Steady-State Concentrations of Coenzyme A, Acetyl-Coenzyme A and Long-Chain Fatty Acyl-Coenzyme A in Rat-Liver Mitochondria Oxidizing Palmitate

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1. Fluorimetric assays are described for CoASH, acetyl-CoA and long-chain fatty acyl-CoA, and are sensitive to at least 50 μmoles of each. 2. Application of these assays to rat-liver mitochondria oxidizing palmitate in the absence and presence of carnitine indicated two pools of intramitochondrial CoA. One pool could be acylated by palmitate and ATP, and the other pool acylated by palmitate with ATP and carnitine, or by palmitoylcarnitine alone. 3. The intramitochondrial content of acetyl-CoA is increased by the oxidation of palmitate both in the absence and presence of L-malate. 4. The conversion of palmitoyl-CoA into acetyl-CoA by β -oxidation takes place without detectable accumulation of acyl-CoA intermediates.

The oxidation of fatty acids by mitochondria involves coenzyme A,* and as an aid in the investigation of the steps regulating this process it seemed desirable to develop techniques for assay of some of the coenzyme A derivatives involved. A requisite of such assays was that they should be sufficiently sensitive to apply to small amounts of mitochondria (1–5 mg. of protein) removed from an incubation mixture of which the metabolic behaviour had been continuously followed with an oxygen electrode. By this means controlled changes of metabolic state could be correlated with changes in the mitochondrial content of CoA and various derivatives. In this paper we describe techniques for these purposes and their application to the problem of 'compartmentation' of CoA within the

mitochondria from rat liver (Chappell & Crofts 1965).

The assays for CoASH, acetyl-CoA and long-chain fatty acyl-CoA described here are all based on the use of oxoglutarate dehydrogenase (EC 1.2.4.2). Under suitable conditions (Garland, 1964) CoASH is completely succinylated (reaction 2) and the reduction of NAD followed fluorimetrically. Long-chain fatty acyl-CoA (C_{10} or C_{12} upwards) is assayed as CoASH after hydrolysis of the thiol ester bond (Bortz & Lynen, 1963; Tubbs & Garland, 1964). Acetyl-CoA is also assayed as CoASH after arsenolysis with phosphate acetyltransferase (EC 2.3.1.8) (reaction 1). The standard (pH 7.0) free-energy change for each reaction is large and negative (less than -7.0 kcal./mole), and the reactions proceed to completion in the presence of sufficient NAD^+ to prevent inhibition of oxoglutarate dehydrogenase by NADH and succinyl-CoA (Garland, 1964). The exceedingly low K_m of oxoglutarate dehydrogenase for CoASH (less than 10^{-7} M ; Massey, 1960) and the high specific activity of phosphate acetyltransferase (1200 units/mg. for arsenolysis of acetyl-CoA) make possible rapid

(Arsenate)
(H_2O)

(1)

(2)

* Enzymic assays for CoASH frequently operate under conditions where acyl-CoA derivatives or CoA disulphides are also assayed after their conversion into CoASH (Michal & Bergmeyer, 1963), and to ensure clear distinction between the thiol form of coenzyme A and its derivatives the following abbreviations are used: CoASH, thiol form of coenzyme A; acyl-CoA, thiol esters of coenzyme A; CoA, coenzyme A and derivatives in general.

enzymic assays of low concentrations (less than $0.1\mu\text{M}$) of CoASH and acetyl-CoA in extracts prepared from mitochondria.

MATERIALS AND METHODS

Chemicals and enzymes. NAD⁺, NADH, ATP, ADP, AMP, CoASH, acetyl phosphate, 2-oxoglutaric acid, alcohol dehydrogenase (EC 1.1.1.1), lactate dehydrogenase (EC 1.1.1.27) and phosphate acetyltransferase were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

DL-Carnitine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and the *O*-palmitoyl ester prepared as described by Bremer (1962). Oxoglutarate dehydrogenase of specific activity 2–5 units/mg. was prepared from pig heart according to the procedure of Sanadi, Littlefield & Bock (1952). Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, and freed from fatty acids by the method of Goodman (1958). CoASH was freed from dephospho-CoASH and AMP by chromatography on a column of DEAE-cellulose (Moffatt & Khorana, 1961), and was then considered to be at least 98% pure on the basis of spectrophotometric assays for adenine content (Stadtman, 1957), thiol content (Ellman, 1959) and CoASH content (Garland, 1964). Dephospho-CoASH was obtained from CoASH by oxidation of the latter with H_2O_2 to the disulphide, treatment of the CoA disulphide with alkaline phosphatase until phosphate liberation was complete, reduction of the dephospho-CoA disulphide with 2-mercaptoethanol, and finally purification by chromatography on a column of DEAE-cellulose (Moffatt & Khorana, 1961). Acetyl-CoA was prepared by the method of Simon & Shemin (1953) and purified by chromatography as for CoASH, and standardized by the extinction values at 258 and $232\text{m}\mu$ (Stadtman, 1957), or by the assay of CoASH liberated by alkaline hydrolysis.

Fluorimetric assays. CoASH was assayed by following the reduction of NAD fluorimetrically on adding oxoglutarate dehydrogenase ($1\text{--}5\mu\text{g.}$) to a reaction mixture of 2.0 ml. final volume containing $100\mu\text{moles}$ of potassium arsenate, pH 7.0, $2\mu\text{moles}$ of MgCl_2 , $1\mu\text{mole}$ of EDTA, $4\mu\text{moles}$ of cysteine, $2\mu\text{moles}$ of potassium oxoglutarate, $0.1\mu\text{mole}$ of NAD⁺ and $0.1\text{--}1.0\mu\text{mole}$ of CoASH. The CoASH-dependent reduction of NAD⁺ is completed in 1–2 min., and acetyl-CoA was then assayed by the addition of $1\mu\text{g.}$ of phosphate acetyltransferase. Initially these assays were attempted with an Eppendorf photometer with fluorescence accessories (Netheler und Hinz G.m.b.H., Hamburg, Germany), but the sensitivity to NADH obtained by Estabrook & Maitra (1962) could not be repeated. So the following modifications were made: (i) the Hg-vapour light source was replaced by a 12v 100w quartz-tungsten-iodine lamp (Philips) powered by a 12v accumulator; (ii) the anode current of the photomultiplier tube was led to earth through a $3 \times 10^6\Omega$ resistance, and the voltage across this resistance measured with a high-impedance d.c. millivoltmeter (Pye model 79 pH-meter; W. G. Pye and Co. Ltd., Cambridge). The output of the millivoltmeter was then recorded with a 0–1mv potentiometric recorder (Honeywell Controls Ltd., Motherwell, Lanarkshire). Sensitivity ranges were introduced by changing the $3 \times 10^6\Omega$ resistor with other values, though the voltage across this resistor was not allowed to exceed 1% of

the voltage across the last stage of the photomultiplier. Zero compensation for the millivoltmeter output was obtained by adjusting the millivoltmeter zero with the controls provided. By using the Eppendorf filters (313 and $366\text{m}\mu$ primary, 420–3000 $\text{m}\mu$ secondary) and an IP21 photomultiplier tube (Radio Corp. of America, Harrison, N.J., U.S.A.) operated at 500v, the recorder could give full-scale (10 in.) response to $1.0\mu\text{M}$ -NADH, with a noise level corresponding to $0.005\mu\text{M}$ -NADH. Calibration was performed either by the oxidation of NADH with lactate dehydrogenase and pyruvate, or by the reduction of NAD⁺ with alcohol dehydrogenase and ethanol, and subsequent reference to spectrophotometric assays of NADH or NAD⁺ (Klingenberg, 1963). The cell holder was water-jacketed and maintained at 30°.

Mitochondria. These were prepared from the livers of male Wistar rats, 150–200g. body weight, by a procedure essentially that of Hogeboom, Schneider & Palade (1948), with 0.25M-sucrose in 5mm-tris-chloride buffer, pH 7.2, as the homogenizing medium. The homogenate was centrifuged at 800g for 10 min., and mitochondria were then isolated from the resultant supernatant by centrifuging at 8000g for 10 min. The mitochondria were then washed twice by resuspending in 0.25M-sucrose in 5mm-tris-chloride buffer pH 7.2, and reisolating by centrifuging at 8000g for 10 min. The final mitochondrial pellet was stored at a protein concentration of 60–80mg./ml. at 0°, and used within 4 hr.

Microsomes were obtained by centrifuging at 100000g for 1 hr. the supernatant remaining after sedimentation of mitochondria from the liver homogenate, and resuspended in homogenizing medium at a protein concentration of 20mg./ml.

Incubation apparatus and procedure. Mitochondrial incubations were carried out at 28° in a medium containing tris-chloride buffer, pH 7.2 (20mm), KCl (80mm) and MgCl_2 (5mm), with additions as indicated in the legends of the relevant Figures. To provide for sampling and oxygen pressure recording, incubations were performed in a horizontally mounted 20 ml. Chance syringe (Fig. 1) fitted with a Repette plunger assembly (Jencons, Hemel Hempstead, Herts.) and stirred with a polythene-coated magnetic follower (A. Gallenkamp and Co. Ltd., London, E.C. 2) and magnetic stirrer (Rank Bros., Bottisham, Cambs.). The syringe was modified as shown in Fig. 1. Use of the Repette automatic device allowed rapid ejection of constant-sized samples through the drilled stopper and needle directly into 10% (w/v) HClO_4 .

The oxygen pressure of the medium was measured polarographically (Chappell, 1964) and the same author's data used in converting oxygen pressure into oxygen concentration.

In general each incubation contained 3–4mg. of mitochondrial protein/ml. in a total volume of 20ml. Each sample was of 1–2ml., according to the experiment, and was ejected into sufficient ice-cold 10% (w/v) HClO_4 to give a final concentration of 3% (w/v). The mixture of HClO_4 and denatured mitochondrial protein was then treated by a scaled-down modification of the procedure used by Tubbs & Garland (1964) for the extraction of CoA derivatives from rat liver. Samples for the assay of CoASH (free thiol) were processed and assayed within 6 hr.

Experimental design. As shown in Fig. 3(a)–(c), or Fig. 9, mitochondria were incubated with continuous recording of

Fig. 1. Apparatus for incubation of mitochondria. (a) A 20ml. Chance syringe (A) is fitted with a Repette plunger assembly (B). The syringe is fitted with a B9 Quickfit stopper (C) (Quickfit and Quartz Ltd., Stone, Staffs.), into which fits a drilled stopper containing a hollow needle (D). The syringe nozzle is removed, permitting approach of the magnetic stirrer (E), which rotates the magnetic follower in the mitochondrial suspension (stippled compartment). The plunger has a Teflon head (F) sealed with a rubber 'O' ring. (b) A cross-section through X-X'. Two B14 Quickfit cones (G) are mounted as shown, and one cone contains an oxygen electrode (H) (Clark type from Electronic Industries Ltd., Richmond, Surrey) mounted on a Teflon adaptor (I). The other cone is sealed with a stopper or could be used to hold an alternative electrode system. In addition to the features drawn, all stoppers and cones are fitted with glass lugs to take tensioned springs or rubber bands to maintain a tight fit. The incubations are maintained at 28° either by adding a glass water jacket through which 28° water is circulated, or more simply by circulating the water through numerous coils of polythene tubing, wrapped around the syringe. When in use, additions are made through the B9 Quickfit cone, and samples taken after sealing this cone with its drilled stopper.

oxygen uptake or fluorescence, with intermittent sampling for assays of CoASH and derivatives. Control experiments, not shown in these or other Figures, were performed to demonstrate that any changes in the concentrations of CoASH and derivatives and oxygen uptake or fluorescence after an addition to the incubation did not occur in the absence of such an addition.

RESULTS

Assay methods. Fig. 2(a) shows typical progress curves for the assay of CoASH and acetyl-CoA. In each case the change in fluorescence was directly proportional to the starting concentration of CoASH or acetyl-CoA. Acetyl-dephospho-CoA is not a substrate for phosphate acetyltransferase (Michal & Bergmeyer, 1963) and it is not assayed.

Fig. 2. Progress curves for enzymic assays of CoASH, acetyl-CoA and dephospho-CoASH. The contents of reaction cuvette were as described in the Materials and Methods section, with the initial presence of 0.44 μM -CoASH in curve 1, 0.16 μM -CoASH and 0.22 μM -acetyl-CoA in curve 2, 0.34 μM -CoASH and 0.30 μM -dephospho-CoASH in curve 3, 0.34 μM -CoASH in curve 4, and 0.30 μM -dephospho-CoASH in curve 5. Oxoglutarate dehydrogenase (5 μg .) was added at points A, and phosphate acetyltransferase (1 μg .) at points B. The vertical bar in (a) represents 0.2 μM -NADH.

Dephospho-CoA is accepted by oxoglutarate dehydrogenase, but the K_m value is $6 \times 10^{-5} \text{ M}$ and therefore at least 600 times that for CoASH. Thus, under the conditions described here, the presence of both dephospho-CoASH and CoASH each at concentrations of less than 1 μM resulted in progress curves as in Fig. 2(b). All progress curves for rat-liver mitochondrial CoASH or derivatives have been of the type expected for CoA (i.e. rapid assay to completion), and no evidence for the presence of dephospho-CoA has been obtained. Further confirmation of this point was obtained by replacing the arsenate buffer of the assay medium with tris-chloride buffer, and then preincubating the assay system plus sample with acetyl phosphate (50mM) and phosphate acetyltransferase (3 μg .) for 15 min. before the addition of 50 μg of oxoglutarate dehydrogenase. Under these conditions CoASH is not recovered whereas dephospho-CoASH is. With this method no evidence was found for the presence of dephospho-CoA in rat-liver mitochondria. The

Table 1. Concentrations of CoASH, acetyl-CoA and long-chain fatty acyl-CoA in subcellular fractions of rat liver

Experimental details are given in the text. Homogenate was prepared from livers of three rats, and separate values are given for each of two experiments.

	In 100000g supernatant (mμmoles/g. of fresh liver)	In mitochondria (mμmoles/mg. of protein)	In microsomes (mμmole/mg. of protein)
CoASH	4.2, 6.0	1.12, 1.30	zero, zero
Acetyl-CoA	30.2, 27.0	0.5, 0.6	zero, zero
Long-chain fatty acyl-CoA	zero, zero	0.1, 0.5	0.11, 0.10

Fig. 3. Effects of carnitine on the oxidation of palmitate in the presence of 0.5 mM-L-malate. Initially the incubation medium also contained tris-phosphate buffer, pH 7.2 (2.5 mM), tris-malonate buffer, pH 7.2 (5.0 mM), defatted bovine plasma albumin (5 mg./ml.) and AMP (1 mM). Rat-liver mitochondria (A) were added to give a mitochondrial protein concentration of 4.0 mg./ml., followed by ATP (0.5 mM) plus palmitate (20 μM) (B), then DL-carnitine chloride (1.0 mM) (C), and then CoASH (5 μM) (D). In (b) and (c) the following were determined: ▲, CoASH; ○, acetyl-CoA; ■, long-chain fatty acyl-CoA.

recovery of CoASH or acetyl-CoA added to mitochondrial extracts is complete.

CoASH and derivatives in subcellular fractions of rat liver. The content of CoASH, acetyl-CoA and

Fig. 4. Effects of carnitine on the oxidation of palmitate in the absence of added L-malate. Initial conditions and additions were otherwise as described in Fig. 3. The additions were rat-liver mitochondria (A), followed by ATP plus palmitate (B), then DL-carnitine chloride (C), then CoASH (D), as in Fig. 3. In (b) and (c) the following were determined: ▲, CoASH; ○, acetyl-CoA; ■, long-chain fatty acyl-CoA.

long-chain fatty acyl-CoA in the mitochondria, microsomes and 100000g supernatant of rat liver is shown in Table 1. The microsomal and mitochondrial contents are related to protein content to permit evaluation of possible microsomal contribution to the mitochondrial results, whereas the 100000g supernatant content is calculated per g. wet wt. of liver to enable an approximate estimate to be made of the cytoplasmic concentration of CoASH.

Changes in concentrations of CoASH and derivatives in mitochondria oxidizing palmitate. (a) Carnitine-dependent changes. As shown by Fritz, Kaplan & Yue (1962), carnitine stimulates the oxidation of palmitate by mitochondria, and this effect is seen in Figs. 3(a) and 4(a). Before the addition of carnitine, the mitochondrial suspension contained 0.2–0.3 mμmole of long-chain fatty acyl-CoA/mg. of mitochondrial protein, and this concentration was unchanged by the addition of carnitine (Figs. 3c and 4c). Associated with carnitine addition there was a fall in the CoASH concentration and a rise in the acetyl-CoA concentration (Figs. 3b and 4b). The addition of CoASH to the incubation medium caused a further increase in the rate of oxidation, and the added CoASH became converted into long-chain acyl derivatives.

Fig. 5. Changes in mitochondrial concentrations of CoASH and derivatives during the oxidation of palmitoylcarnitine in the absence of added L-malate. Initially the incubation medium also contained tris-phosphate buffer, pH 7.2 (2.5 mM), tris-malonate buffer, pH 7.2 (5.0 mM), defatted bovine plasma albumin (5 mg./ml.) and ADP (1.2 mM). Rat-liver mitochondria (A) were added to give 3.9 mg. of mitochondrial protein/ml., followed by palmitoyl-DL-carnitine (29.4 μ M) (B). The vertical bar in (a) represents 0.210 μ g. atom of oxygen/ml., corresponding to the oxygen uptake during palmitoylcarnitine-stimulated respiration. In (b) and (c) the following were determined: $\blacktriangle$, CoASH; $\circ$, acetyl-CoA; $\blacksquare$, long-chain fatty acyl-CoA.

(b) Palmitoylcarnitine-dependent changes. Palmitoylcarnitine is rapidly oxidized by rat-liver mitochondria (Bremer, 1962; Fritz & Yue, 1963). Fig. 5 shows the changes in oxygen uptake and concentrations of CoASH and derivatives occurring after the addition of 29.4 μ M-palmitoyl-DL-carnitine to mitochondria in the absence of malate. Under these conditions the increase in oxygen consumption (0.210 μ g. atom/ml.) corresponded to the oxidation of 50% of the added palmitoyl-DL-carnitine to acetoacetate, which is in keeping with a specificity of carnitine palmitoyltransferase (EC 2.3.1.-) for either the L- or D-isomer. After the addition of palmitoylcarnitine there was a marked conversion of CoASH into acetyl-CoA (Fig. 5b), and a rise in the long-chain fatty acyl-CoA concentration (Fig. 5c). These changes were gradually reversed as the oxidation of palmitoylcarnitine approached completion. Repetition of this experiment in the presence of 0.5 mM-L-malate resulted in a large increase in oxygen consumption compatible with the conversion into citrate of at least 90% of the palmitoylcarnitine oxidized (Fig. 6a). Although the presence of malate decreased the acetyl-CoA

Fig. 6. Changes in mitochondrial concentrations of CoASH and derivatives during the oxidation of palmitoylcarnitine in the presence of 0.5 mM-L-malate. Conditions were otherwise as described in Fig. 5. The additions were rat-liver mitochondria (A), followed by palmitoyl-DL-carnitine (B), as in Fig. 5. The vertical bar in (a) represents 0.298 μ g. atom of oxygen/ml., corresponding to the oxygen uptake during palmitoylcarnitine-stimulated respiration. In (b) and (c) the following were determined: $\blacktriangle$, CoASH; $\circ$, acetyl-CoA; $\blacksquare$, long-chain fatty acyl-CoA.

content to undetectable levels before the addition of palmitoylcarnitine, acetyl-CoA accumulated during the oxidation of palmitoylcarnitine (Fig. 6b).

Studies with non-respiring mitochondria. The experiments described in Figs. 5 and 6 were repeated except that the palmitoylcarnitine addition preceded that of ADP (Figs. 7 and 8). In each case the addition of palmitoylcarnitine caused extensive long-chain acylation of CoA (Figs. 7d and 8d), but a rise in acetyl-CoA concentration did not occur until oxidation was initiated by the addition of ADP. A carnitine-dependent increase of mitochondrial long-chain fatty acyl-CoA in the presence of ATP and palmitate could also be demonstrated in the presence of oligomycin under conditions where changes in reduction of mitochondrial nicotinamide nucleotides were followed fluorimetrically, as described by Chappell & Crofts (1965), and as shown in Fig. 9.

DISCUSSION

Assays. As discussed by Garland (1964), the assay of CoASH with oxoglutarate dehydrogenase is free from interference by acyl-CoA derivatives or CoA disulphides. Further, under the conditions of

Fig. 7. Changes in mitochondrial concentrations of CoASH and derivatives after ADP stimulation of the oxidation of palmitoylcarnitine in the presence of 0.5 mM-L-malate. Conditions were otherwise as described in Fig. 6 except that ADP (C) was added last, after rat-liver mitochondria (A) and palmitoyl-DL-carnitine (B). In (b), (c) and (d) the following were determined: ▲, CoASH; ○, acetyl-CoA; Δ, total mitochondrial CoA extractable in HClO_4 ; ●, excess of total mitochondrial CoA extractable in HClO_4 over the sum of acetyl-CoA and CoASH; ■, long-chain fatty acyl-CoA.

Fig. 8. Changes in mitochondrial concentrations of CoASH and derivatives after ADP stimulation of the oxidation of palmitoylcarnitine in the absence of added L-malate. Conditions were otherwise as described in Fig. 5, except that ADP (C) was added last, after rat-liver mitochondria (A) and palmitoyl-DL-carnitine (B). In (b), (c) and (d) the following were determined: ▲, CoASH; ○, acetyl-CoA; Δ, total mitochondrial CoA extractable in HClO_4 ; ●, excess of total mitochondrial CoA extractable in HClO_4 over the sum of acetyl-CoA and CoASH; ■, long-chain fatty acyl-CoA.

low (approx. $1\mu\text{M}$) concentrations of CoASH the assay can detect the presence of similar quantities of dephospho-CoASH and distinguish between CoASH and dephospho-CoASH by means of the enzyme-reaction progress curves obtained (Fig. 2b). Unlike the acetyl-CoA assay of Wieland & Weiss (1963) the assay described here for acetyl-CoA does not react with acetyl-dephospho-CoA, nor does it suffer from the incomplete recovery described by Pearson (1965) for the Wieland & Weiss (1963) method. Although dephospho-CoA has not been detected by us in rat-liver mitochondria it is present in rat heart muscle (P. B. Garland, D. Shepherd & D. W. Yates, unpublished work). Wieland, Weiss & Eger-Neufeldt (1964) have demonstrated that palmitoyl-dephospho-CoA has different regulatory functions from palmitoyl-CoA, and it would thus seem important that in ascribing regulatory functions to CoASH or dephospho-CoASH (and their acyl derivatives) a distinction should be made between the two compounds, which, through the use of enzymes of insufficient specificity, may be described as 'coenzyme A'.

'Compartmentation' of coenzyme A in rat-liver mitochondria. Freshly isolated rat-liver mitochondria contained a rather variable amount of long-chain fatty acyl-CoA ($0.1\text{--}0.5\text{ m}\mu\text{mole/mg.}$ of mitochondrial protein), and higher concentrations were not obtained by incubation of mitochondria with ATP in the absence of carnitine. This long-chain fatty acyl-CoA content is unable to support respiration at more than 10% of the rate obtained with palmitoylcarnitine, and it may be concluded that this long-chain fatty acyl-CoA content is in a compartment separate from the enzymes of β -oxidation but not separate from palmitoyl-CoA-synthetase activity (Figs. 3, 4 and 9). Since added CoASH can both stimulate the oxidation of palmitate and also be long-chain fatty acylated it presumably has access to the site of palmitoyl-CoA-synthetase activity, which, as shown by Chappell & Crofts (1965), is within an atractyloside-sensitive membrane. The permeability of this membrane to CoASH could explain the variable amount of CoASH that can be acylated by palmitate

Fig. 9. Carnitine-dependent changes in the reduction of mitochondrial nicotinamide nucleotides and changes in the concentrations of CoASH and long-chain fatty acyl-CoA. The incubation mixture had initial additions of pentachlorophenol ($5\mu\text{M}$), tris-malonate buffer, pH 7.2 (5mM), and mitochondria ($3.0\text{mg. of protein/ml.}$). After 3 min. pre-incubation defatted bovine plasma albumin (5mg./ml.) plus oligomycin ($10\mu\text{g./ml.}$) were added (A), followed by palmitate ($10\mu\text{M}$) plus ATP (0.5mM) (B), then DL-carnitine chloride (1.0mM) (C). Samples were removed from the reaction cuvette at times indicated by breaks in the recording of fluorescence in (a). In (b) the following were determined: $\blacktriangle$, CoASH; $\blacksquare$, long-chain fatty acyl-CoA.

and ATP in the absence of carnitine, if it is assumed that CoA found in this compartment in twice-washed mitochondria is derived from adsorbed long-chain fatty acyl-CoA, which is capable of strong binding to protein (Tubbs, 1963). CoASH itself would be expected to be lost from this compartment, and so the amount of CoA found in this compartment would vary with the extent of long-chain fatty acylation of CoASH at the time of preparing mitochondria from rat livers, and with the efficiency of subsequent washing.

The remaining mitochondrial CoA assayed in these experiments is more constant in amount ($1.5\text{--}2.0\text{nmol/mg. of mitochondrial protein}$), and behaves as if it were in a compartment separate from palmitoyl-CoA-synthetase activity but accessible to palmitoylcarnitine (Figs. 5–8), palmitate in the presence of ATP and carnitine (Fig. 9), and the enzymes of β -oxidation, citrate synthesis and the formation of oxaloacetate from malate (see below).

These interpretations both agree with and provide independent support for those of Chappell & Crofts (1965), and likewise permit a more precise definition of the location of carnitine-dependent acyl-transfer between intracellular compartments as initially proposed by Bremer (1962) and Fritz & Yue (1963).

Acetyl-CoA content of rat-liver mitochondria. One feature of our results is the presence of considerable amounts of acetyl-CoA in mitochondria incubated without added substrates (Figs. 4 and 6). This acetyl-CoA behaved as if it were in the CoA pool in the matrix, since its concentration changed with the oxidation of palmitoylcarnitine (Figs. 5–8), the oxidation of palmitate in the presence of ATP and carnitine (Figs. 3 and 4), and the presence of L-malate (Figs. 3–8), but not with the presence of palmitate and ATP in the absence of carnitine (Figs. 3 and 4).

Changes in the mitochondrial content of acetyl-CoA have been implicated in the control of acetoacetate synthesis (Wieland & Weiss, 1963; Tubbs & Garland, 1964), and the extent to which an increase in mitochondrial acetyl-CoA content can influence acetoacetate synthesis can be assessed from Fig. 5. Thus before the addition of palmitoylcarnitine the rate of oxygen uptake was $0.005\mu\text{g. atom/min./mg. of mitochondrial protein}$, and could correspond to a maximal rate of acetoacetate synthesis of $1.44\text{m}\mu\text{moles/min./mg. of mitochondrial protein}$ if the oxygen uptake were due to oxidation of endogenous palmitate. The addition of palmitoylcarnitine caused a tenfold rise in the rate of oxygen uptake and therefore a tenfold rise in the calculated rate of acetoacetate synthesis, yet the mitochondrial acetyl-CoA content was only doubled. It is therefore evident that under these conditions control of the rate of acetoacetate synthesis must be largely exerted on the rate of conversion of acetyl-CoA into acetoacetate, and this control is not directly explicable in terms of changes in mitochondrial acetyl-CoA content.

Hulsman, Siliprandi, Ciman & Siliprandi (1964) have proposed that carnitine activates fatty acid oxidation by lowering the intramitochondrial $[\text{acetyl-CoA}]/[\text{CoASH}]$ ratio. This mechanism cannot be operative in the carnitine-stimulated palmitate oxidation shown in Figs. 3 and 4, since the addition of carnitine and the onset of palmitate oxidation is associated with a marked rise in the $[\text{acetyl-CoA}]/[\text{CoASH}]$ ratio.

Intermediates of β -oxidation. In experiments in which the mitochondrial oxidation of palmitoylcarnitine was stimulated by the addition of ADP (Figs. 7 and 8), the long-chain fatty acyl-CoA content measured before the addition of ADP would presumably be composed of palmitoyl-CoA, and during ADP-stimulated oxidation would be

expected to contain intermediates such as the 2,3-unsaturated, 3-hydroxy- and 3-oxo-acyl-CoA derivatives corresponding to the C₁₆, C₁₄ and C₁₂ levels of β -oxidation. Further, the mitochondrial CoASH content should be lowered owing to the formation of these intermediates and also those of lower chain length that are soluble in acid. Reference to Figs. 7(b-d) and 8(b-d) shows that stimulation of the oxidation of palmitoylcarnitine with ADP (Figs. 7a and 8a) was associated with a fall in the long-chain fatty acyl-CoA content (Figs. 7d and 8d). Further, the total amount of CoASH found in the perchloric acid-soluble fraction of mitochondrial CoA, after treatment of this fraction with alkali and 2-mercaptoethanol (Tubbs & Garland, 1964), was only slightly in excess of the sum of the mitochondrial CoASH and acetyl-CoA, and this slight excess (which must include any succinyl-CoA) was decreased to undetectable levels (less than 0.02 μ mole/mg.) on stimulation of oxidation with ADP (Figs. 7c and 8c). Thus the process of conversion of palmitoyl-CoA into acetyl-CoA appears to occur without detectable accumulation of the 27 possible acyl-CoA compounds thought to be involved (Jaenicke & Lynen, 1960). Similar conclusions were drawn by Drysdale & Lardy (1953), who were unable to demonstrate acyl-CoA intermediates (as their hydroxamates) in the conversion of octanoyl-CoA into acetyl-CoA catalysed by a solubilized system from rat-liver mitochondria, and who suggested the possibility that the true intermediates of β -oxidation were enzyme-bound.

The absence of detectable intermediates in fatty acid oxidation resembles the situation in fatty acid synthesis (Bressler & Wakil, 1961; Martin & Vagelos, 1961; Lynen & Tada, 1961), where the intermediates involved are enzyme-bound as thiol esters of one or more of the proteins involved (Lynen, 1961; Bressler & Wakil, 1962; Alberts, Goldman & Vagelos, 1963; Brodie, Wasson & Porter, 1963). If this were also the case for fatty acid oxidation it could be expected that some of the enzymes involved would have active thiol groups and also thioacyltransferase activity. This is so for 3-oxo-acyl-CoA thiolase (EC 2.3.1.16) (Lynen, 1953; Goldman, 1954) and for enoyl-CoA hydratase (EC 4.2.1.17) (Stern, 1961).

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