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Synthesis, characterisation and high-performance liquid chromatography of C_6–C_{16} dicarboxylyl-mono-coenzyme A and -mono-carnitine esters

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ABSTRACT

The synthesis and purification of the mono-coenzyme A and mono-carnitine esters of the homologous series of straight-chain even-numbered dicarboxylic acids (C_6–C_{16}) is described. The corresponding 3-hydroxyacyl- and 2-enoyl-CoA esters were prepared enzymatically. A reversed-phase high-performance liquid chromatographic (HPLC) system for the analysis of the intact CoA esters is described and their chromatographic behaviour documented. Reversed-phase HPLC systems for the analysis of the 4-bromophenacyl derivatives of the dicarboxylyl-mono-carnitines and the 4-nitrobenzyl derivatives of the free acids are also described. Some preliminary studies of the metabolism of [U-^{14}C]hexadecanedionoyl-mono-CoA by rat liver peroxisomes and rat skeletal muscle mitochondria are described illustrating the application of these methods.

INTRODUCTION

The excretion of dicarboxylic acids of chain length C_6–C_{10} is observed in a number of metabolic states including diabetic ketoacidosis [1], riboflavin deficiency [2–4] and prolonged fasting [5]. In addition, inherited disorders of mitochondrial β-oxidation [6–8] and acquired disorders, such as Jamaican vomiting sickness caused by the ingestion of hypoglycin [9] and valproate intoxication, are characterised by the excretion of dicarboxylic acids [10].

The biogenesis of these compounds is thought to involve Ω- and β-oxidation pathways although the precise sequence of events remains uncertain. Our studies are concerned with the mitochondrial and peroxisomal β-oxidation of [U-^{14}C]hexadecanedionoyl-mono-coenzyme A for which we required methods for the analysis of the probable CoA ester intermediates. Carnitine is intimately involved in fatty acid oxidation both with respect to the passage of fatty acids across the inner mitochondrial membrane and the disposal of the products of fatty acid oxidation [11,12]. We therefore also wished to analyse the carnitine esters of dicarboxylic acids. In the present paper we describe the analysis of these compounds by reversed-phase high-performance liquid chromatography (HPLC)

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and present some preliminary findings on the intermediates of the peroxisomal β-oxidation of [U-^{14}C]hexadecadionoyl-mono-CoA which were measured using simultaneous photodiode array and continuous on-line radiochemical detectors.

EXPERIMENTAL

Materials

Dicarboxylic acids were obtained from Aldrich (Gillingham, UK). Acetyl-CoA, succinyl-CoA, CoA, ATP, NAD^+, acyl-CoA synthetase, acyl-CoA oxidase and crotonase were purchased from Sigma (Poole, UK). Acetonitrile (S grade) was purchased from Rathburn (Walkerburn, UK). HPLC-grade water, Analar-grade chloroform, methanol, Scintran-grade xylene, Triton X-100 and 2,5-diphenyloxazole (PPO) were supplied by BDH (Poole, UK). All other reagents were of the highest available purity unless otherwise specified. [U-^{14}C]Hexadecanedionoyl-mono-CoA was prepared as described previously [13].

Synthesis of O-(4-nitrobenzyl)-N,N'-(diisopropyl)isourea

The synthesis of 4-nitrobenzyl esters of organic acids using O-(4-nitrobenzyl)-N,N'-(diisopropyl)isourea (4-NBDI) was first described by Knapp and Krueger [14]. Although this reagent is commercially available it is expensive and contains many UV-absorbing impurities. We therefore synthesised and purified our own material by the method of Schmidt *et al.* [15] as follows. 4-Nitrobenzyl alcohol (0.1 mol), diisopropylcarbodiimide (0.1 mol) and $CuCl_2$ (10 mg) were dissolved in 10 ml of dimethylformamide, and the solution was mixed for 96 h. The solvent and unreacted diisopropylcarbodiimide were removed by distillation under reduced pressure. The residue was dissolved in 200 ml of petrol 40–80°C (fraction) and applied to an alumina column (20 cm × 3 cm I.D.). 4-NBDI was eluted with a further 500 ml of petrol, the solvent removed *in vacuo* and the product recrystallised from pentane three times. Yellow crystals were obtained and a working solution of 0.1 *M* in dioxane was prepared.

Synthesis and purification of dicarboxylyl-mono-CoA esters

The mono-CoA esters of hexadecanedioic, tetradecanedioic and dodecanedioic acids were synthesised enzymically as follows. The free acid (10 μmol) was incubated with a 1 U of acyl-CoA synthetase in medium containing 10 m*M* $MgCl_2$, 10 m*M* ATP, 3 m*M* CoA, 0.1% (v/v) Triton X-100 and 50 m*M* KH_2PO_4 pH 7.4, in a total volume of 5 ml for 4 h at 30°C. The reaction was terminated by the addition of HCl to bring the pH to 2. The CoA esters of hexanedioic, octanedioic and decanedioic acids were synthesised from the corresponding monochlorides as follows. The free acid (10 mmol) and thionyl chloride (10 mmol) in 20 ml of dioxane were refluxed for 5 h. After cooling unreacted thionyl chloride, HCl and dioxane were removed *in vacuo*. The resultant acyl-mono-chloride was used without further purification and 1 mmol was dissolved in 7.5 ml of tetrahydrofuran

and added to a solution of 50 μmol of CoA in 4.5 ml of degassed water dropwise under nitrogen. The pH was kept at about 8 by the addition of triethylamine and a single phase maintained by the addition of small amounts of tetrahydrofuran or water. The disappearance of free thiol was monitored using nitroprusside. The pH was brought to 2–3 by the addition of HCl and the tetrahydrofuran removed with a rotary evaporator. The resultant mixture was extracted with 30 ml of diethyl ether three times. The aqueous layer was applied to a 500 mg C_{18} reversed-phase cartridge (Bond Elut) and washed with 3 ml of 10% (v/v) acetonitrile in 50 m*M* KH_2PO_4 pH 5.3 to remove unreacted CoA and any CoA disulphide and dephospho-CoA formed during the incubation. The acyl-mono-CoA ester was eluted with 3 ml of 50% (v/v) acetonitrile in 50 m*M* KH_2PO_4 pH 5.3 and freeze-dried. The yields by these procedures were typically 60–70% with respect to CoA.

Dicarboxyl-2-enoyl-CoA esters were synthesised enzymically from the respective saturated acyl-CoA esters. Dicarboxylyl-CoA (2 μmol) was treated with 0.5 U of acyl-CoA oxidase in 25 m*M* Tris buffer pH 7.4 in a total volume of 2 ml at 30°C. The formation of 2-enoyl-CoA was monitored by measuring the increase in absorbance at 225 nm due to the conjugated 2,3 double bond. Similarly the corresponding 3-hydroxydicarboxyl-CoA esters were synthesised enzymically by treating the 2-enoyl-CoA esters with crotonase (0.5 U) in 25 m*M* KH_2PO_4 pH 7.4.

Synthesis and purification of dicarboxylyl-mono-carnitine esters

Hexadecanedionoyl-, tetradecanedionoyl- and dodecanedionoyl-mono-carnitine esters were synthesised and purified as follows. Carnitine · HCl (2 mmol) and dicarboxylic acid monochloride (2 mmol) prepared as described above were dissolved in 2 ml of trifluoroacetic acid. The mixture was stirred for 16 h at 55°C. After cooling, 20 ml of diethyl ether, 3 ml of water and ice chips were added, the mixture was shaken vigorously for 1 min and the ether layer was discarded. Extraction with diethyl ether was repeated to remove free dicarboxylic acid and the carnitine ester was extracted into butanol. The butanol extract was washed with water to remove unreacted carnitine and then taken to dryness. The residue was dissolved in 1 ml of methanol and the dicarboxylylcarnitine precipitated by the addition of diethyl ether. The product was separated, washed with diethyl ether and dried. The yields were 60–70%.

Decanedionoyl-, octanedionoyl- and hexanedionoyl-mono-carnitine were prepared by a modification of the method described by Bohmer and Bremer [16] for succinylcarnitine. Carnitine · HCl (2 mmol) was treated with dicarboxylyl-monochloride (2 mmol) in 2 ml of trifluoroacetic acid for 16 h at 55°C. After cooling to room temperature 10 ml of acetone were added, the mixture was left at 0°C for 5 h and undissolved material was removed by filtration. The dicarboxylyl-carnitine was precipitated by the addition of 50 ml of diethyl ether. The precipitate was dissolved in 0.5 ml of water and the solution repeatedly extracted with diethyl

ether to remove remaining free acid and most of the water. The residue was dried under nitrogen and dissolved in 0.5 ml of methanol. The product was reprecitated by addition of 5 ml of acetone and 30 ml of diethyl ether as above. Finally the product was recovered as an oily residue, washed with diethyl ether and dried. The yields were 50–65%.

HPLC analysis of acyl-CoA esters

A Waters 600 solvent delivery system fitted with a 5 μm LiChrosorb C_{18} column (250 mm × 4.6 mm I.D.) was used for HPLC. 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% acetonitrile (5 min); isocratic 10% acetonitrile (0.1 min); linear gradient to 40% acetonitrile (29.9 min); linear gradient to 50% acetonitrile (5 min); linear gradient to 5% acetonitrile (5 min). The flow-rate was 1.5 ml/min and the total run time 45 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 0.35-s intervals over the range 200–300 nm with a band-width of 1.4 nm. Spectral data were acquired on a 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 0.75 ml flow cell as described previously [17,18]. Since the flow-rate of effluent from the photodiode array detector was 1.5 ml/min the flow-rate of the scintillation fluid (10 g PPO, 330 ml Triton X-100, 150 ml methanol and 670 ml xylene) was 6 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 UV traces. The time lag between the detectors was determined using [U-^{14}C]hexadecadionoyl-CoA.

HPLC analysis of acyl-carnitine esters

Acyl-carnitines were analysed as their 4-bromophenacyl derivatives by treatment with 4-bromophenacyl bromide in acetonitrile as previously described [19].

HPLC was carried out using a Spectra-Physics SP8700 system fitted with a 5 μm Hypersil C_8 reversed-phase column (250 mm × 4.6 mm I.D.) at 30°C. Solvents were deaerated with helium. Analytes were detected by a Pye-Unicam variable-wavelength detector (Model LC3; 8 μl flow cell; 10 mm path length) at 260 nm. Acyl-carnitine esters were resolved by a ternary gradient of acetonitrile, water and 0.15 M triethylamine phosphate (TEAP; pH 5.6) as follows. Starting conditions 60% acetonitrile, 38% water, 2% TEAP; linear gradient to 80% acetonitrile, 18% water, 2% TEAP (6 min); linear gradient to 60% acetonitrile, 33% water, 7% TEAP (4 min); linear gradient to 91% acetonitrile, 0% water, 9%

TEAP (15 min); linear gradient to 95% acetonitrile, 0% water, 5% TEAP (15 min). The flow-rate was 1.2 ml/min. The column was re-equilibrated under starting conditions (acetonitrile–water–TEAP, 60:38:2, v/v) for at least 5 min between runs to maintain reproducibility of retention times. Radioactivity was detected as described above.

Extraction and preparation of 4-nitrobenzyl (NB) ester derivatives of dicarboxylic acids and other organic acids

Undecanedioic acid (10 nmol of internal standard) was added to the sample which was acidified to pH 1–2 with HCl and saturated with NaCl; the organic acids were extracted twice with five volumes of ethyl acetate. The combined extracts were dried with 2 g of Na_2SO_4, the volume reduced to ≈ 2 ml under nitrogen and transferred to a 3 ml Reacti-vial (Pierce) and the volume further reduced to ≈ 100 μl. 4-NBDI (500 μl, 0.1 *M* in dioxane) was added, the vial sealed and heated for 1 h at 80°C followed by 1 h at 90°C with occasional shaking. After cooling, solvents were removed under nitrogen and the residue was dissolved in 1 ml of acetonitrile. Excess 4-NBDI was removed by the addition of 0.5 g of Dowex (50W-X8), mixing for 30 min at 20°C and decanting off the acetonitrile. This procedure resulted in efficient removal of excess 4-NBDI, presumably by reaction with the sulphonic acid groups, and preliminary experiments (results not shown) demonstrated that there were no losses of the derivatised organic acids. This supernatant was analysed by HPLC.

HPLC analysis of dicarboxylic acids

The 4-NB esters of dicarboxylic acids were analysed by HPLC using a Spectra-Physics SP8700 system. A 5 μm, Hypersil reversed-phase C_{18} column (250 mm × 4.6 mm I.D.) maintained at 30°C was used with a flow-rate of 1.3 ml/min and the following linear gradient of acetonitrile in water: starting conditions 40% acetonitrile (1 min); linear gradient to 50% acetonitrile (4 min); linear gradient to 100% acetonitrile (20 min); isocratic 100% acetonitrile (15 min). Analytes were detected by a Pye-Unicam variable-wavelength detector (Model LC3; 8 μl flow cell; 10 mm pathlength) at 265 nm. Radioactivity was detected as described above.

Fast atom bombardment mass spectrometry (FAB-MS)

A Kratos MS 80RF mass spectrometer in FAB ionisation mode was used. Aqueous solutions of samples (2–3 μl) were mixed with 10 μl of glycerol matrix and introduced via a vacuum lock direct insertion probe. A charge transfer gun was used to generate a xenon atom beam of 7 eV kinetic energy and mass spectra were acquired over the range 60–2000 a.m.u. Good quality spectra were obtained from 1–2 nmol of acyl-carnitine and 5–10 nmol of acyl-CoA esters.

Incubation of mitochondrial and peroxisomal fractions

Mitochondrial fractions were prepared from hind-leg muscle of male Wistar rats as described previously [12]. Peroxisomal fractions were prepared from ciprofibrate-treated rats by isopycnic centrifugation on self-generated Percoll gradients as described previously [20]. [U-^{14}C]hexadecadionoyl-mono-CoA was incubated with peroxisomes using non-solubilising (iso-osmotic) conditions in the presence or absence of NAD^+ and an NAD^+-regenerating system was previously described [20]. Mitochondrial incubations were carried out according to Watmough *et al.* [12] except that acyl-CoA as used as substrate.

Preparation of samples for radio-HPLC analysis of acyl-CoA and acyl-carnitine esters

Incubations were terminated by the addition of 100 μl of 0.1 *M* H_2SO_4 to 1 ml of the reaction mixture. Internal standard (20 nmol of undecanedionoyl-CoA or 50 nmol of undecanoyl-carnitine) were added followed by 100 μl of saturated ammonium sulphate. Each sample was extracted twice with ten volumes of ethyl acetate–diethyl ether (1:1, v/v) to remove free carboxylic acids and then extracted with 10 ml of methanol–chloroform (2:1, v/v) for 1 h with continuous agitation. After centrifugation (5000 g_{av}; 5 min) the supernatant was retained, and the pellet was re-extracted with 5 ml of chloroform–methanol and re-centrifuged. The combined supernatants were evaporated to dryness under a stream of nitrogen at 30°C. In the case of peroxisomal incubations this preparation was dissolved in 500 μl of 5% acetonitrile in phosphate buffer (50 m*M*, pH 5.3) and analysed directly as described above for acyl-CoA esters. The recovery of acyl-CoA esters by the procedure is shown in Table I. In the case of mitochondrial incubations the residue was dissolved in 4 ml of methanol–water (4:1, v/v) and insoluble material removed by centrifugation (5000 g_{av}; 5 min). The supernatant was applied to a DEAE-Sephacel column (acetate form; 60 mm × 6 mm I.D.). The eluate was

TABLE I

RECOVERY OF ACYL-CoA AND ACYL-CARNITINE ESTERS

Sample	Amount added (nmol)	Recovery (mean ± S.D., n = 5) (%)				
		Acetyl-CoA	DC_6-CoA	DC_{11}-CoA	DC_{12}-CoA	DC_{16}-CoA
Peroxisomes	5	85.0 ± 2.2	89.6 ± 4.5	91.7 ± 3.8	94.5 ± 2.7	93.2 ± 4.5
Mitochondria	2	68.8 ± 6.9	70.2 ± 4.2	71.8 ± 4.0	72.4 ± 3.8	72.8 ± 3.6
Mitochondria	10	70.5 ± 4.9	72.9 ± 3.6	74.3 ± 3.3	73.7 ± 3.1	74.8 ± 4.7
		Acetyl-CN	DC_6-CN	DC_{12}-CN		DC_{16}-CN
Mitochondria	10	80.7 ± 4.2	77.6 ± 5.4	75.2 ± 6.5		73.1 ± 7.8

retained and reapplied to the column to promote maximal binding. The column was washed with 4 ml of methanol–water (4:1, v/v) and the eluate retained for analysis of acyl-carnitines. The acyl-CoA esters were eluted with 5 ml of methanol–water (4:1, v/v) containing 0.5 *M* ammonium acetate and 10 m*M* acetic acid. The methanol was removed under nitrogen and after the addition of 2 ml of water the sample was freeze-dried. The residue was dissolved in 500 μl of 5% acetonitrile in phosphate buffer (50 m*M*, pH 5.3) and analysed by radio-HPLC as described above. The recoveries by this procedure are shown in Table I. The acyl-carnitine fraction was further treated as follows. After removal of methanol under nitrogen, 2 ml of water were added and the sample was freeze-dried. The residue was dissolved in 1.5 ml of water and applied to a column of Dowex 50X-8W (200–400 mesh; 50 mm × 6 mm I.D.; pyridinium form) and the eluate reapplied to the column as above. The column was washed with 2 ml of 10 m*M*

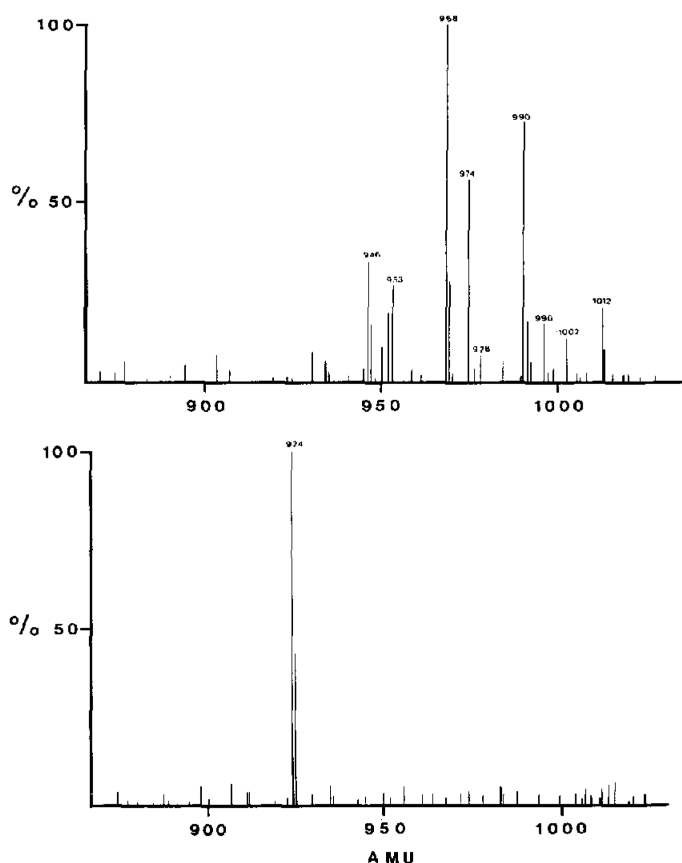

Fig. 1. Octanedionoyl-mono-CoA FAB mass spectra. Upper: without removal of alkali metals; lower: with removal of alkali metals. The assignations of the ions were as follows: 924, $[MH_5]^+$; 946, $[MH_4Na]^+$; 953, $[MH_3LiNa]^+$; 968, $[MH_3Na_2]^+$; 974, $[MHLiNa_2]^+$; 990, $[MH_2Na_3]^+$; 996, $[MHLiNa_3]^+$; 1002, $[MLi_2Na_3]^+$; 1012 $[MHNa_4]^+$.

HCl and 2 ml of water, and the acyl-carnitines were eluted with 4 ml of 1 *M* pyridinium acetate (pH 4.5)–ethanol (1:1, v/v) and freeze-dried. The resultant acyl-carnitines were analysed by radio-HPLC after derivatisation as described above. The recoveries of acyl-carnitines by this procedure are given in Table I.

RESULTS

FAB-MS analysis of dicarboxylyl-CoA and -carnitine esters

The CoA used for the synthesis of CoA esters was the Li_3^+ salt. During preparation Na^+ was added to the reaction mixture, therefore the products contained both these ions. It is known that alkali metals can complicate FAB-MS analysis by adduct formation with the analyte and glycerol matrix (see for example ref. 21), resulting in dilution of the analyte signal with reduction of sensitivity. A variety of methods have been used to overcome this problem including addition of Ag^+ to the glycerol matrix [22] and desalting the sample with ion-exchange resins [23]. Fig. 1 shows the FAB mass spectrum of octanedionoyl-CoA in the region of the molecular ion. The upper spectrum was obtained without removal of the alkali metals from the sample and multiple adducts were clearly present. The lower spectrum was obtained after removal of alkali metals with 250 mg/ml Dowex 50 (hydrogen form) and only the protonated molecular ion was present

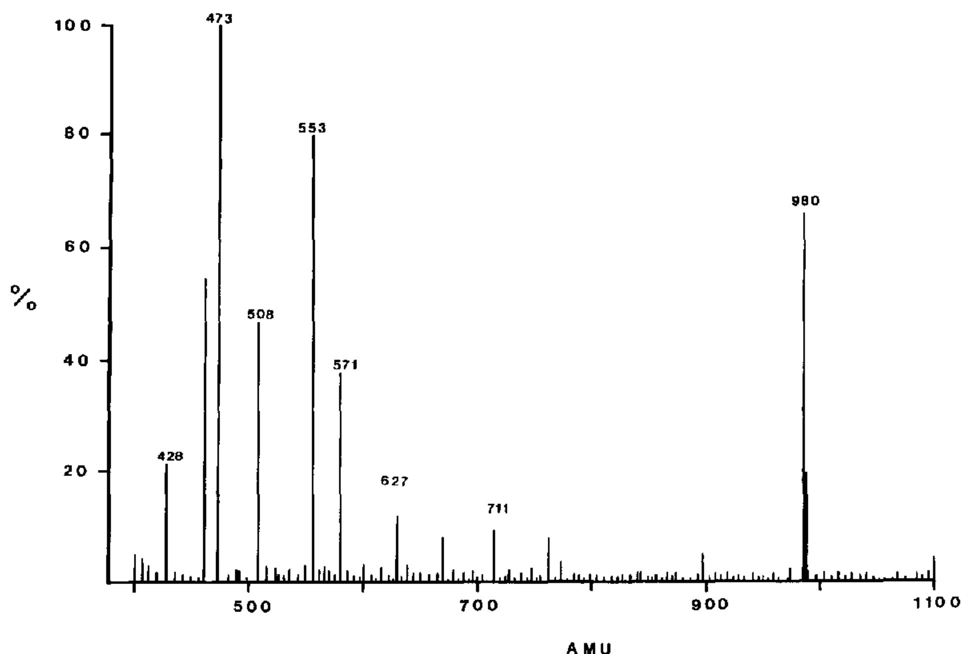

Fig. 2. FAB mass spectrum of dodecanedionoyl-mono-CoA.

and resulted in considerably enhanced sensitivity. Accordingly alkali metals were removed from all samples prior to FAB-MS analysis.

Fig. 2 shows the FAB mass spectrum of dodecanedionoyl-CoA, derived after subtracting an averaged background spectrum to remove signals from the glycerol matrix. The spectrum illustrates the characteristic major fragmentation process of this class of compounds. The ion at 980 a.m.u. corresponds to the molecular ion of the protonated free acid ($[MH_5]^+$). Cleavage of the carbon–oxygen and phosphorus–oxygen bonds resulted in ions at 428, 473, 508 and 553 a.m.u. The 473 and 553 a.m.u. ions are of particular importance because they preserve the identity of the dicarboxylyl group and therefore shift in mass as the acyl group changes. These ions were observed in all the CoA esters examined. Other fragments derived from cleavages at adenine–ribose and ribose–phosphate bonds of the CoA moiety (136 and 330 a.m.u.) were present in all spectra and are not shown. Table II summarises the major characteristic ions with their relative intensities, normalised to the $[MH_2-508]^+$ ion, observed in the FAB mass spectra of the dicarboxylyl-CoA esters studied. In no case was there any evidence for the presence of dicarboxylyl-di-CoA esters.

A typical FAB mass spectrum of dicarboxylyl-mono-carnitine (decanedionoyl-carnitine) after substraction of the matrix signals is shown in Fig. 3. Prominent ions corresponding to the protonated molecular ion (M^+, 346 a.m.u.) and decarboxylation product ($[M-44]^+$, 302 a.m.u.) were observed. Other fragments at 85, 100, 144 and 162 a.m.u. are probably derived from the carnitine moiety. This pattern was a consistent finding in the spectra of all the dicarboxylyl-carnitines analysed. Table III summarises the major ions observed in the compounds studied.

HPLC of dicarboxylyl-mono-CoA esters

Initial attempts to analyse intact dicarboxylyl-mono-CoA esters were made

TABLE II

MAJOR CHARACTERISTIC FRAGMENTS AND THEIR RELATIVE INTENSITIES (NORMALISED TO $[MH_2-508]^+$) OBSERVED IN THE FAB MASS SPECTRA OF DICARBOXYLYL-MONO-CoA ESTERS

Ester	Fragment (relative intensity)				
	$[M+H]^+$ (20–65)	$[MH_2-508]^+$ (100)	$[MH_2-428]^+$ (35–60)	$[M-386]^+$ (15–30)	$[M-408]^+$ (20–40)
DC_{16}-CoA	1036	529	609	nd	nd
DC_{14}-CoA	1008	501	581	nd	nd
DC_{12}-CoA	980	473	553	nd	571
DC_{10}-CoA	952	445	525	565	543
DC_8-CoA	924	417	497	537	515
DC_6-CoA	896	389	469	509	487

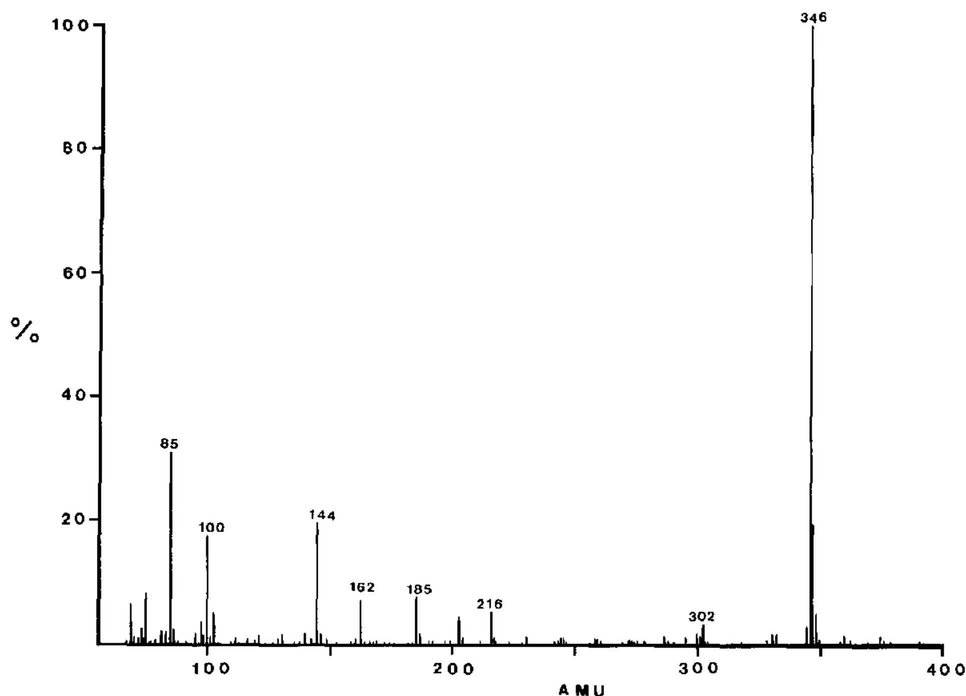

Fig. 3. FAB mass spectrum of dodecanedionoyl-mono-carnitine.

based on the method of Causey *et al.* [17]. This method and its modifications [18], however, did not provide sufficient resolution. The method was modified by changing the gradient profile and reducing the flow-rate. Adequate separation of the saturated acyl- and 2,3-enoyl-CoA esters of chain-length C_6 to C_{16} was

TABLE III

MAJOR IONS OBSERVED IN THE FAB MASS SPECTRA OF DICARBOXYLYL-MONO-CARNITINE ESTERS

Ester	Fragment (relative intensity)	
	M^+ (100)	$[M-44]^+$ (5–10)
DC_{16}-CN	430	386
DC_{14}-CN	402	358
DC_{12}-CN	374	330
DC_{10}-CN	346	302
DC_8-CN	318	274
DC_6-CN	290	246

achieved by this means using a 10 μm LiChrosorb C_{18} column. However, the system still failed to resolve the DC_n–2,3-enoyl-CoA from DC_{n+2}–3-hydroxyacyl-CoA analyte pairs. In an attempt to further improve the resolution of these pairs four columns were investigated: 5 μm Hypersil C_{18} (250 mm × 4.6 mm I.D.), 3 μm Hypersil C_{18} (250 mm × 4.6 mm I.D.), 10 μm LiChrosorb C_{18} (250 mm × 4.6 mm I.D.) and 5 μm LiChrosorb C_{18} (250 mm × 4.0 mm I.D.). Surprisingly, although the 5 μm Hypersil column was superior to LiChrosorb for the resolution of monocarboxyl-acyl-CoA ester [20], it was not capable of resolving dicarboxylyl-CoA esters and severe peak-tailing occurred. No attempt was made to determine whether altering the pH or concentration of mobile phase buffer would overcome this problem. The best resolution was obtained using the 5 μm LiChrosorb column with the gradient of acetonitrile and 50 m*M* phosphate buffer pH 5.3 described in the Experimental section. A typical chromatogram is shown in Fig. 4 and the retention times are listed in Table IV. This system was able to resolve all the CoA esters with the exception of tetradecanedi-2,3-enoyl-CoA and 3-hydroxyhexadecanedionoyl-CoA which proved impossible to separate. Acyl-CoA esters of a given chain-length were eluted in the order 3-hydroxyacyl, enoyl, acyl as has been reported previously for monocarboxylate acyl-CoA esters [18].

HPLC of dicarboxylyl-mono-carnitine esters

As a starting point a derivatised standard mixture of acetyl- and dicarboxylyl-

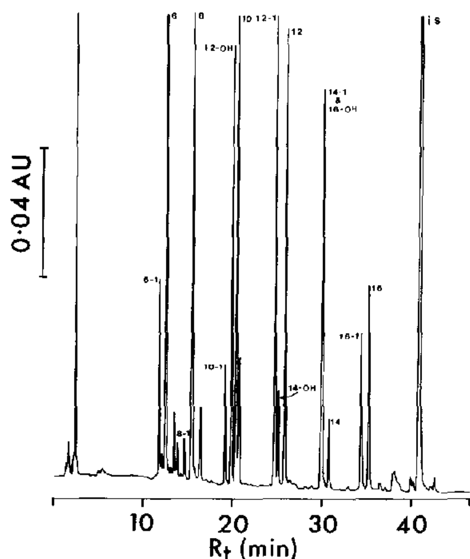

Fig. 4. HPLC separation of a standard mixture of dicarboxylyl-mono-CoA esters. Chromatographic conditions were as described in the text. The carbon numbers are as indicated, 2,3-enoyl-CoA esters are indicated by the suffix 1 and 3-hydroxyacyl-CoA esters are indicated by the suffix OH. The internal standard (is) was tridecanoyl-CoA.

TABLE IV

RETENTION TIMES OF DICARBOXYLYL-MONO-CoA ESTERS

CoA esters were analysed using a 5 μm LiChrosorb C_{18} (250 mm × 4.6 mm I.D.) column eluted with a binary gradient of acetonitrile in 50 m*M* KH_2PO_4 (pH 5.3) as described in the Experimental section.

CoA ester	Retention time (min)	Relative retention time
Acetyl	9.76	0.428
DC_4	4.70	0.206
DC_6	12.76	0.559
$DC_{6:1}$	12.23	0.536
DC_8	15.73	0.689
$DC_{8:1}$	14.88	0.652
DC_{10}	20.36	0.892
$DC_{10:1}$	19.23	0.840
$DC_{10:OH}$	15.50	0.679
DC_{11}	22.81	1.000
DC_{12}	25.27	1.107
$DC_{12:1}$	24.23	1.062
$DC_{12:OH}$	19.79	0.867
DC_{14}	29.73	1.303
$DC_{14:1}$	28.95	1.269
$DC_{14:OH}$	24.57	1.077
DC_{16}	33.76	1.480
$DC_{16:1}$	33.00	1.446
$DC_{16:OH}$	28.99	1.271

mono-carnitine (DC_6–DC_{16}) esters were chromatographed on a 10 μm LiChrosorb C_{18} column (250 mm × 4.6 mm I.D.) developed with a linear ternary gradient of 50:45:5 to 90:5:5 (v/v/v) acetonitrile–water–0.2 *M* TEAP, pH 5.8 over 30 min with an initial isocratic elution for 5 min. The flow-rate was 1 ml/min and the column was maintained at 30°C. Under these conditions the carnitine esters were resolved with poor peak shape and distribution, prolonged retention times and DC_{16}-CN did not elute from the column. The gradient was modified by increasing the starting acetonitrile concentration to 60% (v/v) and the flow-rate to 1.2 ml/min. This allowed the elution of DC_{16}-CN but the peak shape remained poor. The effect of the buffer was studied next. The concentration of TEAP had a marked effect on retention times, peak shape and resolution. At low concentrations ($\leq$ 2 m*M*) there was peak broadening and prolonged retention times whereas at higher concentrations ($\geq$ 12 m*M*) peak shape improved and retention times were reduced. However, buffer pH had very little impact over the range examined (3.0–7.0). Further improvements were achieved by altering the TEAP and acetonitrile gradient and optimum results were achieved using the gradient described in

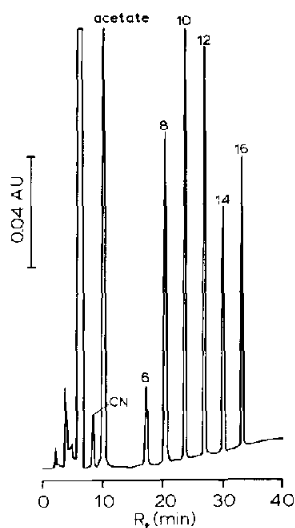

Fig. 5. HPLC separation of 4-bromophenacyl derivatives of a mixture of carnitine (CN, 7.96 min), acetyl-carnitine (acetate, 9.89 min), hexanedionoyl-mono-carnitine (6, 17.03 min), octanedionoyl-mono-carnitine (8, 20.04 min), decanedionoyl-mono-carnitine (10, 23.30 min), dodecanedionoyl-mono-carnitine (12, 26.50 min), tetradecanedionoyl-mono-carnitine (14, 29.59 min) and hexadecanedionoyl-mono-carnitine (16, 32.69 min). Excess derivatising reagent is the large peak running at the beginning of the chromatogram (5.90 min).

the Experimental section. The initial gradient step, 60:38:2 to 80:18:2 (v/v/v) acetonitrile–water–TEAP, was introduced to elute excess derivatising reagent and UV-absorbing compounds in biological samples (nucleotides) before the analytes of interest. Two columns were investigated; 10 μm LiChrosorb C_8 (250 mm × 4.6 mm I.D.) and 5 μm Hypersil C_8 (250 mm × 4.6 mm I.D.). The latter yielded better peak shapes and resolution and, unlike the former, did not deteriorate with use. Fig. 5 shows a typical chromatogram of a standard mixture of carnitine, acetyl- and dicarboxylyl-mono-carnitine esters. The retention times are given in the legend to Fig. 5.

HPLC of 4-nitrobenzyl derivatives of dicarboxylic acids and other organic acids

The 4-NB esters of organic acids were separated with a reversed-phase system using a gradient of acetonitrile and water. Different combinations of various gradients with three types of HPLC column were investigated; 5 μm Spherisorb C_8 (250 mm × 4.6 mm I.D.), 5 μm Spherisorb C_{18} (250 mm × 4.6 mm I.D.) and 5 μm Hypersil C_{18} (250 mm × 4.6 mm I.D.). In general the C_{18} columns were superior to the C_8 column for the present application. Although the resolutions achieved by the Hypersil and Spherisorb columns were comparable, some peak broadening occurred with the Spherisorb column. The addition of buffer to the mobile phase at different pH values was studied but had no effect on either

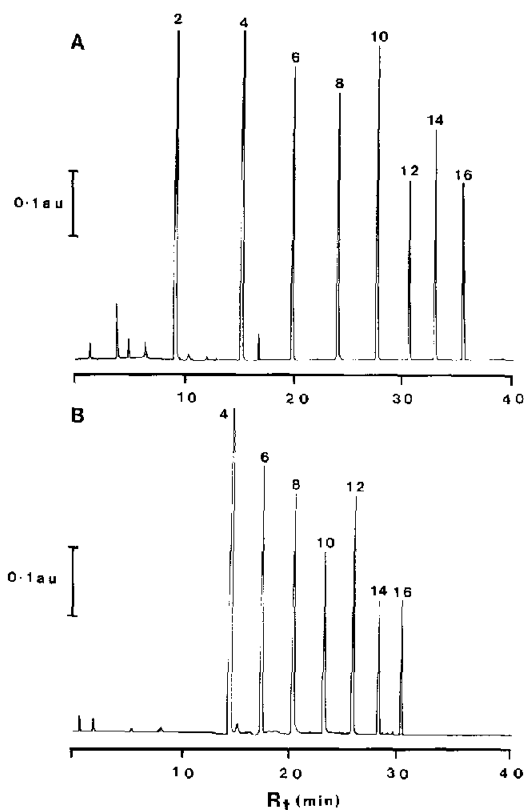

Fig. 6. HPLC separation of 4-nitrobenzyl esters of (A) monocarboxylic and (B) dicarboxylic acids using a 5 μm Hypersil C_{18} column. The chromatographic conditions were as described in the text. The carbon numbers are as indicated.

resolution or peak shape and were omitted in subsequent experiments. Optimum conditions were obtained using the 5 μm Hypersil C_{18} column and the gradient described in the experimental section. Typical chromatograms illustrating the resolution of 4-NB esters of mono- and dicarboxylic acids are shown in Fig. 6.

Optimisation of organic acid derivatisation with O-(4-nitrobenzyl)-N,N′-(diisopropyl)isourea

The effects of reaction time, temperature, solvent and molar ratio of 4-NBDI to analyte were investigated. The effects of reaction time and temperature were studied by preparing samples containing 5 μmol of either octanoic, octanedioic or citric acids in 100 μl of dichloromethane in 3 ml Reacti-vials. 4-NBDI (5 equiv.) was added as a solution in dichloromethane and the total volume brought to 500 μl. A series of six vials of each acid were maintained at 70, 80 or 90°C. The progress of esterification was monitored at 20-min intervals when a vial was

TABLE V

EFFECTS OF SOLVENT ON EFFICIENCY OF DERIVATISATION BY O-(4-NITROBENZYL)-N,N'-(DIISOPROPYL)UREA

Results are expressed as peak-area ratios relative to the internal standard, 4-NB-undecanedioate.

Acid	Peak-area ratio			
	Dioxane	Tetrahydrofuran	Dichloromethane	Acetonitrile
Lactate	1.02	1.03	0.89	0.66
Formate	0.78	0.86	0.82	0.54
Acetate	0.94	0.93	0.82	0.80
Malate	1.92	1.86	0.96	0.92
Octanoate	0.96	0.94	0.85	0.85
Octanedioate	1.91	1.83	1.44	1.13
Citrate	2.82	2.64	1.38	1.26

removed, 5 μmol of chromatographic internal standard (undecanedionoyl-4-NB) were added and the sample was analysed by HPLC. Maximal conversions were obtained after 1 h at 80°C, 1.5 h at 80°C and 1.5 h at 90°C for octanoate, octanedioate and citrate, respectively. Esterification of citrate resulted in the formation of multiple products initially (presumably mono-, di- and triesters) which were then converted to a single product upon heating at 90°C for 1.5 h. However when reactions were maintained at 90°C several unknown peaks (possibly due to decomposition of 4-NBDI) were formed. To overcome this problem different combinations of reaction time and temperature were studied. Optimal results were obtained by heating at 80°C for 1 h followed by 1 h at 90°C. Under these conditions single products were formed from all acids studied and no by-products were formed.

The effects of solvent on the derivatisation were studied, and acetonitrile, dichloromethane, tetrahydrofuran and dioxane were compared. The results are shown in Table V. It can be seen that the efficiency of derivatisation is strongly solvent-dependent, the best results being obtained using dioxane for all the acids studied and this solvent was used in subsequent experiments. The minimum molar ratio of the carboxylic acid/reagent was determined by derivatising multiple mixtures of octanoate, octanedioate and citrate (5 μmol of each) at different concentrations of 4-NBDI. The progress of the reactions was monitored at reagent sample ratios of 3, 5 and 10. The rate of reaction was considerably increased when 10 equiv. of the reagent were used. However, after 2 h no significant differences were observed at the three different reagent concentrations. Since excess reagent must be removed prior to HPLC analysis it would seem preferable to use the lesser amount. Table VI summarises the efficiency of extraction and derivatisation of the organic acids studied together with the retention times.

TABLE VI

RECOVERY AND RELATIVE RETENTION TIMES OF ORGANIC ACID 4-NITROBENZYL ESTERS

4-Nitrobenzyl esters were analysed using a 5 μm Hypersil C_{18} (250 × 4.6 mm I.D.) column eluted with a binary gradient of acetonitrile in water as described in the Experimental section.

Acid	Recovery (%)	Relative retention time
Acetic	74.8 ± 11.5	0.37
cis-Aconitic	59.7 ± 1.1	0.80
Butyric	89.8 ± 4.1	0.61
Citric	28.6 ± 1.3	0.65
Decanedioic	98.9 ± 0.3	0.95
Decanoic	100.4 ± 1.0	1.12
Dodecanedioic	100.2 ± 0.2	1.05
Dodecanoic	103.7 ± 0.5	1.24
Ethylmalonic	101.7 ± 0.8	0.67
Fumaric	65.4 ± 9.7	0.66
Glutaric	88.5 ± 0.8	0.63
Hexadecanedioic	103.1 ± 0.9	1.24
Hexadecanoic	106.8 ± 2.4	1.44
Hexanedioic	90.0 ± 0.2	0.70
Hexanoic	92.0 ± 3.3	0.80
3-Hydroxybutyric	44.1 ± 7.7	0.27
3-Hydroxy-3-methylglutaric	90.2 ± 1.0	0.55
Isocitric	48.0 ± 4.4	0.61
Lactic	81.3 ± 5.1	0.24
Malic	40.2 ± 3.5	0.51
Malonic	46.5 ± 2.2	0.55
2-Methylglutaric	100.9 ± 0.8	0.69
Methylmalonic	96.7 ± 1.1	0.57
Octanedioic	96.0 ± 0.1	0.82
Octanoic	98.8 ± 2.0	0.97
Succinic	72.0 ± 0.3	0.60
Tetradecanedioic	101.0 ± 1.0	1.15
Tetradecanoic	104.9 ± 0.1	1.34
Undecanedioic	100.0	1.00

Radio-HPLC analysis of dicarboxylyl-CoA and -carnitine esters

The objective of the studies described above was to develop a comprehensive range of analytical methods to study the metabolism of hexadecanedioic acid by isolated mitochondria and peroxisomes. These studies will be published in detail elsewhere but to illustrate the application of the methods we report here some preliminary studies of the β-oxidation of [U-^{14}C]hexadecadionoyl-mono-CoA by rat skeletal muscle mitochondria and rat liver peroxisomes. The results presented in Fig. 7 show the acyl-CoA intermediates generated by incubation of purified

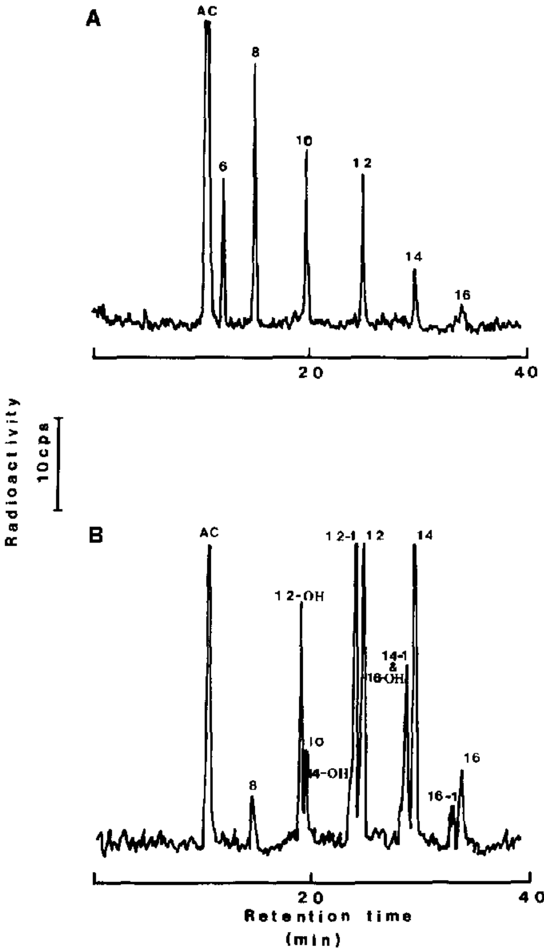

Fig. 7. Intermediates of β-oxidation of 22 μM [U-^{14}C]hexadecadionoyl-mono-CoA in the presence (A) and absence (B) of an NAD$^+$-regenerating system by purified rat liver peroxisomes. Peaks are as designated in the legend to Fig. 4.

peroxisomes with 22 μM [U-^{14}C]hexadecanedionoyl-mono-CoA in the presence (A) and absence (B) of an NAD$^+$-regenerating system for 2 min. In the presence of NAD$^+$ the C_6–C_{16} saturated acyl-CoA intermediates and acetyl-CoA were detected. However, in the absence of NAD$^+$ 3-hydroxyacyl- and 2,3-enoyl-CoA intermediates were seen with a concomitant lowering of flux (12.9 ± 2.9 *versus* 8.0 ± 3.3 nmol acetate per min per mg protein; mean ± S.D., n = 3). Fig. 8 shows the acyl-CoA esters (A) and acyl-carnitine esters (B) generated by the incubation of [U-^{14}C]hexadecanedionoyl-mono-CoA with skeletal muscle mitochondria. We concluded from these studies that chain-shortened, unsaturated

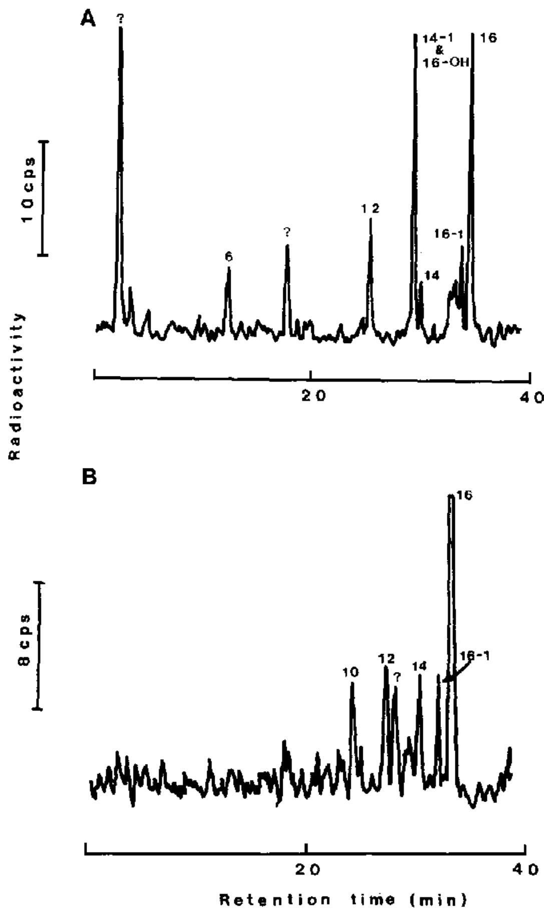

Fig. 8. CoA (A) and carnitine (B) esters generated by incubation of 22 μM [U-^{14}C]hexadecadionoyl-mono-CoA with rat skeletal muscle mitochondria. Peaks are as designated in the legend to Fig. 4.

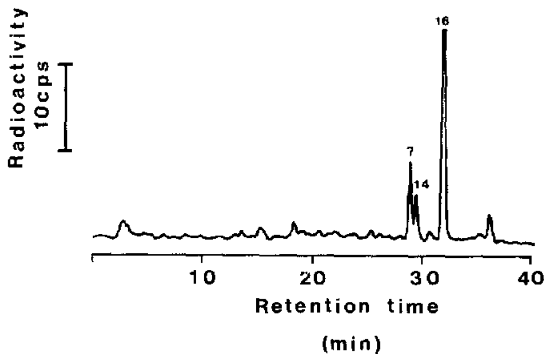

Fig. 9. Radio-HPLC analysis of the organic acids generated from the incubation of 22 μM [U-^{14}C]hexadecadionoyl-mono-CoA with rat liver peroxisomes. The carbon numbers are as shown.

and 3-hydroxydicarboxylyl-CoA esters are poor substrates for carnitine acyl-CoA transferases. In some experiments (Fig. 9) free dicarboxylates generated by incubation of [U-^{14}C]hexadecadionoyl-mono-CoA with peroxisomes were analysed; however, only tetradecanedioate and hexadecanedioate were detected.

DISCUSSION

A number of methods have been devised for the measurement of CoA and its esters in various tissues (see ref. 23). However, there is no report in the literature of the analysis of intact medium- and long-chain dicarboxyl-mono-CoA esters. Acyl-CoA esters can be fractionated by acid precipitation. This procedure allows measurement of three classes: CoA, short-chain (acid-soluble) esters and long-chain (acid-insoluble) esters. This designation is arbitrary and does not account for partial solubility of medium-chain esters and the effects that a substituent (*e.g.* hydroxy or oxo) might have on solubility. CoA and acyl-CoA esters can be measured by enzyme-linked assays, for example acetyl-CoA using citrate synthase and succinyl-CoA using 3-oxoacid-CoA transferase and 3-hydroxyacyl-CoA dehydrogenase [24]. Acyl-CoA esters can also be analysed by indirect chromatographic methods, for instance by paper chromatography after conversion to acyl hydroxymates [25] or by gas chromatography (GC) of liberated acids after hydrolysis [26]. The former method lacks resolution and the latter is insensitive.

A variety of direct methods for the analysis of acyl-CoA esters have been devised. Paper, thin-layer and column chromatography are time-consuming, suffer from poor resolution and have limited analytical application. The applicability of FAB-MS for the analysis of CoA esters in biological samples has yet to be demonstrated, HPLC, on the other hand, has proven to be an attractive method for the analysis of acyl-CoA esters. This method is non-destructive and allows the analysis of intact acyl-CoA esters without derivatization. The rapid analysis permits good recoveries of labile esters such as succinyl-CoA. Baker and Schooley [27,28] were the first to develop an HPLC method for acyl-CoA esters. This method used tetrabutylammonium phosphate as anion-pair reagent; using a gradient of methanol in water and a constant concentration of ion-pair reagent they achieved baseline separation for a homologous series of acyl-CoA esters (C_2–C_{20}) and CoA on a C_8 column. Replacement of methanol by acetonitrile improved isocratic separation of aromatic acyl-CoAs (benzoyl- and phenylacetyl-CoA). This system has not proved very popular because the ion-pair reagent is corrosive and column performance rapidly deteriorates.

Ingebretsen and co-workers [29–31] described an assay for CoA, dephospho-CoA and acetyl-CoA in neutralized perchloric acid extracts of biological samples using a C_{18} column with a solvent system of phosphate buffer–methanol. The development of this system led to the methods of Corkey *et al.* [32] and De Buysere and Olson [33] for the analysis of short-chain acyl-CoAs. Using a complex phosphate–methanol gradient with a C_{18} column the latter group achieved

excellent separation of 3-hydroxy-3-methylglutamyl-CoA succinyl-CoA and acetyl-CoA–acetoacetyl-CoA. Causey *et al.* [17] employed a gradient of phosphate buffer–methanol to separate the CoA ester intermediates of 3-methyl-2-oxopentanoate metabolism in a reversed-phase system. They also investigated the effect of pH and concentration of phosphate buffer on the retention times of a series of short-chain acyl-CoA esters and showed that retention times were increased by increasing the buffer concentration but that pH had little effect [17].

Since acetonitrile is a stronger solvent than methanol in reversed-phase systems it has been used to affect the resolution of long-chain acyl-CoA esters. Woldegiorgis *et al.* [34] separated a mixture of C_{12} to C_{18} saturated and unsaturated acyl-CoA esters using a gradient of phosphate buffer–acetonitrile. Causey and Bartlett [35] modified this system to resolve a mixture of even-chain saturated acyl-CoA esters from C_2 to C_{16} within 30 min. This system was further modified to separate all the acyl-CoA intermediates of hexadecanoate β-oxidation [18]. In the present study we extend the range of CoA esters analysed to the dicarboxylyl esters. We achieved separation of all C_6–C_{16} saturated, 2,3-enoyl and 3-hydroxyacyl esters studied with the exception of tetradecane-2,3-dienoyl-CoA and 3-hydroxyhexadecanedionoyl-CoA. The results shown in Figs. 7 and 8 demonstrate the application of the method to the analysis of the intermediates generated by incubation of [U-^{14}C]hexadecadionoyl-mono-CoA with rat liver peroxisomes and skeletal muscle mitochondria, respectively. If the NAD^+-regenerating system is omitted from the incubation (Fig. 7B), flux is slowed with concomitant lower production of acetyl-CoA (*cf.* Fig. 7A). Furthermore under these conditions 2-enoyl-CoA and 3-hydroxyacyl-CoA esters accumulate. Very similar effects have been observed when the intermediates of peroxisomal [U-^{14}C]hexadecanoate oxidation were examined [20], suggesting that the peroxisomal β-oxidation of dicarboxylates and monocarboxylates may be modulated by the prevailing intracellular redox state. The results from the skeletal muscle mitochondrial incubation are of interest, since there was an accumulation of 2-enoyl-CoA ($DC_{16:1}$). In addition the rate of [U-^{14}C]hexadecadionoyl-mono-CoA was slow in comparison to the rate of oxidation of [U-^{14}C]hexadecanoate ($<10\%$, result not shown) unlike liver [13], suggesting that there are some differences in the properties of the muscle and liver β-oxidation pathways, presumably with respect to the substrate specificities. This area clearly warrants further study.

Our study of the β-oxidation of dicarboxylic acids required a method for analysis of acyl-carnitine esters generated from the oxidation of hexadecanedioate. A number of indirect chromatographic methods have been devised. These involve GC [36] and GC–MS [37] analysis of liberated acids following hydrolysis of acyl-carnitines and HPLC analysis of acyl-CoA esters formed from enzymatic conversion of acyl-carnitines [38]. The former is insensitive and it is difficult to avoid contamination from other acyl-containing compounds; the latter is dependent upon the specificity of carnitine acyltransferases.

Several direct methods for the analysis of acyl-carnitines have been devised.

LC–MS [39] and FAB-MS [40] require expensive instrumentation and paper and thin-layer chromatography lack resolution and sensitivity. NMR spectroscopy [41] is insensitive and inappropriate for trace analysis of analytes in complex matrices. The radioisotopic exchange technique was devised to increase the detectability of carnitine esters by labelling the acyl-carnitine pool. This method has been used to detect short-chain acyl-carnitines separated by thin-layer chromatography and HPLC [42,43]. In order to label the acyl-carnitine pool effectively the carnitine esters in the sample must be substrate for carnitine acetyltransferase or acyltransferase(s). Although by adding carnitine octanoyltransferase or carnitine palmitoyltransferase to the system medium-chain and long-chain acyl-carnitines could also be measured, the applicability of this method for the analysis of dicarboxyl-mono-carnitine esters has not been studied. The GC–MS method of Lowe and Rose [44] involves conversion of acyl-carnitines into their corresponding volatile lactones. Excellent separation of acyl-carnitines is obtained by this procedure, however, the application of this method to the analysis of dicarboxyl-mono-carnitines has not been reported and would presumably require further derivatization of the free Ω-carboxylic group. As we demonstrates here, HPLC is a powerful method for the separation of carnitine esters. To enhance detectability and to improve the chromatographic properties of acyl-carnitines in reversed-phase mode their 4-bromophenacyl derivatives are prepared [45]. HPLC has the additional advantage in that it is more suited to radiochemical detection which we required for our studies of peroxisomal and mitochondrial β-oxidation. The results presented in Fig. 8 show that some of the intermediates of muscle mitochondrial β-oxidation of [U-^{14}C]hexadecanedionoyl-mono-CoA are converted to the corresponding carnitine derivatives.

Methods commonly used for HPLC analysis of carboxylic acids can be classified in four main groups, namely ion-exchange and ion-exclusion chromatography, separation based on solvophobic interactions, ion-pair chromatography and reversed-phase separation [46]. The choice of the method depends on many factors including the nature of the acid to be analysed. In general, separation of a homologous series of carboxylic acids can probably be achieved best by reversed-phase separation of their derivatives. To improve the chromatographic properties of carboxylic acids and to enhance UV and fluorescence detection they must be converted into the corresponding ester. Many chromogenic and fluorogenic groups have been developed for this purpose. The factors limiting the application of some of these methods include the laborious derivatisation procedure, the interference with the subsequent chromatographic analysis by excess derivatising reagent or by-products, instability of the derivatives and carcinogenic properties of some of the derivatising reagents. Furthermore the applicability of many of these methods to organic acid analysis in biological samples has yet to be demonstrated. Moreover, the quantitative derivatisation by these methods have often been evaluated based on UV absorption, so incomplete conversion of fatty acids or formation of non-UV-absorbing by-products in small amounts could not be

detected. This problem may not be significant in UV absorption-based analysis. However, our intention was to analyse radiolabelled organic acids by on-line radioactive monitoring as well as UV detection. The underivatised acids or non-UV-absorbing by-products can be eluted and complicate the quantitation and interpretation of radio-chromatograms.

Our aim was to find a chromogenic reagent capable of derivatising a wide range of carboxylic acids. Initially we studied 4-bromophenacyl bromide, since this reagent has been used successfully for derivatisation of carnitine esters. However preliminary results were not encouraging since the 4-bromophenacyl esters of some short-chain dicarboxylic acids, especially succinic and fumaric acids, were insoluble in almost all solvents. Therefore different derivatisation methods were investigated. Fumarate and succinate have been analysed as their 4-nitrobenzyl esters [47], so these derivatives were studied in detail. Three derivatizing reagents have been described for the preparation of 4-nitrobenzyl esters: 4-nitrobenzyl bromide or chloride [48], 1,4-nitrobenzyl-3,4-tolyltriazene [49] and O-(4-nitrobenzyl)-N,N′-(diisopropyl)isourea [17]. Esterification using 4-nitrobenzyl bromide or chloride requires a phase transfer catalyst such as crown ether. Using crown ether provides excellent yields for the preparation of phenacyl ester (*e.g.*, see ref. 50), but for preparation of benzyl esters less satisfactory results have been reported (*e.g.*, see refs. 51 and 52). Triazene compounds have been reported to be carcinogenic [53] making them less than desirable reagents. 4-NBDI appeared to be the reagent of choice and, provided that conditions are carefully optimised with respect to solvent, temperature and time, good yields are obtained. The results shown in Fig. 9 demonstrate the application of the method to analysis of dicarboxylates in biological matrices. The method can also be applied to hydroxy acids (Table V and VI) although oxo acids such as pyruvate and acetoacetate were poorly derivatised (results not shown).

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