeze-dried
↓
Residue dissolved in 500 μl of h.p.l.c.-grade water and 200 μl of the mixture analysed by radio-h.p.l.c.

Scheme 1. Isolation of acyl-CoA esters from mitochondrial incubations

Each sample was transferred to an extraction tube containing 100 μl of saturated $(NH_4)_2SO_4$ and placed in a boiling-water bath for 2 min, and then allowed to cool to room temperature. Each sample was extracted twice with 10 vol. of diethyl ether to remove free hexadecanoate and then extracted for 1 h with 8 ml of methanol/chloroform (2:1, v/v) with continuous agitation. After centrifugation (40000 $g_{av.}$·min) the supernatant was retained. The pellet was resuspended in 3 ml of methanol/chloroform (2:1, v/v) and re-centrifuged. The solvent was removed from the combined supernatants with a stream of N_2 at 60 °C until the volume was about 2 ml. The pH was adjusted to 6–7 by adding 2.0 ml of 1 M-ammonium acetate, and the sample freeze-dried.

The residue was extracted with 2.4 ml of methanol, centrifuged (90000 $g_{av.}$·min) and the supernatant retained. The pellet was resuspended in 0.8 ml of methanol, re-centrifuged, and the combined supernatants diluted to a final volume of 4.0 ml with 0.8 ml of h.p.l.c.-grade water. This was applied to a column (100 mm × 6 mm) of DEAE-Sephacel (acetate form) and washed with 5 ml of methanol/water (4:1, v/v). The fraction containing acyl-CoA esters and CoA was eluted with 6 ml of methanol/water (4:1, v/v) containing 0.5 M-ammonium acetate and 10 mM-acetic acid, diluted with 2–3 ml of h.p.l.c.-grade water and freeze-dried. The residue was dissolved in 500 μl of h.p.l.c.-grade water and 200 μl was analysed by radio-h.p.l.c.

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

A Waters 600 solvent-delivery system with a Lichrosorb 10RP18 column (250 mm × 4.6 mm) was used for h.p.l.c. The column was maintained at 30 °C. Samples were introduced with a Waters U6K injector. All solvents were deaerated with helium. Acyl-CoA esters were resolved by the following binary gradient of acetonitrile in 50 mM-KH_2PO_4, pH 5.3: isocratic 5% (v/v), 5 min; isocratic 10%, 0.1 min; linear gradient 10–30%, 9.9 min; linear gradient 30–50%, 30 min; linear gradient 50–5%, 5 min. The flow rate was 2.0 ml·min^{-1} and the total run time 52 min. The column was re-equilibrated for at least 10 min under the starting conditions between analyses to maintain reproducibility of retention times.

Analytes were detected with a Waters 990 photodiode-array detector (8 μl flow cell; 10 mm path length). Spectra were acquired at 27 ms intervals over the range 200–300 nm with a bandwidth of 1.4 nm. Spectral data was acquired on an NEC APC IV microcomputer using dedicated software. Radioactivity associated with eluted compounds was detected on-line with an LKB Betacord radioactivity monitor fitted with a 1 ml flow cell as described previously (Causey *et al.*, 1986; Watmough *et al.*, 1988). Since the rate of flow of effluent from the photodiode-array detector was 2.0 ml/min, the flow rate of the scintillation fluid (10 g of PPO, 330 ml of Triton X-100, 150 ml of methanol and 670 ml of xylene) was 8 ml/min. The photodiode-array detector and radioactivity monitor were connected in series, and analogue signals from each detector were acquired by a Waters chromatography data station (model 840). This allowed the generation of superimposable radiochemical and u.v. traces. The time lag between the detectors was determined by using [U-^{14}C]hexadecanoyl-CoA.

RESULTS AND DISCUSSION

Optimization of the h.p.l.c. system

The chromatographic conditions described by Causey & Bartlett (1986) do not resolve long-chain-fatty-CoA esters adequately. A shallower gradient of acetonitrile gave considerably improved resolution of all the saturated acyl-CoA esters which are intermediates of β-oxidation as well as resolving heptadecanoyl-CoA added as internal standard (Fig. 1*a*). 2-Enoyl-CoA esters have retention times about 1 min less than those of saturated acyl-CoA esters of the same chain length (Fig. 1*b*). A hydroxy substituent at the 3-position of the acyl group decreases the retention time by an extent which is slightly greater than the effect of the loss of an ethylene unit (Fig. 1*c*). Thus 3-hydroxy(C_n)acyl-CoA is only partially resolved from 2-enoyl(C_{n-2})-CoA (e.g. 3-hydroxyhexadecanoyl-CoA and tetradec-2-enoyl-CoA). The order of elution of acyl-CoA esters of given chain length is as follows: acyl-CoA, 2-enoyl-CoA, 3-oxoacyl-CoA, 3-hydroxyacyl-CoA. β-Oxidation of hexadecanoyl-CoA results in the formation of seven families of acyl-CoA esters which have overlapping retention times. Representative chromatograms of standard compounds are shown in Figs. 1(*a*)–1(*d*), and a complete listing of retention times is given in Table 1.

Identification of acyl-CoA intermediates of mitochondrial β-oxidation

Photodiode detection enables the acquisition of absorption spectra at intervals throughout the chromato-

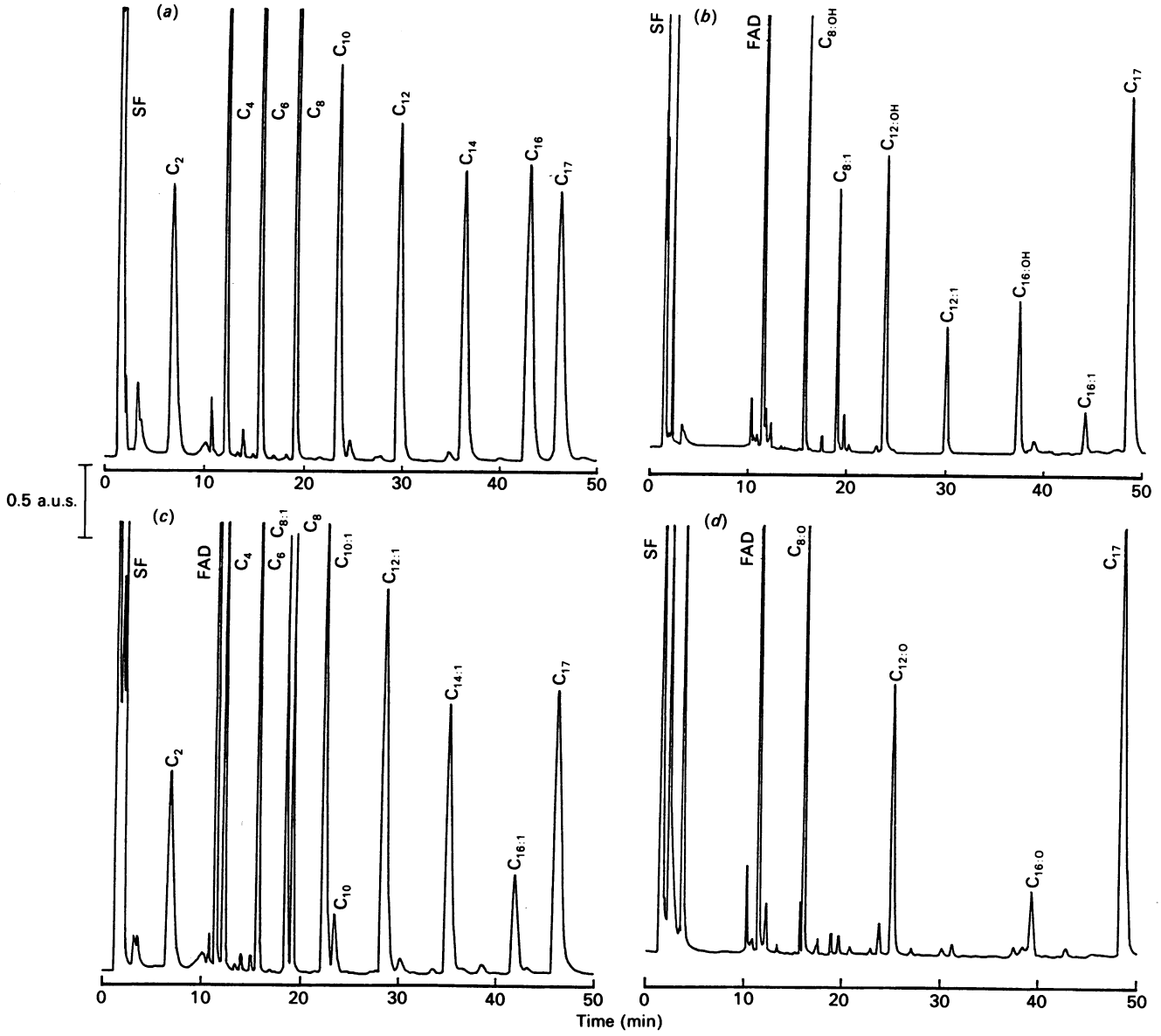

Fig. 1. Separation of standard acyl-CoA esters by h.p.l.c.

(*a*) Saturated acyl-CoA esters; (*b*) 3-hydroxyacyl-CoA esters; (*c*) 2-enoyl-CoA esters; (*d*) 3-oxoacyl-CoA esters. The carbon numbers are as indicated (C_2, C_6 etc.). 2-Enoyl-CoA esters are indicated by ':1', 3-hydroxyacyl-CoA esters are indicated by ':OH', and 3-oxoacyl-CoA esters are indicated by ':O'. Chromatographic conditions are as described in the Experimental section, SF, solvent front; a.u.s., absorbance units scale.

graphic run. The double bond of a 2-enoyl-CoA ester is conjugated with the thioester bond, which shifts the thioester absorbance maximum from 232 nm to 263 nm, superimposing it on the absorbance due to the adenine group of CoA at 260 nm. This increases the molar absorption of a 2-enoyl-CoA ester at 260 nm and decreases it at 232 nm compared with saturated acyl-CoA esters. These characteristic spectral properties enabled identification of 2-enoyl-CoA esters even where they were poorly resolved from saturated or 3-hydroxyacyl-CoA esters (Fig. 2). However, some analytical problems remain. The use of a 1.0 ml flow cell and the 3:1 dilution of the column eluent with scintillant in the radiochemical monitor results in some band broadening. Thus the partially resolved complex of peaks at 36 min in Fig. 2 (peaks 4–6) is recorded by the photodiode-array detector (flow-cell volume 8 μl) and chromatographic fidelity is maintained. However, definition is lost by the radiochemical monitor. This complicates determination in mitochondrial acyl-CoA esters, since the complex comprises a mixture of 3-hydroxyhexadecanoyl-CoA (ϵ_{260} = 16000 litre·mol^{-1}·cm^{-1}; sp. activity identical with that of hexadecanoate) and tetradec-2-enoyl-CoA (ϵ_{260} 22000 litre·mol^{-1}·cm^{-1}; sp. activity 7/8 of that of hexadecanoate). The molar ratios of these analytes were calculated from peak-area ratios, taking account of the difference in molar absorption coefficients. Clearly the sensitivity of radiochemical detection depends on the carbon number of the metabolite derived from a universally labelled substrate. Since the molar specific ac-

Table 1. Retention times of acyl-CoA esters

Chromatographic conditions are given in the Experimental section. nd, not detected.

Chain length	Ester...	Relative retention time			
		Saturated	2-Enoyl	3-Hydroxy-acyl	3-Oxo-acyl
C_2		0.150			
C_4		0.267	0.250	0.186	0.166
C_6		0.340	0.320	0.258	nd
C_8		0.414	0.402	0.327	0.341
C_{10}		0.508	0.485	0.411	0.422
C_{12}		0.643	0.617	0.491	0.518
C_{14}		0.789	0.761	0.624	0.659
C_{16}		0.931	0.908	0.765	0.807
C_{17}		1.000			

tivity of acetyl-CoA is only 1/8 of that of palmitoyl-CoA, simple inspection of a radiochromatogram can be misleading.

To our knowledge, the present study provides the first unambiguous demonstration of acyl-CoA intermediates of β-oxidation in intact respiring mitochondria. Mitochondria were incubated under uncoupling conditions to allow maximum rates of electron transport and with the citrate cycle inhibited by fluorocitrate to minimize endogenous respiration. With these conditions the only intermediate consistently detected was tetradecanoyl-CoA, although in experiments with higher protein concentrations (Table 3 below) small amounts of dodecanoyl-CoA could be detected. Tetradecanoyl-CoA was only detected during maximal flux through β-oxidation. There is no evidence for any kinds of acyl-carrier protein for β-oxidation (by analogy with the fatty acid synthetase complex), and it must be assumed that the concentrations of all the other acyl-CoA intermediates in the β-oxidation of hexadecanoate were below the limits of detection. These results are in broad agreement with those of Stanley & Tubbs (1974, 1975), who used radio-g.l.c. to detect methyl esters of fatty acids released by the alkaline hydrolysis of compounds formed by rat liver mitochondria incubated with [16-^{14}C]hexadecanoylcarnitine in the presence of 10 mM-oxaloacetate. Those authors detected hexadecanoate, tetradecanoate and dodecanoate, and the highest concentrations formed coincided with maximum flux through the pathway.

Preparation of an acyl-CoA fraction from mitochondrial incubations

Since short-chain ($< C_8$-fatty-acyl-CoA esters are acid-soluble, a neutralized $HClO_4$ extract can be used for their analysis (Causey *et al.*, 1986). However, long-chain-fatty-acyl-CoA esters are precipitated with protein and other material in $HClO_4$-quenched samples. Causey & Bartlett (1986) reported good recovery of butyryl-CoA, octanoyl-CoA and tetradecanoyl-CoA standards from mitochondrial fractions using a modification of the method of Mancha *et al.* (1975) and stressed the requirement for the addition of $(NH_4)_2SO_4$. However, in this procedure other acylated classes such as acylcarnitine are also extracted (Bhuiyan & Bartlett, 1988), which complicates the interpretation of the radiochromatograms. Thus there are compounds generated in mitochondrial incubations with [U-^{14}C]hexadecanoate which are radioactive but non-u.v. absorbing at 260 nm. Furthermore, in analyses of samples prepared by the method of Mancha *et al.* (1975), the early part of the u.v. chromatograms are obscured by the presence of a large number of 260 nm-absorbing compounds, presumably nucleotides.

Extraction with diethyl ether of the acidified sample removes excess substrate, and an ion-exchange resin separates acyl-CoA esters from acylcarnitine esters. Pande *et al.* (1986) reported an assay for CPT in which the [1-^{14}C]hexadecanoylcarnitine formed was separated from the substrate ([1-^{14}C]hexadecanoyl-CoA) by DEAE-Sephacel chromatography. The combination of a polar matrix (Sephacel) and a mobile phase containing 80% (v/v) methanol prevents non-specific binding of long-chain-acyl-CoA to the matrix, which occurs when resins which have hydrophobic matrices, such as Dowex, are used. Preliminary studies showed that 2.5 nmol of [U-^{14}C]hexadecanoyl-CoA could be recovered efficiently (86%) from a DEAE-Sephacel column eluted with 0.2 M-LiCl in methanol/water (4:1, v/v). Furthermore, hexadecanoyl[*methyl*-^{3}H]carnitine could be eluted quantitatively by water under conditions where acyl-CoA esters were retained. However, lithium salts are very soluble in methanol, and their subsequent separation from acyl-CoA esters is difficult. Ammonium acetate (0.5 M) in methanol/water (4:1, v/v) gave similar recoveries and can be removed by freeze-drying. The chromatograms shown in Fig. 3 illustrate the improved sample clean-up by this procedure.

Recovery and reproducibility of the extraction of acyl-CoA esters

The recoveries of butyryl-CoA, octanoyl-CoA and heptadecanoyl-CoA from quenched mitochondrial incubations were determined. Preliminary experiments showed variable loss of long-chain acyl-CoA esters. Since the solvent-extraction step, the DEAE-Sephacel step and the freeze-drying cycles involved no chain-length-dependent losses, an alternative explanation was sought. Rat liver mitochondrial fractions contain long-chain acyl-CoA hydrolase activity (Berge & Farstad, 1979). Quenching incubation with acetic acid lowers the pH to about 3 without completely denaturing all mitochondrial proteins. The subsequent diethyl ether extraction to remove the substrate also removes some acetic acid, causing the pH to rise sufficiently for hydrolase activity. However, with the inclusion of treatment of 100 °C to inactivate acyl-CoA hydrolase and other enzymes which might distort the pattern of acyl-CoA esters observed (Scheme 1), the recoveries were essentially the same for all chain lengths and were reproducible (Table 2).

It was necessary to establish that the sample preparation and internal standardization gave reproducible results with radiolabelled acyl-CoA esters generated in mitochondrial incubations. Five replicate incubations were quenched after 90 s, when flux through β-oxidation was maximal. The samples were internally standardized with 20 nmol of heptadecanoyl-CoA and prepared for h.p.l.c. analysis. The results were reproducible irrespective of the chain-length and concentration of the acyl-CoA esters (Table 3).

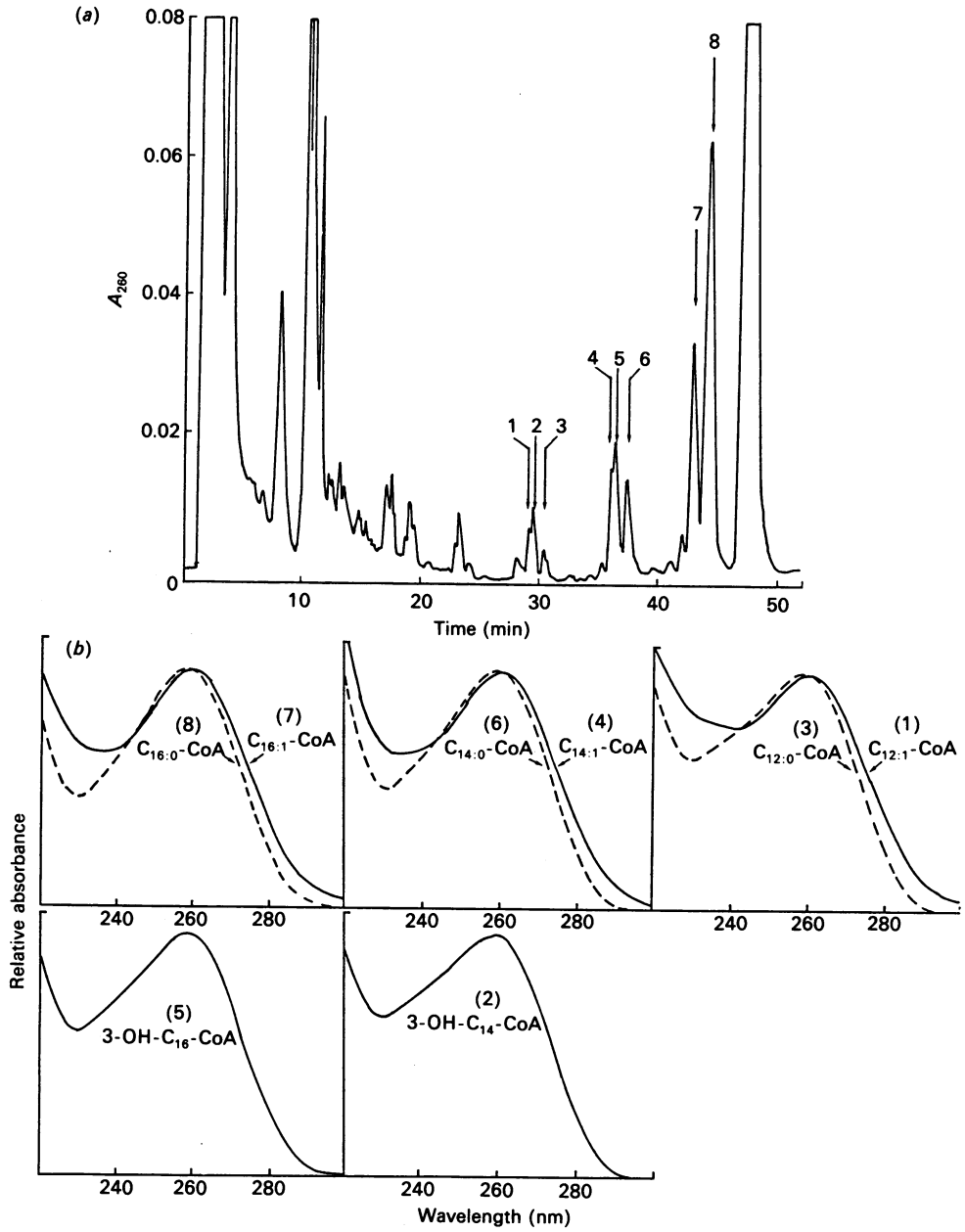

Fig. 2. H.p.l.c. analysis of acyl-CoA esters using photodiode-array detection

Mitochondrial fractions were incubated with palmitate in the presence of rotenone for 90 s, and the acyl-CoA ester fraction was isolated as described in the Experimental section. (*a*) Chromatogram of analytes derived from two pooled incubations detected at 260 nm. (*b*) Spectra were acquired at the points indicated in the chromatogram: 1, dodec-2-enoyl-CoA; 2,3-hydroxytetradecanoyl-CoA; 3, dodecanoyl-CoA; 4, tetradec-2-enoyl-CoA; 5, 3-hydroxyhexadecanoyl-CoA; 6, tetradecanoyl-CoA; 7, hexadec-2-enoyl-CoA; 8, hexadecanoyl-CoA.

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

The appearance of radioactive acyl-CoA esters with respect to time and the effect of rotenone were examined during the oxidation of a limiting amount of [U-^{14}C]hexadecanoate. Duplicate incubations were quenched at various times between 15 and 240 s, and the flux through β-oxidation was assessed by measurement of acid-soluble metabolites (Fig. 4*a*). The rate was maximal between 60 and 90 s after the addition of substrate (36 nmol of [U-^{14}C]hexadecanoate), and oxidation was essentially complete after 180 s. Rotenone decreased the maximal rate, and the reaction did not go to completion over the time course studied. In the absence of rotenone hexadecanoyl-CoA, tetradecanoyl-CoA and acetyl-CoA were detected (Figs. 4*a*, 4*e* and 4*g* respectively). Maximal concentrations of hexadecanoyl-CoA, tetradecanoyl-CoA and acetyl-CoA occurred after 90 s. In the presence of rotenone, which inhibits NADH dehydrogenase by about 95% (Slater, 1967) and therefore lowers the [NAD^+]/[NADH] ratio in the matrix of respiring mitochondria, hexadec-2-enoyl-CoA, 3-hydroxyhexadecanoyl-CoA and tetradec-2-enoyl-CoA were detected and attained steady-state concentrations

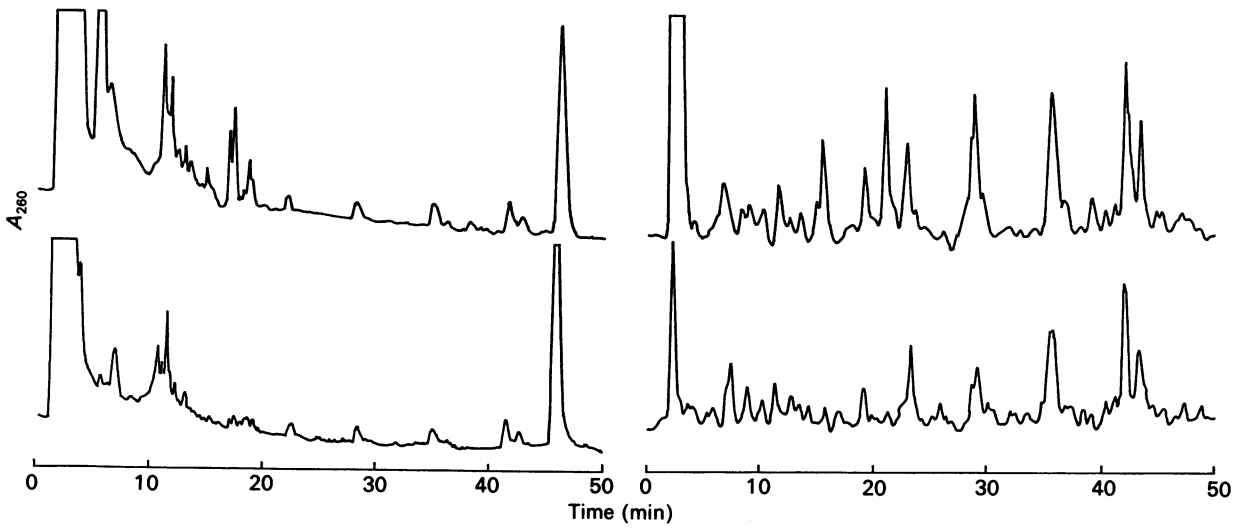

Fig. 3. Radio (right) and u.v. (left) chromatograms of extracts of incubations of mitochondrial fractions with [U-^{14}C]palmitate

Upper panels, without prior purification with DEAE-Sephacel; lower panels, with prior purification with DEAE-Sephacel.

Table 2. Recovery of acyl-CoA esters from mitochondrial incubations

Either 20 or 5 nmol each of butyryl-CoA, octanoyl-CoA and heptadecanoyl-CoA were added to quenched mitochondrial incubations (6.0 mg of protein). Samples were prepared for h.p.l.c. analysis as described in the Experimental section, except that hexanoyl-CoA was used as a chromatographic internal standard and was added after sample preparation. The percentage recovery was determined by reference to a standard mixture by peak areas of the u.v. chromatogram. The results are expressed as means ± S.E.M. ($n = 6$).

Amount of analyte (nmol)	Recovery (%)		
	Butyryl-CoA	Octanoyl-CoA	Heptadecanoyl-CoA
5	65.3 ± 1.0	66.7 ± 1.6	69.3 ± 7.2
20	68.6 ± 1.5	68.7 ± 1.6	76.8 ± 2.9

Table 3. Precision of the radiochemical determination of acyl-CoA esters formed during replicate mitochondrial incubations

Values shown are means ± S.E.M. for five independent incubations. The incubation conditions were as described in the Experimental section; the incubation time was 1.5 min in each case. The extent of β-oxidation was assessed by measurement of total acid-soluble radioactivity and was 21.3 ± 1.4 nmol of acetyl units · mg of protein^{-1}, which corresponded to 19.3% utilization of the added [U-^{14}C]palmitate. The concentrations of the intermediates was calculated from the theoretical molar specific radioactivities and assumed no dilution by endogenous intermediates.

Intermediate	Radioactivity (d.p.m./ of protein)	Concentration (nmol/mg of protein)	Percentage
Hexadecanoyl-CoA	21899 ± 1396	2.35 ± 0.15	53.7 ± 2.3
Tetradecanoyl-CoA	1942 ± 144	0.24 ± 0.02	5.4 ± 0.3
Dodecanoyl-CoA	563 ± 113	0.08 ± 0.02	1.8 ± 0.3
Acetyl-CoA	1986 ± 143	1.70 ± 0.12	39.0 ± 2.4

after 120 s (Figs. 4*c*, 4*d* and 4*f* respectively), although the concentration of tetradecanoyl-CoA continued to increase. Acetyl-CoA was not detected at any time in the presence of rotenone. Dodec-2-enoyl-CoA and 3-hydroxytetradecanoyl-CoA occurred at concentrations which were too low to determine accurately. The flux through β-oxidation decreases with decreasing [NAD^+]/[NADH] ratios and the formation of 3-hydroxyacylcarnitine esters has been demonstrated under such conditions (Bremer & Wojtczak, 1972; Lopez-Cardozo *et al.*, 1978; Latipää *et al.*, 1986). 3-Hydroxyalkanoic and alk-2-enoic acids were detected after hydrolysis of total lipid extracts (containing acyl-CoA and acylcarnitine esters) from mitochondria slowly oxidizing hexadecanoylcarnitine in the presence of rotenone and 10 mM-carnitine (Stanley & Tubbs, 1975). Similarly the formation of 3-hydroxy acids has been demonstrated in anoxic rabbit heart and anoxic rabbit heart mitochondria (Moore *et al.*, 1980, 1982*a*,*b*). Furthermore, anoxia also increases the concentrations of total 3-hydroxyacyl-CoA and total 2-enoyl-CoA in rat hearts (Latipää *et al.*, 1988).

3-Hydroxyacyl-CoA esters are only slowly converted into 3-oxoacyl-CoA esters by 3-hydroxyacyl-CoA dehydrogenase when NADH dehydrogenase is inhibited by rotenone. Since the equilibrium constant of the enoyl-CoA hydratase is close to unity, 2-enoyl-CoA esters also accumulate. Although ETF:QO is not inhibited by rotenone, the acetyl-CoA dehydrogenases are strongly product-inhibited by the enoyl-CoA esters formed, and such feedback inhibition may be more important than effects due to changes in [CoA]. Furthermore, porcine kidney medium-chain acyl-CoA dehydrogenase binds, and is inhibited by, a number of acyl-CoA esters. The binding of 3-oxoacyl-CoA esters is particularly tight (3-oxooctanoyl-CoA: $K_d = 55$ nM), although all acyl-

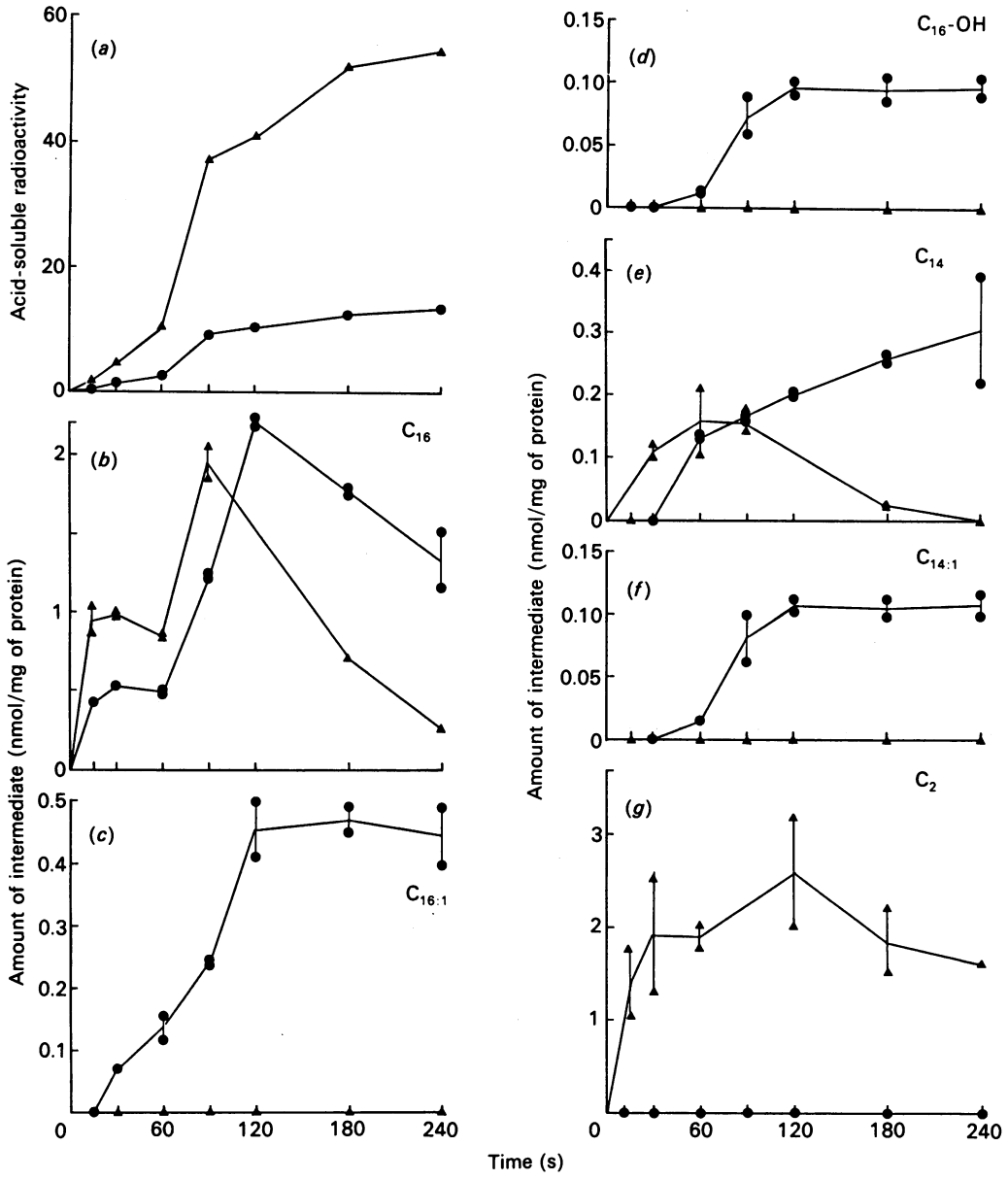

Fig. 4. Time course of the appearance of intermediates of β-oxidation of U-^{14}C]palmitate by mitochondrial fractions in the presence (●) or absence of rotenone (▲) as described in the Experimental section

(*a*) Acid-soluble metabolites; (*b*) hexadecanoyl-CoA; (*c*) hexadec-2-enoyl-CoA; (*d*) 3-hydroxyhexadecanoyl-CoA; (*e*) tetradecanoyl-CoA; (*f*) tetradec-2-enoyl-CoA; (*g*) acetyl-CoA.

CoA esters studied were bound to some extent (Powell *et al.*, 1987). The failure to detect acetyl-CoA (Fig. 4) suggests that the capacity for ketone-body formation exceeds the rate of formation of acetyl-CoA under these conditions.

The acyl-CoA dehydrogenases transfer electrons to the CoQ (coenzyme Q) pool via ETF and ETF:QO. Electrons can be transferred reversibly between the NAD^+/NADH pool and the ETF/$ETFH_2$ pool by CoQ (Frerman, 1987). During rapid oxidation of other substrates (NADH or succinate), some ETF may accept an electron to form ETF_{sq}, which is a potent inhibitor of medium-chain-fatty-acyl-CoA dehydrogenases (Beckmann *et al.*, 1981) and, by analogy, other acyl-CoA dehydrogenases. This might provide a mechanism for modulating the flux through β-oxidation when there is competition by other substrates for the respiratory chain. Indeed, there is a good correlation between the flux through β-oxidation and the redox state of ETF in intact rat liver mitochondria over a wide range of [NAD^+]/[NADH] ratios (Kunz, 1988). Competition between NADH and $ETFH_2$ for the CoQ pool with consequent impairment of long-chain acyl-CoA dehydrogenases may explain the accumulation of tetradecanoyl-CoA and dodecanoyl-CoA when the flux is maximal.

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