Intermediates in Fatty Acid Oxidation

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1. Aqueous extracts of acetone-dried liver and kidney mitochondria, supplemented with NAD⁺, CoA and phenazine methosulphate, efficiently convert fatty-acyl-CoA compounds into acetyl-CoA; the process was followed with an O₂ electrode. 2. Label from [1-¹⁴C]octanoyl-CoA appears in acetyl-CoA more rapidly than that from [8-¹⁴C]octanoyl-CoA. 3. Oxidation of [8-¹⁴C]octanoyl-CoA was terminated by addition of neutral ethanolic hydroxylamine and the resulting hydroxamates were separated chromatographically. Hydroxamate derivatives of 3-hydroxyoctanoyl-, hexanoyl-, butyryl- and acetyl-CoA were obtained. 4. These and other observations suggest that oxidation of octanoyl-CoA by extracts involves participation of free intermediates rather than uninterrupted complete degradation of individual molecules to acetyl-CoA by a multi-enzyme complex. 5. Intact liver mitochondria studied by the hydroxamate technique were also shown to form intermediates during oxidation of labelled octanoates. In addition to octanoylhydroxamate, [8-¹⁴C]octanoate gave rise to small amounts of hexanoyl-, butyryl- and 3-hydroxyoctanoyl-hydroxamate. In contrast with extracts, however, where the quantity of intermediates found was a significant fraction of the precursors, mitochondria oxidizing octanoate contained much larger quantities of octanoyl-CoA than of any other intermediate.

The oxidation of fatty acids by mitochondria (' β -oxidation') involves repeated operation of a sequence of processes (oxidation, hydration, oxidation and cleavage) that results in the production of acetyl units and shortening of the chain length. Early work with ω -phenyl fatty acids (e.g. Dakin, 1908, 1909*a,b*) supported the existence of products of partial degradation, but most studies with naturally occurring fatty acids have failed to demonstrate intermediates. In the equally repetitive process of fatty acid synthesis the intermediates are not normally liberated from the enzymes (Lynen, 1961; Bressler & Wakil, 1962).

Intravenous administration to rats of palmitic acid (incorporated into triglyceride) labelled in C-6 or C-11 failed to show detectable differences in the production of ¹⁴CO₂ and Weinman *et al.* (1950) concluded that a significant amount of intermediate-chain-length fatty acids could not have accumulated *in vivo*. However, Elovson (1965) detected [1-¹⁴C]-palmitate after injecting rats with [3-¹⁴C]stearate, and concluded that 10–20% of the stearate had escaped from the β -oxidation system after removal of only one acetyl unit. Garland *et al.* (1965) showed that during mitochondrial oxidation of palmitoylcarnitine virtually all of the CoA was present either as acetyl-CoA or HClO₄-insoluble (long-chain) fatty acyl-CoA,

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and accordingly proposed that significant amounts of short-chain acyl-CoA intermediates were not formed. Garland & Yates (1967) suggested that, because of the repetitive nature of fatty acid oxidation, a mechanism involving free intermediates was unlikely (by 'free' intermediates we mean those not so tightly bound to enzymes as to prevent rapid equilibrium with the surrounding medium). By using [1-¹⁴C]octanoate and extracts of acetone-dried mitochondria Drysdale & Lardy (1953) did not detect any intermediate (other than acetyl-CoA), but this precursor would be expected to label only C₈ intermediates.

However, when rat salivary-gland mitochondria, depleted of NAD, oxidized palmitate in the presence of carnitine, β -hydroxypalmitoylcarnitine accumulated (Horak & Pritchard, 1971), and a soluble fatty acid oxidase preparation from peanuts formed a substance tentatively identified as β -hydroxypalmitoyl-CoA when oxidizing palmitate in the absence of NAD⁺ and NADP⁺ (Rebeiz & Castelfranco, 1964). Therefore under certain circumstances the formation of free intermediates may occur during fatty acid oxidation. The object of the present study was to determine whether β -oxidation, like fatty acid synthesis, is mediated by a multi-enzyme complex without the release of free intermediates, or whether such compounds normally accumulate.

A computer-based model for the depolymerization

Fig. 1. Computer plot of a theoretical model of depolymerization of a linear tetramer from one end

(a) Disappearance of substrate and appearance of intermediates and final product (monomer). In terms of octanoyl-CoA oxidation, curve (A) corresponds to acetyl-CoA production; curve (B) represents octanoyl-CoA disappearance; curves (C) and (D) describe the concentration of the C_6 and C_4 intermediates respectively. (b) Liberation of ^{14}C as a percentage of total labelled precursor for the system shown in Fig. 1(a). Precursor in curve (A) is $[1-^{14}C]$ octanoyl-CoA and in curve (B) is $[8-^{14}C]$ octanoyl-CoA.

of a linear tetramer from one end by three equivalent cleavages to form four monomers is shown in Fig. 1(a). This model, which may resemble in some respects the cleavage of octanoyl-CoA to acetyl-CoA, assumes that the probability of each intermediate participating in further cleavage events is proportional to its relative concentration. It can be seen that during the first quarter of the degradative process there is extensive consumption of the 4-unit species accompanied by the gradual appearance in turn of 3-unit and 2-unit intermediates. Because the attack is always from one end it follows that label introduced into the reactive end of the polymer (e.g. the carboxyl group) will appear in the products (i.e. acetyl-CoA) sooner than will label at the opposite end. Fig. 1(b) shows the liberation of label from opposite ends of the polymer into the product expressed as a fraction of the total reaction (i.e. total depolymerization).

An alternative hypothesis assumes that the depolymerization of each molecule goes to completion once it has been initiated. The biological analogue of this alternative is a multi-enzyme complex that does

not release the acyl-CoA substrate into the medium until it is completely converted into acetyl-CoA. In this instance carboxyl- and methyl-labelled octanoate should both produce acetyl-CoA which at all stages in the process has a specific radioactivity equal to 25% of that of the precursor.

Accordingly by measurement of the specific radioactivity of acetyl-CoA (determined as citrate) during the first quarter of the oxidative process it should be possible to distinguish between a mechanism in which there are no free intermediates (specific radioactivity at all times about 25% that of precursor) and one in which free intermediates are obligatory. In the latter case $[1-^{14}C]$ octanoyl-CoA should give rise to product with a specific radioactivity in excess of one-quarter that of the precursor, whereas $[8-^{14}C]$ octanoyl-CoA should give rise to a product with a specific radioactivity substantially less than 25% that of the precursor.

Time-course experiments can yield unequivocal evidence for one mechanism or the other only if the test system is free of rate-limiting permeability barriers. A soluble system capable of rapid oxidation

of acyl-CoA derivatives has been developed and used to examine the question: are free intermediates involved in the catabolism of fatty acids?

Experimental

Materials

CoA, NAD⁺, citrate lyase (EC 4.1.3.6), inorganic pyrophosphatase (EC 3.6.1.1) and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Carnitine hydrochloride, hexanal and ethyl bromoacetate were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Hexanoic acid and octanoic acid were obtained from Fluka A.G., Buchs, Switzerland, and phenazine methosulphate was supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. 5,5'-Dithiobis-(2-nitrobenzoic acid) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., as were most other chemicals used. Fluoro-citric acid was a gift from Sir Rudolph Peters, and highly purified 'outer' carnitine acyltransferase (EC 2.3.1.23) (West *et al.*, 1971) and palmitoyl-CoA were kindly provided by Mr. M. R. Edwards. Citrate synthase (EC 4.1.3.7) was isolated from ox heart by the method of Srere (1969). Acyl-CoA synthetase (EC 6.2.1.2) was prepared by the method of Pearson & Tubbs (1967) to a specific activity of about 1 unit/mg of protein (throughout the paper one unit of an enzyme refers to the amount which can, under appropriate conditions, form 1 μ mol of product/min). [1,5-¹⁴C]Citric acid (21.6 mCi/mmol) and sodium [1-¹⁴C]octanoate (17 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and sodium [8-¹⁴C]octanoate (5.1 mCi/mmol) was supplied by C.E.A., 91 Gif-sur Yvette, France.

Preparations

Acetyl-CoA and butyryl-CoA were prepared from the anhydrides and CoA in dilute NaHCO₃ at pH 8. Hexanoyl-, octanoyl-, decanoyl- and lauroyl-CoA were prepared from the corresponding thioglycollates by ester exchange. The thioglycollates were prepared as described by Chase & Tubbs (1972) and were crystallized from light petroleum (b.p. 40–60°C). Lauroyl- and decanoyl-CoA were prepared by the method described by Chase & Tubbs (1972) for palmitoyl-CoA.

Octanoyl-CoA. Octanoyl thioglycollate (268.3 mg) was dissolved in 1 ml of 2-methylpropan-2-ol and 1 M-NaOH was added to give a pH of about 7. This solution was added to 52.8 mg of CoA (17-fold molar excess of ester) and the pH was adjusted to 8–9 (measured by using BDH-Whatman wide-range indicator paper). After incubation at 30°C for 45 min the pH was lowered to about 3 with HClO₄ and the 2-methylpropan-2-ol removed with a stream of N₂

at 30°C. The pH was adjusted to below 1 with HClO₄ at 0°C and the reaction mixture was saturated with NaCl to promote precipitation of octanoyl-CoA. The residual thioglycollic ester and acid were removed by seven extractions with an equal volume of diethyl ether at 0°C. The product was collected by centrifugation as a solid disc at the interface and the free CoA was removed with the infranatant. The octanoyl-CoA was dissolved in the minimum amount of 0.1 M-NaHCO₃ and stored frozen at about pH 5. The yield was 46.2 μ mol of octanoyl-CoA and a negligible amount of CoA.

The same procedure applied to hexanoyl-CoA synthesis failed to yield an insoluble CoA ester; the NaCl step was therefore omitted and the product was contaminated with a small amount of free CoA.

Radioactive octanoyl-CoA. Enzymic synthesis was performed in the following mixture, in a final volume of 3.91 ml; sodium [1-¹⁴C]- or [8-¹⁴C]-octanoate, 9.8 μ mol (50–100 μ Ci); CoA, 11.9 μ mol; ATP, 50 μ mol; MgCl₂, 50 μ mol; 0.1 ml of Tris-HCl buffer (1 M, pH 8.2); inorganic pyrophosphatase, 3 units; and acyl-CoA synthetase, 2 units. Reaction at 30°C was followed by removing 50 μ l samples and measuring octanoyl-CoA by means of carnitine acyltransferase and 5,5'-dithiobis-(2-nitrobenzoate) (Tubbs & Garland, 1969). Reaction appeared to be complete in 15–17 min. Omission of the pyrophosphatase decreased the yield by two-thirds. Reaction was stopped by two drops of HClO₄ (72%, w/v) and the mixture was chilled to 0°C. After 10 min it was neutralized to about pH 6 with 1 M-KHCO₃. Recovery of the product in the supernatant was 90%. Since chromatographic separation of ATP and octanoyl-CoA presents difficulties, the phenol-extraction procedure of Seubert *et al.* (1968) was used before chromatographic separation of the product on a column (12 cm $\times$ 2 cm) of DEAE-Sephadex A-25 [Pharmacia (G.B.) Ltd., Ealing, London W.5, U.K.] (formate form) (Cha & Parks, 1964). The column was developed with a linear gradient between 0.1 M-formic acid (250 ml) and a solution 0.75 M in both formic acid and sodium formate (250 ml). The peak fractions (identified and measured by means of absorption at 260 nm, the presence of radioactivity and response in the carnitine acyltransferase assay) were pooled and freed of Na⁺ by passage through a column of Zeo-Karb 225 (SRC 14) (The Permutit Co. Ltd., Gunnersbury Ave., London W.4, U.K.) (H⁺ form). The effluent was concentrated by rotary evaporation and evaporated to dryness in a desiccator *in vacuo* over NaOH and silica gel. A weakly acid solution was stored in the deep freeze. The specific radioactivity of [1-¹⁴C]-octanoyl-CoA was 12.96×10^6 c.p.m./ μ mol and of [8-¹⁴C]octanoyl-CoA 9.63×10^6 c.p.m./ μ mol.

Hexanoyl-L-carnitine. This was prepared by using the method of Chase & Tubbs (1972) for palmitoyl-carnitine.

Ethyl 3-hydroxyoctanoate. This was prepared by a Reformatsky reaction from freshly distilled hexanal, zinc wool and ethyl bromoacetate in dry benzene-diethyl ether (Adickes & Andresen, 1943). The ester distilled at 128–131°C at 1700 N/m² and the hydroxamate prepared from it migrated as a single spot on paper chromatography in benzene–acetic acid–water (5:4:5, by vol.) with R_F 0.18–0.21.

Ethyl trans-oct-2-enoate. This was prepared by a modified Knoevenagel reaction (Galat, 1946) from ethyl hydrogen malonate and hexanal. The hydroxamate produced from the product migrated as a single spot (R_F 0.73) in the above benzene–acetic acid–water system. This material was chromatographically indistinguishable from the hydroxamate derived from the product of dehydration of ethyl 3-hydroxyoctanoate with POCl₃ (Kon & Nargund, 1932).

Methods

Acetone-dried mitochondria. Fresh ox liver (1 kg) was homogenized at 5°C with 2.5 vol. of 0.25 M-sucrose. The dispersion was centrifuged at 400–500 g for 6 min and the residue discarded; the supernatant was centrifuged for 12 min at 23 000 g at 3°C. The supernatant was discarded and the residue, suspended in 250 ml of 0.15 M-KCl, was poured slowly with vigorous stirring into 10 litres of acetone at –10°C. The precipitated mitochondria were collected on a Buchner funnel and washed with cold acetone. The material was dried in a current of air and finally *in vacuo* over silica gel (yield 135.5 g). Pig kidney mitochondrial acetone-dried powder was prepared in a similar manner.

Acetone-dried powder extract. Acetone-dried powder (1.5 g) was stirred with 10 ml of sodium phosphate buffer (0.01 M, pH 7.2) for 30 min at 0°C. The suspension was centrifuged at 10 000–11 000 g for 25 min at 3°C. The clear amber extract (8.5–8.9 ml) was passed through a column (3 cm × 22 cm) of Sephadex G-25 equilibrated with the same buffer. The central portion of the protein effluent (10 ml) was collected and contained 15–18 mg of protein/ml.

Incubation conditions. Extracts (12–14 mg of protein) were incubated in an O₂-electrode vessel (Rank Bros., Bottisham, Cambs., U.K.) in the presence of CoA (0.2 mM) and NAD⁺ (0.3 mM) in a volume of 3 ml including 30 μl of 0.1% (w/v) Methylene Blue and 10 μl of 1% (w/v) phenazine methosulphate. The reaction was initiated by addition of substrate (acyl-CoA); the maximum rate of O₂ consumption was attained 2.5–5 min after addition of substrate. In most cases the final concentration of sodium phosphate buffer (pH 7.3) was 28 mM. Incubation was performed in the dark because bright light produced a significant blank rate of O₂ uptake.

Experiments with hydroxylamine. Relative salt-free neutral 1 M-hydroxylamine was prepared by mixing

equal volumes of 2 M-KOH and 2 M-hydroxylamine hydrochloride, each in dry methanol. After chilling to –10°C, the supernatant was used for the preparation of 0.1 M-hydroxylamine reagent by dilution with ethanol.

Acyl-CoA derivatives were converted into hydroxamates by adding the incubation mixture to 5 ml of cold 0.1 M-hydroxylamine reagent. The apparatus was rinsed with 1 ml of water, and this was also added to the hydroxylamine. After 10 min at room temperature the mixture was kept at 0°C for 20 min and then centrifuged. The protein precipitate was washed with 1 ml of ethanol and the combined supernatants were concentrated in a rotary evaporator (bath temperature 30°C) to about 0.3–0.5 ml. This solution was transferred with washings of 1 M-sodium malonate buffer, pH 5.1 (2 ml in all), to a glass-stoppered test tube, adjusted to pH 5 if necessary, and extracted with 3–4 ml of peroxide-free diethyl ether five times. The combined extracts were dried over Na₂SO₄ and evaporated under a stream of N₂ at 30°C. Samples were finally dried *in vacuo* and dissolved in a known volume of methanol for chromatography.

Paper chromatography was carried out on Whatman no. 1 paper prewashed with acetic acid–ethanol–water (2:1:1, by vol.), descending in the ‘machine direction’ of the paper. Solvent systems were adapted from those described by Thompson (1951) and were the upper phase of various mixtures of benzene, acetic acid and water. The appearance of opaque false fronts tended to slow the process and disturb the separations. When such fronts appeared the paper was dried in a current of air and chromatography was restarted. Radioactive samples were spotted as a 2 cm streak and after development and drying a 4 cm strip centred on the streak was taken from the chromatogram and cut into pieces (4 cm × 1 cm) which were placed in vials (1.3 cm × 5 cm) (Hospital and Laboratory Supplies Ltd., London EC1M 6BB, U.K.) and covered with about 4 ml of toluene containing 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene. These small tubes were stoppered and placed in conventional screw-cap bottles (2.8 cm × 6 cm) for radioactivity counting in a Nuclear–Chicago Unilux IIA liquid-scintillation spectrometer.

Hydroxamate standards were prepared with neutral hydroxylamine and anhydrides of acetic acid and butyric acid and thioglycollic acid esters of hexanoate and octanoate. Alkaline hydroxylamine (Fink & Fink, 1949) was used to prepare hydroxamates from oxygen esters. The following R_F values were observed in benzene–acetic acid–water (5:4:5, by vol.): acetate, 0.003–0.013; butyrate, 0.106–0.140; hexanoate, 0.45–0.49; octanoate, 0.78–0.80.

Chromatography of water-soluble organic acids. This was carried out essentially as described by La Noue *et al.* (1970) except that a column (20 cm × 1 cm)

of Amberlite CG 400 (type I) (formate form) was used as the exchange medium. The linear formate gradient (200ml) was followed by acid ammonium formate and the fraction size was about 4ml. The profile of organic acids under these conditions was comparable with that reported by La Noue *et al.* (1970). β -Hydroxybutyric acid normally appeared ahead of acetate and acetoacetate, which were not resolved. The appearance of citrate closely coincided with the NH_4^+ front. Radioactivity in the effluent was determined by adding 0.2ml of each fraction to 3ml of a solution prepared from 1 part of Triton X-100 and 3 parts of toluene and containing 0.5% (w/v) 2,5-diphenyloxazole.

To isolate a 'citrate fraction' from each sample of the time-course experiment the above method was modified. A resin bed of 120–125mm $\times$ 5mm was prepared and neutral citrate-containing samples in about 2–3ml were percolated on to the column and washed in with an equal volume of water. When the fluid had just entered the resin, the column was washed with 10ml of 2M-formic acid followed by 25ml of 3M-HCl. The water, formate and HCl effluents were collected separately and counted for radioactivity. The HCl fraction was evaporated to dryness and analysed for citrate.

Determination of citrate. This was done by the citrate lyase method (Moellering & Gruber, 1966) in HClO_4 filtrates of reaction mixtures containing phenazine methosulphate, but was complicated by the occurrence of non-enzymic NADH oxidation. This background oxidation could be largely eliminated by addition of sodium ascorbate, and spectrophotometric analysis was carried out in a 2ml reaction mixture containing: 40mM-triethanolamine buffer, pH 7.85; 25mM-sodium ascorbate; malate dehydrogenase, 2 μl (7 units); citrate lyase, 20 μl [0.8 unit in 0.3mM- ZnCl_2 and 0.45M- $(\text{NH}_4)_2\text{SO}_4$]. Recoveries with standards were between 97 and 103%; the concentrations of fluorocitrate used did not interfere.

Acetoacetate. This was determined by the method of Mellanby & Williamson (1963).

Protein. Protein in chromatographic fractions was measured by the method of Warburg & Christian (1941) as described by Layne (1957) and in extracts and mitochondria by the method of Gornall *et al.* (1949).

Determination of CoA and its esters. Free thiol (mainly CoA), acetyl-CoA and other acyl-CoA compounds were determined sequentially in the same cuvette (Tubbs & Garland, 1969) by addition of 5,5'-dithiobis-(2-nitrobenzoate), citrate synthase and carnitine acyltransferase to samples in the presence of oxaloacetate and carnitine.

Mitochondrial experiments. Rat liver mitochondria were isolated, washed twice and resuspended in 0.25M-sucrose containing 10mM-Tris-HCl buffer,

pH 7.4. Incubations in the O_2 electrode were carried out in 5ml containing 20mM-Tris-HCl buffer, pH 7.35, 3.3mM- MgCl_2 , 3.3mM-potassium phosphate buffer, pH 7.2, 80mM-KCl, 3mM-ATP, 1mM-ADP, 2.5mM-potassium malonate, 1.2mM-sodium malate and sufficient fluorocitrate to abolish totally oxidation of added citrate.

Reaction was initiated by adding a fatty acid substrate. The carboxyl-labelled octanoate was added as 499nmol containing 1.0 μCi of ^{14}C and the methyl-labelled material was added as 504nmol containing 0.51 μCi of ^{14}C . When hexanoate or hexanoylcarnitine was used their final concentration was 0.15mM and label was supplied by the addition of 1 μCi of ^{14}C in 58.8nmol of [1- ^{14}C]octanoate or 0.51 μCi of ^{14}C in 88.2nmol of [8- ^{14}C]octanoate. When added, the concentration of rotenone was 1 $\mu\text{g}/\text{ml}$. Mitochondrial protein concentration was 3.5mg/ml and incubation was carried out at 20°C. Hydroxamates were prepared and isolated as described above for experiments with extracts.

Calcium-treated mitochondria. Rat kidney mitochondria prepared in 0.25M-sucrose containing 10mM-Tris-HCl buffer, pH 7.4, but suspended at the final centrifugation in cold water were stirred with 25vol. of 1mM- CaCl_2 and fluorocitrate (about 10 μM) for 5min at room temperature. The following additions (final concns.) were then made: Tris-HCl buffer, pH 7.3 (20mM), MgCl_2 (3mM), NAD^+ (0.2mM) and cytochrome *c* (0.04mM) followed by malate (1mM) and octanoyl-CoA (0.27mM).

Results

Fatty acid oxidation in extracts

As extracts do not have permeability barriers they are well suited to the study of the characteristics of fatty acid oxidation. Drysdale & Lardy (1953) showed that fatty acid activation and oxidation enzymes are present in aqueous extracts of acetone-dried mitochondria. In the present study the activation step was circumvented by supplying preformed fatty acyl-CoA derivatives; hydrogen-donating steps were coupled directly to molecular O_2 by means of phenazine methosulphate and the process was followed polarographically in an O_2 electrode.

Some oxidation of fatty acyl-CoA occurred on incubation with a buffered extract of acetone-dried mitochondria in the presence of phenazine methosulphate only, but the addition of CoA and NAD^+ greatly increased both the rate and amount of O_2 uptake. Fig. 2 shows that the rate of O_2 consumption was slow in the absence of added NAD^+ but that the maximal rate could be restored by supplementation. The addition of Methylene Blue slightly increased the rate of O_2 consumption and tended to sustain O_2 utilization in the presence of small

Fig. 2. Effect of NAD⁺ on fatty acyl-CoA oxidation by ox liver mitochondrial extracts

Curve (A) contains the complete mixture (see the Methods section); curve (B) is with NAD⁺ absent; curve (C) is as for curve (B), but is supplemented with NAD⁺ (0.125mM) at point 3. At point 1, 10μl of 1% phenazine methosulphate was added and at point 2 octanoyl-CoA (0.25mM).

Fig. 3. Effect of chain length of fatty acyl-CoA on the rate of O₂ consumption by ox liver mitochondrial extract

For conditions, see the Methods section. Addition of further substrate during oxidation did not alter the rate. Final concentrations (mM) of substrate are given in parentheses.

Table 1. Oxidation of CoA esters by extracts of ox liver mitochondria

Except as indicated conditions were the standard ones described in the Methods section. The amount of protein used was 13.0mg, and incubations were continued for 8.5 min in a final volume of 2.95–2.99ml. The incubation was terminated by adding the sample to 50μl of HClO₄ (60% w/v) followed after 10 min at 0°C by 0.42ml of 1M-KHCO₃. The precipitate was removed by centrifugation and washed with 0.2ml of water, which was added to the supernatant before analysis.

Substrate	Constituents of reaction mixture at termination					O ₂ consumption (μg-atoms)	CoA added (μmol)	Acetyl equivalent (μmol)
	Concn. (mM)	Acetyl-CoA (μmol)	Higher acyl-CoA (μmol)	Acetoacetate (μmol)	Citrate (μmol)			
Nature								
Butyryl-CoA	0.344	0.702	0.541	0.034	*	1.13	0.70	2.03
Octanoyl-CoA	0.217	0.407	0.422	0.056	*	1.18	0.70	2.56
Lauroyl-CoA	0.155	0.250	0.149	0.039	0	0.77	0.70	2.77
Lauroyl-CoA and malate†	0.155	0.001	0.176	0	0.35	1.19	0.70	2.77

* Analyses not performed.
† In this experiment 3mM-L. malate, fluorocitrate (about 10 μM) and citrate synthase (about 10 units) were added.

Fig. 4. Water-soluble products of oxidation of $[8-^{14}\text{C}]$ octanoyl-CoA by ox liver mitochondrial extract

A 5 ml mixture containing 0.065 mM-octanoyl-CoA was incubated until the O_2 consumption was equal to about 90% of that required to convert completely the substrate into acetyl-CoA. The reaction was stopped with HClO_4 and the denatured protein removed by centrifugation after neutralization with KHCO_3 . The supernatant was divided into two equal parts. One portion was saponified with 0.25 M-KOH at 20°C for 80 min, acidified with HClO_4 to below pH 1 and extracted with an equal volume of light petroleum (b.p. $40-60^\circ\text{C}$) four times to remove residual octanoic acid before neutralization and chromatography on Amberlite CG 400 anion-exchange resin (formate form) (a). The second portion was treated for 15 min at room temperature with about 10 units of citrate synthase and 1 mM-oxaloacetate in 150 mM-tris-HCl buffer, pH 8.2, followed by 1 M-neutral salt-free hydroxylamine for 10 min. The solution, adjusted to pH 5, was extracted three times with an equal volume of ether to remove hydroxamates. The aqueous phase was neutralized to pH 6-7 and subjected to ion-exchange chromatography (b). A total of 175896 c.p.m. was recovered from the saponified sample and 132705 c.p.m. was recovered from the hydroxylamine-treated ether-extracted preparation.

amounts of phenazine methosulphate. Mg^{2+} had little effect on O_2 consumption and was usually omitted. The effect of the chain-length of the substrate on the rate of O_2 consumption is shown in Fig. 3. Hexanoyl-CoA was the optimum substrate but C_4 - C_{12} fatty acyl-CoA derivatives were well utilized, and palmitoyl-CoA was oxidized at a significant rate.

The end product of oxidation was largely acetyl-CoA. Table 1 shows that, although small amounts of acetoacetate were detectable in the absence of a citrate trap and citrate was formed in the presence of one, the principal product in the absence of added malate was acetyl-CoA. The identity of the acetyl-CoA was confirmed by the results in Fig. 4, which show that direct saponification of a reaction mixture after incubation yielded a substance which appeared principally in fractions 12 and 13 on ion-exchange chromatography as expected of acetic acid, whereas treatment of a portion of the same solution, before saponification, with citrate synthase and oxaloacetate converted this material into a substance which appeared in fractions 52-60 and which had the

properties of citric acid. From the technical standpoint it should be noted that essentially quantitative conversion of acetyl-CoA into citrate occurred in the presence of an appropriate citrate trap and that in the presence of fluorocitrate citrate becomes an acetyl sink.

The rate of oxidation of octanoyl-CoA ($0.045 \mu\text{g}$ -atom of O_2 /min per mg of protein) was remarkably high, corresponding to about 66% of the specific activity of octanoate oxidation by intact mitochondria, and the O_2 equivalent required to convert the added substrate into acetyl-CoA made up about 90% of the observed O_2 consumption when the process was allowed to go to completion.

Time-course experiments

These were performed in an apparatus similar to that described by Garland *et al.* (1965) with 0.375 mM- $[1-^{14}\text{C}]$ - and $[8-^{14}\text{C}]$ -octanoyl-CoA as substrates in a total volume of 15 ml. Incubation was terminated when the consumption of O_2 was equivalent to that required to convert 27-28% of the added substrate

Scheme 1. Time-course experiments

After incubation of 0.375 mM- ^{14}C octanoyl-CoA with mitochondrial extracts samples were treated as indicated and analysed for the compounds shown in *italics*.

Fig. 5. Accumulation of acetyl-CoA in incubations of ox liver mitochondrial extract with [1-¹⁴C]octanoyl-CoA (a) and [8-¹⁴C]octanoyl-CoA (b) as a function of O₂ consumption

Samples were prepared for acetyl-CoA analysis as described in Scheme 1. The amounts refer to the initial vol. of 15 ml. —, represent lines of best fit and ----, are at two standard errors of estimate (Moroney, 1953) on each side of the regression line.

into acetyl-CoA. The samples were analysed for free thiol (CoA), acetyl-CoA, higher acyl-CoA and citrate as outlined in Scheme 1. The time-course of acetyl-CoA formation is shown in Fig. 5. The accumulation of acetyl-CoA was linear with respect to O₂ consumption, and application of the Z-test of Fisher (1934) indicated the regression lines obtained with [1-¹⁴C]- and [8-¹⁴C]-octanoyl-CoA were not statistically different. Thiol disappearance was also linear and was not statistically different with the two substrates. The sum of free thiol, acetyl-CoA and higher acyl-CoA during the course of the experiment remained approximately constant at all stages (i.e. with [1-¹⁴C]octanoyl-CoA, 0.547 ± 0.007 mM, and with [8-¹⁴C]octanoyl-CoA, 0.567 ± 0.0075 mM).

Further purification of the citrate produced in the

samples by the subsequent action of citrate synthase was essential because even after ether extraction the water-soluble, non-citrate radioactive products of [8-¹⁴C]octanoyl-CoA utilization were much more abundant than the corresponding products from the 1-¹⁴C-labelled precursor (see Fig. 9). In this experiment the mean radioactivity of the citrate fraction of all samples studied (0.75–10 min of incubation) was $60.6 \pm 5.3\%$ of the total radioactivity recovered in the water-soluble products of [1-¹⁴C]octanoyl-CoA oxidation, whereas only $8.3 \pm 1.1\%$ of the radioactive products appeared in the citrate fraction with the 8-¹⁴C-labelled precursor; thus much larger amounts of radioactivity appeared in the citrate fraction from the 1-¹⁴C-labelled precursor relative to acetyl-CoA produced than was the case with the

8- 14 C-labelled substrate (Fig. 6). Enzymic estimation of citrate was possible after purification in six samples taken between 1.5 and 9.0 min (Scheme 1), and the specific radioactivity of citrate derived from [1- 14 C]-octanoyl-CoA was 154.8 ± 11.2 c.p.m./nmol (31.8% of precursor) whereas that derived from [8- 14 C]-octanoyl-CoA was 28.4 ± 3.2 c.p.m./nmol or 8% of the precursor.

Hydroxamates formed in the experiments with extracts

Fatty acyl-CoA compounds and neutral hydroxylamine form hydroxamates without interference from oxygen esters (Jaenicke & Lynen, 1960) and because of their weakly acidic character (Wise & Brandt, 1955) these fatty acyl hydroxamates can be extracted into ether at pH 5 without major contamination from stronger organic acids (e.g. citrate) although free fatty acids do appear in the ether.

Experiments were performed chiefly with [8- 14 C]-octanoyl-CoA diluted with unlabelled octanoyl-CoA, hexanoyl-CoA or butyryl-CoA. The final concentration of acyl-CoA ranged from 0.312 mM to 0.335 mM and the molar ratio of the unlabelled to labelled acyl-CoA preparations was maintained at about 28. Incubation was allowed to proceed until 66–68% of the dissolved O_2 had been consumed before reaction was stopped with hydroxylamine reagent.

Chromatogram (a) in Fig. 7 showed that unincubated [8- 14 C]octanoyl-CoA yielded virtually a single product; a similar preparation from [1- 14 C]-octanoyl-CoA was almost identical. When [8- 14 C]-octanoyl-CoA was incubated with mitochondrial extract (Fig. 7b) a number of substances were formed which corresponded in their mobilities to 3-hydroxyoctanoyl-, hexanoyl- and acetyl-hydroxamate, and these were presumably derived from the corresponding CoA esters. The addition of a substantial pool of hexanoyl-CoA (Fig. 7c) only barely increased the amount of 14 C in the 3-hydroxyoctanoyl- and hexanoyl-hydroxamate regions, but the amount of 14 C in the butyryl- and acetyl-hydroxamate areas became greater. The addition of butyryl-CoA accentuated still further the disappearance of tracer quantities of octanoyl-CoA (Fig. 7d), but substantial amounts of radioactivity appeared in the butyryl- and acetyl-hydroxamate regions.

As noted above NAD^+ was required for maximal activity of extracts (Fig. 2) and it was decided to study the nature of the substances accumulating in the absence of added NAD^+ . Incubation of octanoyl-CoA in the absence of NAD^+ was continued until 69–70% of the dissolved O_2 was consumed (about 30 min, compared with 15 min in the presence of NAD^+). This quantity of O_2 , which corresponded to about 1.5 g-atoms of O_2 /mol of substrate added, was greater than the amount required to convert the substrate into 2-octenoyl- or 3-hydroxyoctanoyl-

CoA. Chromatograms (e) and (f) (Fig. 7) show the hydroxamates formed when [8- 14 C]- and [1- 14 C]-octanoyl-CoA, respectively, were used as substrates. In both chromatograms there is clear evidence of increased accumulation of 3-hydroxyoctanoylhydroxamate and possibly a shoulder of the 2-octenoyl compound in the precursor region. As might be expected from the O_2 -consumption results a small accumulation of hexanoylhydroxamate was formed from the ω -labelled precursor.

Hydroxamates formed in experiments with rat liver mitochondria

Since CoA and its acyl derivatives do not readily penetrate mitochondria, simple extension of the above experiments to mitochondria was not possible. Further, any intermediate of fatty acid oxidation must be formed from intramitochondrial CoA, and this is present in much smaller quantities [about 2 nmol/mg of protein (Garland *et al.*, 1965), or a total of about 35 nmol/incubation mixture in the present experiments] than can be provided in experiments with extracts. To ensure a reasonable concentration of highly labelled precursor (intramitochondrial

Fig. 6. Comparison of the radioactivity incorporated into citrate from [1- 14 C]octanoyl-CoA (curve A) and [8- 14 C]octanoyl-CoA (curve B) relative to the acetyl-CoA formed in incubations of ox liver mitochondrial extracts

The experiment was the same as that of Fig. 5 and Scheme 1.

Fig. 7. Distribution of radioactive products from $[^{14}\text{C}]$ octanoyl-CoA utilization by mitochondrial extracts

Chromatograms (a)–(e) were derived from $[8\text{-}^{14}\text{C}]$ octanoyl-CoA. (a) Unincubated precursor developed in the upper phase of benzene–acetic acid–water (10:3:10, by vol.); (b) octanoyl-CoA (935nmol) incubated with extract; solvent system benzene–acetic acid–water (10:7:10, by vol.); (c) octanoyl-CoA (33nmol) incubated with hexanoyl-CoA (936nmol); solvent system as in (b); (d) octanoyl-CoA (33nmol) incubated with butyryl-CoA (970nmol); solvent system benzene–acetic acid–water (1:1:1, by vol.); (e) octanoyl-CoA (807nmol) incubated in absence of NAD^+ ; solvent system benzene–acetic acid–water (5:4:5, by vol.); (f) as (e), but with $[1\text{-}^{14}\text{C}]$ octanoyl-CoA. Total radioactivity (c.p.m.) per chromatogram was: average 26800 (16170–38300). The following marker substances occupied the position of the numbered bars: (1) free octanoic acid; (2) octanoylhydroxamate; (3) 2-*trans*-octenoylhydroxamate [not well resolved from the trailing edge of region (2)]; (4) hexanoylhydroxamate; (5) 3-hydroxyoctanoylhydroxamate; (6) butyrylhydroxamate; (7) acetylhydroxamate. Markers for each chromatogram are shown. The arrow represents the solvent front. O, represents the origin.

octanoyl-CoA) the sodium octanoate added had a specific radioactivity 6–10 times greater than that of the octanoyl-CoA added in the experiments with extracts. O_2 consumption was allowed to proceed (except in the rotenone experiments) until it corresponded to that required to convert 200nmol of octanoate into citrate.

Some of the results are shown in Fig. 8. Formation of octanoyl-CoA was clearly evident [region (2) in Figs. 8a, 8b and 8c]. Hexanoyl-CoA [region (4),

Fig. 8a] was formed in detectable quantities which were increased somewhat by the addition of hexanoyl-carnitine [region (4), Fig. 8b]. Similar results were observed with the $8\text{-}^{14}\text{C}$ -labelled precursor and added hexanoate, although, as expected, no evidence of a radioactive hexanoylhydroxamate region was seen under any condition when $[1\text{-}^{14}\text{C}]$ octanoate was used as a precursor. The addition of rotenone clearly increased this peak (Fig. 8c), again only with the $8\text{-}^{14}\text{C}$ -labelled precursor. This response may be compared

Fig. 8. Distribution of radioactive products from sodium $[8-^{14}\text{C}]$ octanoate utilization by rat liver mitochondria

Chromatograms of ether-soluble materials after treatment with neutral hydroxylamine and extraction at pH 5.1 developed in benzene-acetic acid-water (5:4:5, by vol.). Chromatograms were developed 38 cm from the origin (O) and radioactivity (c.p.m.) is plotted as a percentage of the counts between 0 and 32 cm (free octanoate lies in the region 33–38 cm). Total radioactivity (c.p.m.) recovered per chromatogram was: average 62231 (25624–81344); and radioactivity (c.p.m.) in the region 0–32 cm was: average 10877 c.p.m. (7654–13307). (a) $[8-^{14}\text{C}]$ Octanoate (88 nmol) and 416 nmol of unlabelled octanoate as substrate; (b) hexanoylcarnitine (750 nmol) as substrate with 88 nmol of $[8-^{14}\text{C}]$ octanoate; (c) as in (a), but with added rotenone. Marker compounds occupied the position of the numbered bars as in Fig. 7. For explanations of regions (8) and (9), see the text. The arrow represents the solvent front.

with the effect of excluding NAD^+ in experiments with extracts (Figs. 7e and 7f). However, the accumulation of 3-hydroxyoctanoylhydroxamate was very small from either $[1-^{14}\text{C}]$ - or $[8-^{14}\text{C}]$ -octanoate, and the presence of 2-octenoylhydroxamate was questionable. What appeared to be a significant accumulation of butyrylhydroxamate [Fig. 8a, peak (6)] was augmented when hexanoylcarnitine or hexanoate replaced unlabelled octanoate. The reason for this is not clear. The addition of butyrate also increased this peak with $[8-^{14}\text{C}]$ octanoate as precursor.

The peak in the (8) region of Fig. 8 is thought to be an artifact for the following reasons. It was not seen when octanoyl-CoA (which was chromatographically purified) was used as a substrate in experiments with extracts. However, when the sodium salts of $[1-^{14}\text{C}]$ - and $[8-^{14}\text{C}]$ -octanoate were converted into hydroxamates (via the acyl chlorides and the ethyl esters) the ether extracts at pH 5.1 exhibited peaks in the (8) region which ranged between 1.7 and 3% of the total radioactivity (c.p.m.) recovered from the paper. Further, in experiments where large

proportions of the labelled octanoates underwent conversion into citrate (which is poorly extracted, and appears at 0–1 cm), the amount of the peak in

region (8) increased when expressed as a percentage of total radioactivity on the paper. These observations have been interpreted to mean that radioactivity found in region (8) is due to a metabolically inert contaminant of both octanoate precursors. The radioactivity in region (9) of Fig. 8(c) is of unknown identity. It appeared only in the presence of rotenone and corresponds in mobility to free acetoacetic acid. However, no evidence of a discrete radioactive substance was seen at 8–10 cm, where 3-hydroxybutyrate, the expected companion of acetoacetate, would appear.

Water-soluble products of [1-¹⁴C]- and [8-¹⁴C]-octanoyl-CoA oxidation

In the time-course experiment it was pointed out that a much larger part of the water-soluble radioactivity appeared in citrate when [1-¹⁴C]octanoyl-CoA was used as a substrate than was the case with [8-¹⁴C]octanoyl-CoA (Figs. 9a and 9b; see also Fig. 6). These samples were subjected (Scheme 1) to treatment with hydroxylamine followed by ether extraction before ion-exchange chromatography (the ether extracts contained a significant amount of radioactivity, which was shown on chromatography for [8-¹⁴C]-octanoyl-CoA to correspond closely to that shown in Fig. 7b). Experiments with pig kidney mitochondrial extracts incubated in the presence of malate and saponified directly to yield free acids showed a somewhat different profile (Figs. 9c and 9d) from that of the hydroxamates, but here also the [8-¹⁴C]-octanoyl-CoA yielded much more non-citrate radioactive material than did [1-¹⁴C]octanoyl-CoA. It seems highly likely that a significant part of the non-citrate radioactivity in Fig. 9(d) corresponds to (saponified) intermediates of fatty acid oxidation, and that these are formed by kidney as well as liver extracts.

Mitochondria from rat liver, kidney or heart pre-incubated under hypo-osmotic conditions with CaCl₂

Fig. 9. Distribution of water-soluble radioactive products from [1-¹⁴C]octanoyl-CoA (a and c) and [8-¹⁴C]-octanoyl-CoA (b and d) produced by extracts of mitochondria from ox liver (a and b) and pig kidney (c and d)

The samples represented by (a) and (b) were the terminal samples from the time-course experiment of Figs. 5 and 6 and were prepared for chromatography as described in Scheme 1. The samples represented by (c) and (d) were terminal samples from a similar type of experiment, in which, however, incubation was carried out in the presence of 10 mM-malate and added citrate synthase. The samples were taken when an O₂ consumption equivalent to that required to convert 19% of substrate into acetyl-CoA had occurred. Reaction was terminated by saponification in 0.16 M-KOH at room temperature for 40 min; the samples were acidified with HClO₄ and extracted with 4 × 4 ml of heptane. The aqueous phase, in all cases neutralized to about pH 6.5, was subjected to chromatography on Amberlite CG 400 (formate form). Recovery of radioactivity from the column was 83% in (a), 77% in (b), 87% in (c) and 96% in (d); the total radioactivity (c.p.m) recovered was: (a), 38263, (b), 32086, (c), 126268 and (d), 160769. The citrate fraction makes up 66.4% in (a), 24.6% in (b), 82.1% in (c) and 17.9% in (d), and is the material in fractions 52–60.

and fluorocitrate are capable of rapidly oxidizing octanoyl-CoA in the absence of carnitine. When rat kidney mitochondria treated in this way were incubated with [1-¹⁴C]- or [8-¹⁴C]-octanoyl-CoA and malate until O₂ consumption equivalent to that required to convert 20% of the added substrate into citrate had occurred, the water-soluble products of oxidation after direct saponification and chromatography closely resembled those observed with pig kidney extracts in Figs. 9(c) and 9(d). Thus, when [1-¹⁴C]octanoyl-CoA was a substrate, 68% of the water-soluble radioactivity appeared as citrate whereas for [8-¹⁴C]octanoyl-CoA the corresponding value was 27% (recovery of radioactivity from both columns was 95%). These results suggest that such damaged kidney mitochondria, like soluble extracts, oxidize fatty acids by way of intermediates.

Discussion

A water-soluble extract of acetone-dried mitochondria has been described which, with minimal supplementation, is capable of rapidly oxidizing a wide range of acyl-CoA derivatives. In the presence of phenazine methosulphate the process can be coupled to O₂ consumption, and an O₂ electrode has been used to monitor metabolic activity. The process catalysed consisted primarily of repeated oxidative cleavage of acyl-CoA substrates to yield acetyl-CoA. In Table 1 it is possible to account for 88–103% of the CoA added (as acyl-CoA and free CoA) in the products (free CoA, acetyl-CoA and higher acyl-CoA), and acetyl-CoA is the greatest single product. In many experiments radioactive acetyl-CoA was trapped as citrate. Comparison of the products accumulating when the extent of oxidation of [8-¹⁴C]-octanoyl-CoA is greatly different (compare Fig. 4b, where the O₂ consumption is equal to about 90% of that required to convert the added substrate into acetyl-CoA, with Fig. 9b, where O₂ uptake was equivalent to only 28% of that requirement) showed that in the early stages a much larger amount of non-citrate radioactivity appeared than was the case at later times, and it may be inferred that intermediates evident early in the process were converted into acetyl-CoA as catabolism proceeded. Similarly a comparison of [1-¹⁴C]- and [8-¹⁴C]-octanoyl-CoA as substrates (cf. Figs. 9a and 9b) shows that the methyl-labelled compound gave rise to much larger amounts of non-citrate radioactivity than did the carboxyl-labelled material. This observation is consistent with the notion that intermediates with less than eight carbon atoms accumulated during catabolism.

As might be expected very small amounts of acetoacetate were produced in these experiments. The equilibrium of the thiolase reaction at the prevailing concentration of free CoA is very unfavourable

for ketone-body synthesis (Bremer & Aas, 1969; Greville & Tubbs, 1968), and also the 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) required for acetoacetate synthesis is known to be relatively unstable in crude extracts of acetone-dried powders (Lynen *et al.*, 1958; Caldwell & Drummond, 1963).

The metabolic conversion of octanoyl-CoA into acetyl-CoA requires three repetitive cycles and nominally involves 11 intermediates. If each cycle includes one relatively slow step, the overall system will resemble the three-cycle model described in the introduction provided that the intermediates can diffuse away from the catalytic sites. If they do not escape from the enzyme-bound condition it becomes necessary to postulate a multi-enzyme complex to account for continuing formation of product. The results from the time-course experiment showed that although the overall yield of product appeared to be identical with carboxyl- and methyl-labelled substrates (Fig. 5), the acetyl groups formed early in the degradation process were much more highly labelled by [1-¹⁴C]octanoyl-CoA than by [8-¹⁴C]octanoyl-CoA (Fig. 6) and the specific-radioactivity measurements over the first quarter of the process provided strong support for the theoretical model in which free intermediates were obligatory for the conversion of octanoyl-CoA into acetyl-CoA.

This conclusion was supported by paper-chromatographic separation of acyl-CoA compounds as the corresponding hydroxamate derivatives. When [¹⁴C]-octanoyl-CoA was used as a precursor there was evidence for the formation of the 3-hydroxyoctanoyl derivative, and the amount of this substance formed from carboxyl- or methyl-labelled precursor could be greatly increased by the omission of NAD⁺ from the incubation mixture. Thus, as might be expected, there was an NAD⁺ requirement for the utilization of 3-hydroxyoctanoyl-CoA, and the limited conversion of the substrate into hexanoyl-CoA (Fig. 7e, region 4) presumably occurred as a consequence of small amounts of bound NAD in the extracts.

The effect of creating pools (300 nmol/ml) of unlabelled hexanoyl-CoA or butyryl-CoA in the presence of tracer quantities of [8-¹⁴C]octanoyl-CoA (11 nmol/ml) was investigated. These experiments (Figs. 7c and 7d) showed extensive utilization of [8-¹⁴C]octanoyl-CoA (whose O₂ equivalent for complete conversion into acetyl-CoA is about one-sixth of the O₂ consumption that occurred). The extracts appeared to utilize the longer-chain (labelled) precursor very effectively and preferentially in spite of a 28–30-fold molar preponderance of the pool constituent. At the same time, label from [8-¹⁴C]-octanoyl-CoA appeared in the shorter saturated homologues (C₆ and C₄ for the hexanoyl-CoA pool and C₄ for the butyryl-CoA pool), thereby providing clear evidence for chain shortening and the formation of some free intermediates.

The experiments with extracts have shown that a wide range of acyl-CoA compounds can be converted into acetyl-CoA and that this process may go to completion. The rates of appearance of label from carboxyl- and methyl-labelled octanoyl-CoA in acetyl-CoA during the early stages of catabolism were such as strongly to suggest the obligatory participation of free intermediates in the process. The distribution of radioactivity in the water-soluble organic acids produced during catabolism of the carboxyl- and methyl-labelled precursors was consistent with this mechanism. Paper chromatography of the fatty acyl hydroxamates provided evidence that 3-hydroxyoctanoyl-CoA was formed from octanoyl-CoA and that this intermediate could be caused to accumulate by omission of NAD^+ . The same technique has shown also that labelled C_6 and C_4 saturated intermediates are produced from $[8\text{-}^{14}\text{C}]$ octanoyl-CoA. These observations may be taken to mean that oxidation of fatty acyl-CoA derivatives by extracts proceeds through a number of obligatory intermediates.

It was of interest, therefore, to determine whether intermediates could be detected in mitochondrial oxidations. By the criterion of the organic acid profile in anion-exchange chromatography, mitochondria rendered permeable to octanoyl-CoA by means of treatment with hypo-osmotic Ca^{2+} yielded chromatograms with $[1\text{-}^{14}\text{C}]$ - and $[8\text{-}^{14}\text{C}]$ -octanoyl-CoA as substrates which were virtually indistinguishable from those (Figs. 9c and 9d) obtained with extracts, and it was concluded that participation of free intermediates in fatty acid oxidation in preparations of this type was highly probable. However, these results do not exclude the existence of a labile complex in intact mitochondria. Because time-course experiments of the type described above were not meaningful in mitochondria limited by permeability boundaries, an effort was made to apply hydroxamate chromatography to the detection of intermediates in rat liver mitochondria incubated with $[1\text{-}^{14}\text{C}]$ - and $[8\text{-}^{14}\text{C}]$ -octanoate in place of the corresponding acyl-CoA compounds. Octanoyl-CoA was found in much larger quantities than any other thioester; however, traces of hexanoyl-CoA were obtained with $[8\text{-}^{14}\text{C}]$ octanoate as substrate, and this fraction was enhanced by addition of hexanoylcarnitine, hexanoate or rotenone. Radioactivity corresponding to butyryl-CoA was also observed, which was increased by hexanoylcarnitine and hexanoate. In contrast to the experiments with extracts very little 3-hydroxyoctanoyl-CoA was obtained even when rotenone was present.

Mitochondria therefore apparently form detectable intermediates but their distribution is different from that produced by extract preparations. Calculations from the radioactivity results of the amount of hydroxamate formed indicated that about 28–40 nmol

of hydroxamate had been produced in the experiment of Fig. 8; this value (1.6–2.3 nmol/mg of protein) corresponds closely to the estimate of mitochondrial CoA (about 2 nmol/mg of protein) based on the results of Garland *et al.* (1965). It would appear that most of the mitochondrial CoA was in the form of ester under the conditions of these experiments. The absence of appreciable quantities of 3-hydroxyoctanoylhydroxamate and the very low concentration of hexanoyl-CoA relative to octanoyl-CoA in the mitochondria, compared with the ratios prevailing with extract preparations, suggest that different factors are regulating the pool size of intermediate formed in the two preparations. Indeed, the possibility remains that a labile multi-enzyme complex for β -oxidation exists in intact mitochondria, and the occurrence of low concentrations of intermediate may represent limited dissociation of intermediates from such a complex. It is of course possible that some of the hydroxamates obtained in experiments with intact mitochondria may have been derived from intermediates tightly bound to enzymes of β -oxidation, since the quantities observed were no greater than feasible amounts of the enzymes themselves.

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