The Localization of Some Coenzyme A-Dependent Enzymes in Rat Liver Mitochondria

By B. A. HADDOCK, D. W. YATES AND P. B. GARLAND*

Department of Biochemistry, University of Bristol, Bristol, BS8 1TD, U.K.

(Received 20 April 1970)

1. CoA, acetyl-CoA, L-carnitine and acetyl-L-carnitine when added to rat liver mitochondria equilibrate with approximately two-thirds of the total intra-mitochondrial water. The mitochondrial space calculated to be freely permeable to these solutes was identical with that obtained for sucrose. 2. Acetyl-CoA is rapidly deacylated by rat liver mitochondria at 0°C, and special precautions are required to measure its mitochondrial permeation. 3. Rat liver mitochondria were separated into fractions that correspond to the inner membrane, the outer membrane, and the soluble proteins of the matrix and intermembrane compartment. Soluble enzymes considered to be located in the matrix were citrate synthase (EC 4.1.3.7), palmitoyl-CoA dehydrogenase (EC 1.3.2.2), electron-transferring flavoprotein, medium-chain-length ATP-specific fatty acyl-CoA synthetase (EC 6.2.1.2), L-3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.35) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16). Carnitine palmitoyltransferase (EC 2.3.1.-) is largely associated with the inner-membrane fraction. A long-chain-length ATP-specific fatty acyl-CoA synthetase (EC 6.2.1.3) is associated with the outer-membrane fraction.

The purpose of this paper is to present experimental results on the penetration of mitochondria from rat liver by CoA, L-carnitine and their acetyl esters. In addition, we describe the distribution of some of the individual enzymes of fatty acid oxidation between mitochondrial fractions corresponding to the inner membrane, the outer membrane and the soluble proteins of the matrix and intermembrane compartments. Preliminary reports of this work have been published (Yates & Garland, 1966; Garland & Yates, 1967; Yates & Garland, 1967*a,b*) and also incorporated in two review articles (Tubbs & Garland, 1968; Garland, Haddock & Yates, 1969).

MATERIALS AND METHODS

Reagents. Dithiothreitol was purchased from Calbiochem, London W.1, U.K.; DL-carnitine and ethanedioxybis(ethylamine)tetra-acetate (EGTA) from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; tritiated water (25mCi/ml) from The Radiochemical Centre, Amersham, Bucks., U.K.; 5,5'-dithiobis-2-(nitrobenzoic acid) from Aldrich Chemical Co. Inc., through R. N. Emanuel Ltd., London S.E.1, U.K.; NAD⁺, NADH, ATP, GTP, CoA, oxaloacetic acid, 2-oxoglutaric acid, L-malic acid and sodium pyruvate from Boehringer Corp. (London) Ltd., London W.5, U.K.; oligomycin, antimycin

A, rotenone and tris base from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; silicone fluid type 702 from Edwards High Vacuum Ltd., Crawley, Sussex, U.K.; silicone fluid type 175/200 10 CS and silicone 'Repelcote' [2% (v/v) solution of dimethyldichlorosilane in carbon tetrachloride] from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; Blue Dextran from Pharmacia, Uppsala, Sweden; 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene from Thorn Electronics, Tolworth, Surrey, U.K.; bovine plasma albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; deca-2,4,6,8-tetraenoic acid from K & K laboratories (Hollywood, Calif., U.S.A.); and palmitoylchloride from the Hormel Institute (University of Minnesota, Minneapolis, Minn., U.S.A.). All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and were of the highest grade available. Water distilled twice from an all-glass apparatus was used throughout.

Preparations. Bovine plasma albumin was freed from fatty acids by the method of Goodman (1958). Mitochondria were prepared from the livers of Wistar rats of either sex as described by Jones & Jones (1969). Acetyl-CoA was synthesized from CoA and acetic anhydride by the method of Simon & Shemin (1953), acetyl-DL-carnitine from carnitine and acetylchloride by the method of Bremer (1962), acetoacetyl-CoA from diketene and CoA by the method of Wieland & Rueff (1953), and palmitoyl-CoA from palmitoylchloride and CoA by the method of Seubert (1960). Carnitine acetyltransferase (EC 2.3.1.7) was prepared from pigeon breast muscle by the method of Chase, Pearson & Tubbs (1965).

Assays. Protein was assayed either by a biuret

* Present address: Department of Biochemistry, University of Dundee, Dundee DD1 4HN, U.K.

method (Gornall, Bardawill & David, 1949) after solubilization of mitochondria in 0.1% (v/v) Triton X-100, or by the method of Lowry, Rosebrough, Farr & Randall (1951). Sucrose (Kulka, 1956), acetyl-CoA (Srere, Brazil & Gonen, 1963), L-carnitine (Pearson & Tubbs, 1964a) and acetyl-L-carnitine (Pearson & Tubbs, 1964b) were assayed spectrophotometrically. CoA was assayed fluorimetrically (Garland, Shepherd & Yates, 1965); this procedure was also used in a fluorimetric modification of the assay for carnitine by coupling the release of CoA in the carnitine acetyltransferase reaction (1) to the reduction of NAD⁺ by oxoglutarate dehydrogenase (reaction 2).

The radioactivity of the tritiated water of solutions under study (see below) was measured in 20 μ l samples added to 10 ml of a scintillation mixture containing 100 g of naphthalene, 7 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of peroxide-free dioxan, by using a Nuclear-Chicago series 720 liquid-scintillation counter. The counting efficiency was 30% and all samples were measured in duplicate.

Enzyme assays. The following enzyme assays were used: malate dehydrogenase (EC 1.1.1.37, Ochoa (1955); monoamine oxidase (EC 1.4.3.4), Schnaitman, Erwin & Greenawalt (1967); L-3-hydroxyacyl-CoA dehydrogenase, Lynen & Wieland (1955); 3-ketoacyl-CoA thiolase, Stern (1955); citrate synthase, Srere *et al.* (1963); carnitine palmitoyltransferase, Yates & Garland (1970); ATP-specific decatetraenoyl-CoA synthetase (EC 6.2.1.2), Garland, Yates & Haddock (1970); GTP-specific decatetraenoyl-CoA synthetase, (EC 6.2.1.-), Garland *et al.* (1970); palmitoyl-CoA synthetase, Yates, Shepherd & Garland (1966). Cytochrome *b*₅ was assayed with a dual-wavelength spectrophotometer, by measuring the extinction change at 557 minus 575 nm after the addition of NADH (0.1 mM) to a suspension of the mitochondrial fraction in 2.0 ml of an air-saturated solution at 30°C containing potassium phosphate buffer, pH 7.5 (75 mM), rotenone (0.25 μ g/ml) and defatted bovine plasma albumin (5 mg/ml). In this, as in all other spectrophotometric assays except low-temperature spectroscopy, the light-path of the cuvette was 1.0 cm. For the reduced-minus-oxidized extinction difference of cytochrome *b*₅ at 557 minus 575 nm we have taken Klingenberg's (1958) ϵ_{mM} value of 20.

The assay of electron-transferring flavoprotein (ETF) is an assay that also measures the flavoprotein of acyl-CoA dehydrogenases (Garland, Chance, Ernster, Lee & Wong, 1967). The relative contribution of these species is not known with certainty, but in view of the fact that in ox heart mitochondria the major part of the total mitochondrial flavoprotein can be accounted for as ETF (Beinert, 1967), we have tentatively assumed that this assay measures largely ETF. In the remainder of the paper we refer to this assay as measuring ETF; this is convenient but the underlying assumption must not be overlooked. In greater detail, the assay starts at the point where cytochrome *b*₅ had been reduced by NADH and assayed as just described. The wavelength pair was

then altered to 476 and 515 nm and re-balanced, and the following additions were made: antimycin A (1 μ g/ml), succinate (5 mM) and lastly palmitoyl-CoA (10 μ M). The decrease in extinction at 475 minus 515 nm after the addition of palmitoyl-CoA was used to calculate the ETF concentration, by using $\epsilon_{\text{mM}} = 8.0$ for the reduced-minus-oxidized difference. The addition of succinate and antimycin A before palmitoyl-CoA reduces other pigments (succinate dehydrogenase, cytochrome *b*), which could also be reduced by palmitoyl-CoA (Chance *et al.* 1967).

Cytochromes *a*+*a*₃ were assayed in the same cuvette as cytochrome *b*₅ and ETF. After the latter assay was completed, the wavelength pair was altered to 605 and 630 nm and re-balanced. The change in extinction on addition of a few small grains of Na₂S₂O₄ was then observed and used to calculate the cytochrome *a*+*a*₃ concentration on the basis of $\epsilon_{\text{mM}} = 16$ for the reduced-minus-oxidized difference (Chance, 1957).

Spectra of submitochondrial fractions. These were obtained on a wavelength-scanning spectrophotometer constructed on the general lines of that described by Yang (1954), (see also Garland *et al.* 1970). For room-temperature studies the entrance and exit slit widths were 2 mm, corresponding to a spectral band-width of approx. 2.7 nm. For spectra at the temperature of liquid N₂ a cell holder and a vacuum-flask assembly were constructed as described by French & Lawrence (1968). The cuvettes each had a light-path of 0.2 cm. A slit width of 0.5 mm, corresponding to a spectral band-width of 0.68 nm, was used. Spectra at room temperature were obtained to check that the dual-wavelength spectrophotometric assays for cytochrome *b*₅, cytochromes *a*+*a*₃, and ETF, were not subject to interference from other spectral changes. Such spectra are not published here, but they did demonstrate the specificity of the dual-wavelength spectrophotometric assays.

Calculations of recoveries in submitochondrial fractions. To establish the location of an enzyme activity in a particular fraction it is necessary not only to show an increase in specific activity in that fraction over the starting material but also to show that a high proportion of the total activity is recovered in the fraction. In view of this, we have calculated the percentage recovery of enzyme activity within a fraction from the following equation.

$$\text{Percentage recovery} = \frac{\text{activity in fraction}}{\text{activity in starting material}} \times 100$$

Similar considerations apply in those instances where a mitochondrial component was assayed not as a catalytic enzyme activity but as an absolute amount of a chromophoric group (e.g. cytochrome *b*₅).

Experimental design for the determination of permeable spaces. Mitochondria were suspended in a suitable medium (see below) containing Blue Dextran, tritiated water, sucrose and the solute under test. After a brief incubation, the mitochondria were separated from the medium by centrifugation. The amounts of tritiated water, Blue Dextran, sucrose and solute in the supernatant and mitochondrial pellet were used to calculate the intra- and extra-mitochondrial water of the pellet, and also the percentage of the intramitochondrial water permeated by sucrose. Calculations were made by the method of Bartley & Davies (1954), and the assumption

was made that the solute under test would equilibrate completely with a fraction of the intramitochondrial water rather than incompletely with all the intramitochondrial water. The percentage of the intramitochondrial water calculated to be in equilibrium with the added solute is called the 'permeable space' for that solute. Other assumptions were that Blue Dextran, a polysaccharide of molecular weight greater than 2×10^6 , neither penetrated nor was absorbed by the mitochondria, and that tritiated water equilibrated isotopically with the initially unlabelled intramitochondrial water.

Two procedures of experimental design were used, and they are termed 'slow' and 'fast' according to the speed with which the mitochondria were separated from the incubation medium and extracted with HClO_4 . Both procedures were carried out at 0–4°C except where stated otherwise.

Slow procedure. Of a mitochondrial suspension (containing 25–30 mg of protein/ml of 0.25 M-sucrose) 0.5 ml was diluted in a 10 ml polythene centrifuge tube with 4.5 ml of a medium containing KCl (80 mM), tris-chloride buffer, pH 7.2 (20 mM), sucrose (20 mM), Blue Dextran (1%, w/v), EDTA (1 mM) and tritiated water ($4 \mu\text{Ci/ml}$). In some experiments 0.25 M-sucrose was substituted for KCl and the tris-chloride concentrations lowered to 5 mM. Further solutes were added as indicated below. The mitochondria suspended in this medium were left at 0°C or 30°C for 10 min and then centrifuged at 15000g for 10 min in the 8×50 ml angle rotor of a MSE 18 centrifuge. The supernatant was removed and added to 0.2 ml of 70% (w/v) HClO_4 . The mitochondrial pellet was suspended in 2 ml of 5% (w/v) HClO_4 , and recentrifuged at 15000g for 10 min. The two supernatants obtained in this way were assayed spectrophotometrically for Blue Dextran at 610 nm, where the value $E_{1\%}^{1\text{cm}}$ is 10.0 under these conditions of pH. Each supernatant was then neutralized by adding 0.1 vol. of 1 M- K_2HPO_4 followed by drops of 4 M-KOH until the pH was 6.8 as measured with an external indicator. All the relevant volumes and additions were noted. The neutralized extracts were then assayed for tritiated water, sucrose and the solute under study.

Fast Procedure. This is based on the methods of Werkheiser & Bartley (1957) and Klingenberg & Pfaff (1966). First 1 ml of 30% (v/v) glycerol containing 5% (w/v) HClO_4 was placed in a 5 ml nitrocellulose centrifuge tube previously treated with 'Repelcote', followed by 1.5 ml of a mixture of 40% (v/v) MS 200/10CS and 60% (v/v) 702 silicone fluids. Then 2 ml of the mitochondrial incubation mixture as described above for the slow procedure was layered over the silicone layer. The density of the glycerol- HClO_4 layer was 1.09 g/ml, that of the silicone fluid 1.04 g/ml. In experiments where CoA was the solute under study, 2-mercaptoethanol (10 mM) was added to the glycerol- HClO_4 layer. The tubes were centrifuged at 76000g (30000 rev./min) in the SW 39 rotor of a Beckman-Spinco Model L2 centrifuge for 10 min. In such experiments the centrifugation was commenced 8 min after addition of the mitochondria to the incubation medium. Immediately after centrifugation the top layer was removed and added to 0.1 ml of 70% (w/v) HClO_4 . In the experiments involving CoA 0.01 ml of 2-mercaptoethanol solution (2.0 M) was also added to the acidified top layer. The silicone fluid layer

was washed with 2–3 ml of water, removed from the centrifuge tube and discarded. The washing diminished the risk of contaminating the bottom layer of glycerol- HClO_4 , which was then removed from the pellet of denatured mitochondria. The two acidic supernatants were then treated as described for the slow procedure.

Control experiments. Rat liver mitochondria contain approx. 2 nmol of CoA/mg of protein, and the degree of acylation varies with the experimental conditions (Garland *et al.* 1965). It was therefore necessary when studying mitochondrial penetration by CoA or acetyl-CoA to perform parallel experiments in which these solutes had not been added. This enables a correction to be made for the endogenous amount of CoA and acetyl-CoA in the mitochondria. No such control was required in the experiments with carnitine or acetylcarnitine; application of the sensitive fluorimetric assay demonstrated that the content of carnitine, either as such or as acid-soluble acyl-derivatives, was less than 0.1 nmol/mg of protein.

Subfractionation of mitochondria. All procedures were carried out at 0°C. Freshly prepared mitochondria (about 200 mg of protein) were left suspended in 30 ml of tris-phosphate buffer, pH 7.5 (10 mM-phosphate), for 7 min. Then 10 ml of a solution containing KCl (0.48 M), ATP (2 mM), MgSO_4 (2 mM) and adjusted to pH 7.5 with tris, was added. After a further 10 min the suspension was exposed in 10 ml samples for 10 s to the full output of an MSE 60W ultrasonic probe; 20 ml of the resultant suspension was layered on a discontinuous sucrose gradient consisting of 0.76 M-sucrose (11 ml) on 1.32 M-sucrose (22 ml), and then centrifuged in the SW25 rotor of a Beckman-Spinco L2 centrifuge at 24000 rev./min for 3.5 h. Five discrete bands separated during centrifugation, and were named as shown in Fig. 1.

RESULTS

Space measurements. As measured by the slow procedure, the mitochondrial pellet obtained after the incubation and centrifugation contained typically 7 μl of water/mg of protein, of which 2 μl were attributable, on the basis of the Blue Dextran content, to extramitochondrial water. Thus the intramitochondrial water was 5 μl /mg of protein. Similar results were obtained with the rapid procedure.

The permeation of several solutes into the intramitochondrial water was studied in the experiments shown in Table 1, by using the slow procedure. In agreement with the reports of Werkheiser & Bartley (1957), Tedeschi & Harris (1955), Malamed & Recknagel (1959), Klingenberg & Pfaff (1966) and Brierley & O'Brien (1965), 60–80% of the total mitochondrial water was calculated to have equilibrated with the extramitochondrial concentration of sucrose. The values for carnitine, acetylcarnitine and CoA were very similar to those for sucrose. The apparent non-penetration of the mitochondria by acetyl-CoA was unexpected, and raised the possibility that the incomplete recovery of acetyl-CoA from the

separated mitochondria was influencing the results. Experiments were therefore done in which the incubation medium used in the study of acetyl-CoA penetration was set up and assayed for acetyl-CoA as a function of time. It was found that under the conditions of the incubation (2°C, 0.5mM- or 1.0mM-acetyl-CoA) the mitochondria catalysed the disappearance of 1–2nmol of acetyl-CoA/min per mg of protein. It can be readily estimated that

Fig. 1. Density-gradient centrifugation and fractions. The initial layers from above downwards were L1 (20ml of mitochondrial suspension as described in the Materials and Methods section), L2 (11ml of 0.76M-sucrose) and L3 (22ml of 1.36M-sucrose). The fractions (and their various designations) obtained by centrifugation were S1 (soluble proteins from the intermembrane and matrix spaces), S2 (small fragments of outer membrane and soluble proteins), M1 (outer membrane), S3 (contains <1% of total mitochondrial protein) and M2 (intact mitochondria, inner membrane and unreleased soluble proteins).

such a rate would result in a decrease of the calculated acetyl-CoA permeable space of mitochondria to a low or even negative value if the time between mitochondrial separation and fixation occupied 3–5min, as it inevitably did in the slow procedure.

When the fast procedure was used the permeable space for acetyl-CoA approached that for sucrose (Table 2). During the transit of the mitochondria from the incubation phase to the bottom layer of glycerol-perchloric acid, there was a time-interval in which loss of acetyl-CoA could occur. This time-interval was assumed to last about 15s (Pfaff, 1965), and correction was accordingly made for the loss of acetyl-CoA on the basis of a loss rate of 1.5nmol/min per mg of protein. When this correction was made there was a closer agreement between the acetyl-CoA space and the sucrose space. The cause of acetyl-CoA disappearance is not known, but could be due to deacylation. It is therefore apparent, as suggested by Pfaff (1967),

Table 2. Penetration of mitochondria by CoA and acetyl-CoA

The experiments were carried out at 0–3°C, with the 25mM-sucrose medium and fast procedure (see the Materials and Methods section). The concentration of CoA or acetyl-CoA was 1.0mM. Results are expressed as means $\pm$ S.E.M., with number of observations in parentheses.

Solute	Percentage of intramitochondrial water equilibrated with solute
Sucrose	62.6 $\pm$ 6.3 (6)
CoA	57.0 $\pm$ 5.0 (2)
Acetyl-CoA	51.3 $\pm$ 3.8 (2)
Acetyl-CoA (corrected for loss)	60.0 $\pm$ 4.5 (2)

Table 1. Penetration of mitochondria by various solutes

For details of the experimental procedure, see the Materials and Methods section. The slow procedure was used. Results are expressed as mean $\pm$ S.E.M., with numbers of observations in parentheses.

Concn. of sucrose (mM)	Temp. of medium (°C)	Solute studied	Concn. (mM)	Percentage of intramitochondrial water equilibrated with solute
25	0	Sucrose		79.8 $\pm$ 7.3 (8)
25	0	L-Carnitine	2	75.3 $\pm$ 2.8 (4)
25	0	Acetyl-L-carnitine	2	79.4 $\pm$ 1.1 (2)
250	0	Sucrose		69.7 $\pm$ 8 (7)
250	0	L-Carnitine	1.0	64.0 $\pm$ 3 (2)
250	0	L-Carnitine	1.5	62.0 (1)
250	0	L-Carnitine	2.0	68.0 $\pm$ 11 (3)
25	25	Sucrose		82.5 $\pm$ 6.5 (2)
25	25	L-Carnitine	2.0	73.7 $\pm$ 1.7 (2)
25	0	Acetyl-CoA	1	2.0 $\pm$ 6.1 (3)
25	0	CoASH	1	74.2 $\pm$ 2.4 (4)

Table 3. *Distribution of marker enzymes and some enzymes of fatty acid oxidation in rat liver mitochondrial fractions*

For experimental procedures, see the Materials and Methods section. The results given below are for a single experiment that was typical of four others. Units of specific activity: * $\mu\text{mol}/\text{min}$ per mg of protein; † nmol/min per mg of protein; ‡ nmol/mg of protein. % indicates percentage of total activity present in that fraction. n.d., Component not detectable; —, component not assayed.

Fraction (see Fig. 1)	Malate dehydrogenase		Monoamine oxidase		Cytochrome b_5		Cytochrome $a+a_3$		L-3-Hydroxy- acyl-CoA dehydrogenase		3-Ketoacyl- CoA thiolase		Citrate synthase		Carnitine palmitoyl- transferase		ETF	
	% Protein	*	†	%	†	%	†	%	†	%	†	%	†	%	†	%	†	%
Sonicated mitochondria	100	3.0	3.5	100	0.054	100	0.087	100	705	100	212	100	153	100	9.3	100	0.114	100
S1	29.3	4.5	n.d.	0	0.013	7	n.d.	0	454	18	356	47	182	35	n.d.	0	0.127	33
S2	8.4	1.5	17.4	41	—	—	—	—	353	4	201	8	59	3	—	—	—	—
S3	6.0	0.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
M1	8.7	n.d.	14.5	36	0.287	46	0.034	4	216	3	6	0.2	16	0.9	n.d.	0	n.d.	0
M2	47.6	3.7	1.9	25	0.043	39	0.164	90	1050	71	202	45	205	63	17.6	98	0.161	68

that the low value for the acetyl-CoA permeable space obtained by the slow procedure can be attributed to methodological errors.

Measurements of the permeation of mitochondria by long-chain fatty esters of CoA and carnitine were not attempted. The ability of these hydrophobic molecules to bind to protein and also to form micelles invalidates the calculations of permeable spaces, since these depend on the assumption that neither absorption nor micelle formation occurs. However, it seems likely that the permeation of mitochondria by such long-chain fatty esters would, provided lytic effects were avoided, be the same as that of the acetyl esters.

Distribution of enzymes in submitochondrial fractions. As markers for the outer mitochondrial membrane we have used cytochrome b_5 (Sottocasa, Kuylensstierna, Ernster & Bergstrand, 1967) and monoamine oxidase (Schnaitman *et al.* 1967). By using the assay system of Schnaitman *et al.* (1967) we have been unable to detect any monoamine oxidase activity in a microsome fraction obtained from rat liver. As a marker for the inner mitochondrial membrane we have used cytochrome $a+a_3$, and for the matrix proteins, malate dehydrogenase activity. The distribution of these enzymes is shown in Tables 3 and 4, and it is apparent that fraction M2 corresponded to an inner membrane-matrix fraction, fraction S1 to a matrix fraction, and fractions M1 and S2 to an outer-membrane fraction. Alternative confirmation of the effectiveness of the separation procedure was also obtained by electron microscopy. The techniques and results were similar to those of Sottocasa *et al.* (1967). It was noted that fraction S2 contained numerous small particles, not readily resolved. These are possibly fragments of outer membrane that did not sediment in the time available.

Distribution of some of the enzymes associated with β -oxidation of fatty acids. From Table 3 it can be seen that thiolase, citrate synthase and ETF exhibited a distribution pattern similar to malate dehydrogenase, and so it may be concluded that these enzymes are associated with the mitochondrial matrix. 3-Hydroxyacyl-CoA dehydrogenase was more tightly bound to the inner membrane than were the other enzymes of the matrix. Carnitine palmitoyltransferase activity was found exclusively in the inner membrane-matrix fraction M2. No activity was found in the soluble matrix fraction S1 or outer membrane fraction.

Table 4 indicates the distribution of acyl-CoA synthetases in the submitochondrial fractions. The palmitoyl-CoA synthetase activity was present largely in the outer-membrane fraction. The presence of activity in the other fractions was due in part to cross-contamination and perhaps also to acceptance of palmitate by medium-chain-length

Table 4. *Distribution of fatty acyl-CoA synthetase in rat liver mitochondrial fractions*

For experimental procedures, see the Materials and Methods section. The results of two typical experiments are shown, one experiment used for the measurement of palmitoyl-CoA synthetase, the other for decatetraenoyl-CoA synthetase. Results of assays of marker enzymes are not given, but these were very similar to those of Table 3. Specific activity is expressed as nmol/min per mg of protein. % indicates percentage of total activity present in that fraction; n.d., component not detectable; —, component not assayed.

Fraction (see Fig. 1)	Palmitoyl-CoA synthetase (AMP)		Decatetraenoyl-CoA synthetase (AMP)		Decatetraenoyl-CoA synthetase (GDP)	
	Sp. activity	%	Sp. activity	%	Sp. activity	%
Sonicated mitochondria	5.8	100	0.128	100	0.059	100
S1	0.2	1	0.173	39	n.d.	—
S2	11.3	25	0.143	14	n.d.	—
S3	—	—	—	—	—	—
M1	22.5	29	0.216	17	n.d.	0
M2	3.3	31	0.071	24	0.039	30

Fig. 2. Spectra of outer-membrane and microsome fractions at liquid- N_2 temperature. Reduction of cytochromes was obtained by adding a few grains of $Na_2S_2O_4$ to the sample immediately before freezing. The spectra are reduced-minus-oxidized difference spectra, and the subcellular fractions were suspended in 0.25 M-sucrose. Spectrum (a): outer-membrane fraction, 1.8 mg of protein/ml. Spectrum (b): microsomal fraction, 3.0 mg of protein/ml. The microsomes were prepared by centrifugation at 120000g for 1 h of the first supernatant remaining after sedimentation of the mitochondria during the mitochondrial preparation.

acyl-CoA synthetases in the other fractions. Microsomes had a specific activity in the palmitoyl-CoA synthetase assay that was close to that found

for whole mitochondria (6.0 nmol/min per mg of protein). Thus the three- to four-fold higher specific activity found in the outer membrane cannot be accounted for by contamination of this fraction by microsomes.

The ATP-specific decatetraenoyl-CoA synthetase activity followed the distribution of malate dehydrogenase, and therefore behaved as an enzyme of the matrix. The occurrence of low amounts of this activity in the S2 and M1 fractions can be attributed to acceptance of decatetraenoate by palmitoyl-CoA synthetase. The GTP-specific decatetraenoyl-CoA synthetase also behaved as a matrix enzyme; the lack of activity in fraction S1 is attributable to the fact that this fraction contained 7.5 mM-potassium phosphate, a concentration that could severely inhibit the enzyme (Galzigna, Rossi, Sartorelli & Gibson, 1967).

Low-temperature spectroscopy of cytochrome b_5 . Fig. 2 shows dithionite-reduced-minus-oxidized difference spectra of the outer-membrane fraction (M1) and a microsomal fraction. The relative heights of the two peaks of the split α -band were much the same for each preparation, and it could not be concluded, as has been with guinea-pig liver mitochondria, that the outer membrane and microsomal cytochrome b_5 were spectroscopically distinct (Parsons, Williams, Thompson, Wilson & Chance, 1967). Further spectroscopic studies of the other submitochondrial fractions confirmed the findings made at room temperature by dual-wavelength spectroscopy (Tables 3 and 4).

DISCUSSION

Permeable-space measurements. The finding that the calculated penetration of mitochondria by CoA, acetyl-CoA, carnitine and acetyl-carnitine is indistinguishable from that for sucrose is not

unexpected, and confirms the general rule that the inner mitochondrial membrane is impermeable to low-molecular-weight solutes (Klingenberg & Pfaff, 1966). The recognition of inadequate recoveries of acetyl-CoA, and the use of conditions to avoid this loss, have resolved the discrepancy between the permeable spaces for CoA and acetyl-CoA that were described earlier (Garland & Yates, 1967).

A contrary conclusion about the penetration of rat liver mitochondria by carnitine has been drawn by Hilton & Dallam (1969). These authors measured the uptake of radioactive carnitine by rat liver mitochondria, where 'uptake' was equated with association of radioactivity with mitochondria on centrifugation. The chemical nature of the radioactivity associated with the mitochondrial pellet was not established. It was reported that the uptake of radioactive carnitine increased with both the duration and temperature of the mitochondrial incubation. The uptake was also enhanced by conditions favourable for oxidative phosphorylation. Although Hilton & Dallam (1969) concluded that an enzyme-mediated transport or binding had occurred, it seems that they did not exclude the possibility that the observed 'uptake' of radioactive carnitine was in fact the conversion of carnitine into long-chain fatty acyl-carnitine. More recently, Brosnan & Fritz (1970) have reported that tritiated carnitine penetrated the total water of rat liver mitochondria. Experimental details were not given.

Distribution of CoA-dependent enzymes. It was possible to conclude, on the basis of enzyme-latency phenomena and of permeable-space measurements, that rat liver mitochondria contained an externally situated long-chain fatty acyl-CoA synthetase (Yates *et al.* 1966). It was also concluded that most of the carnitine palmitoyltransferase activity was attached to the inner membrane and that this membrane separated the endogenous mitochondrial CoA and enzymes of β -oxidation from the intermembrane and extramitochondrial spaces (Yates & Garland, 1966). Experimentally independent support for these conclusions is provided by the more direct approach of physically subfractionating the mitochondria. Norum, Farstad & Bremer (1966) demonstrated the association of a long-chain fatty acyl-CoA synthetase with the outer mitochondrial membrane, and of carnitine palmitoyltransferase with the inner membrane. Our results confirm this (Tables 3 and 4). Our results on the location of both GTP- and ATP-specific medium-chain-length acyl-CoA synthetases within the matrix fraction are also in agreement with those of Aas & Bremer (1968) and van den Bergh, Modder, Souverijn & Pierrot (1969).

The enzymes of β -oxidation have been assayed as a multienzyme system by Beattie (1968), who

reported on the ability of mitochondrial subfractions to catalyse the reduction of formazan by palmitoyl-CoA. It was concluded that the enzymes of β -oxidation were located within the matrix. Since this assay would measure the activity of whatever step was rate-limiting in the reduction of formazan by palmitoyl-CoA, it could not exclude the possibility that individual enzymes of β -oxidation in other fractions were not being assayed. In this respect our own results on the individual activities are preferable (Table 3), but on the other hand suffer from the drawback of a very limited chain-length specificity. These drawbacks of rate assays do not occur in the absolute assay of ETF (equated with palmitoyl-CoA-reducible flavoprotein), and the absence of this essential component for β -oxidation from the outer membrane provides further evidence against the idea that the outer membrane is the site of β -oxidation (Allmann, Galzigna, McCaman & Green, 1966).

Relationships between the intramitochondrial locations of enzymes and the metabolic behaviour of whole mitochondria. Ideally there should be no inconsistencies between the conclusions drawn from structural studies and operational studies of the organization of mitochondrial enzymes (Yates & Garland, 1967a; Tubbs & Garland, 1968; Garland *et al.* 1969). Our views on the intramitochondrial organization of CoA-dependent enzymes have been reviewed elsewhere (Garland *et al.* 1969) and need not be repeated here. There is general agreement on this subject with other groups (Beattie, 1968; van den Bergh *et al.* 1969; Bremer & Norum, 1967; Norum *et al.* 1966; Aas & Bremer, 1968; Brdiczka, Pette, Brunner & Miller, 1968). There is also agreement between the conclusions drawn from studies of enzyme distribution in subfractionations and from operational studies such as those of permeability (Klingenberg & Pfaff, 1966; this paper, Tables 1 and 2), enzyme latency (Fritz & Yue, 1963; Yates & Garland, 1966, 1970; Tubbs & Garland, 1968), and direct spectrophotometric assay of acyl-CoA synthetases within intact mitochondria (Yates & Garland, 1967b, 1970). However, a number of points of uncertainty still remain, and these will now be mentioned.

Are there one or two carnitine palmitoyltransferase activities? Two separate carnitine palmitoyltransferase activities must be postulated to explain the carnitine-dependent oxidation of extramitochondrial palmitoyl-CoA (Fritz & Yue, 1963) and evidence has been presented in support of this idea (Yates & Garland, 1966, 1970). Nevertheless, and in agreement with Norum *et al.* (1966) and Bremer & Norum (1967) we were unable to detect a dual distribution of carnitine palmitoyltransferase in mitochondrial subfractions (Table 3). Our failure

to detect a solubilized type I carnitine palmitoyl-transferase (Table 3) may arise from a need for more drastic conditions to dislodge the enzyme from the outer aspect of the inner mitochondrial membrane. Alternatively, the type I activity may indeed be solubilized but subsequently inactivated during the lengthy fractionation procedure.

Are there two intramitochondrial long-chain ATP-specific fatty acyl-CoA synthetases? The relatively high specific activity of acyl-CoA synthetase in the outer-membrane fraction and the contamination of inner membrane by outer membrane make it difficult to state whether there is even one, let alone two, long-chain ATP-specific fatty acyl-CoA synthetase activities in the matrix and inner-membrane fractions. That there is at least one such activity can be deduced from the mitochondrial oxidation of palmitate. The proposal of two intramitochondrial activities (van den Bergh, 1967; van den Bergh *et al.* 1969) in an attempt to explain carnitine-dependent and independent modes of palmitate oxidation by rat liver mitochondria still lacks direct experimental support. Claims for two such enzymes based on assays with medium- and short-chain-length fatty acids are clearly irrelevant (van den Bergh *et al.* 1969): the problem of carnitine dependence does not apply to the oxidation of these acids. It does not seem that the organization of long-chain fatty acyl-CoA synthetase activity within the mitochondria can be resolved until much purer inner-membrane fractions are studied, and the possibility of novel routes of acyl-CoA synthesis is fully explored (Garland *et al.* 1969).

B.A.H. was supported by the Science Research Council, and D.W.Y. by the British Insulin Manufacturers. We are grateful to the Medical Research Council for research expenses and to the Royal Society and to the North Atlantic Treaty Organisation (grant no. 318) for grants for construction of a dual-wavelength and scanning spectrophotometer respectively.

REFERENCES

- Aas, M. & Bremer, J. (1968). *Biochim. biophys. Acta*, **164**, 157.
- Allman, D. W., Galzigna, L., McCaman, R. E. & Green, D. E. (1966). *Archs Biochem. Biophys.* **117**, 413.
- Bartley, W. & Davies, R. E. (1954). *Biochem. J.* **57**, 37.
- Beattie, D. S. (1968). *Biochem. biophys. Res. Commun.* **30**, 57.
- Beinert, H. (1967). In *The Enzymes*, vol. 7, p. 467. Ed. by Boyer, P., Myrback, K. & Lardy, H. New York and London: Academic Press.
- Brdiczka, D., Pette, D., Brunner, G. & Miller, F. (1968). *Eur. J. Biochem.* **5**, 294.
- Bremer, J. (1962). *J. biol. Chem.* **237**, 3628.
- Bremer, J. & Norum, K. (1967). *Eur. J. Biochem.* **1**, 427.
- Brierley, G. P. & O'Brien, R. L. (1965). *J. biol. Chem.* **240**, 4527.
- Brosnan, J. T. & Fritz, I. B. (1970). *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 267.
- Chance, B. (1957). In *Methods in Enzymology*, vol. 4, p. 273. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.
- Chance, B., Ernster, L., Garland, P. B., Lee, C. P., Light, P. Ann., Ohnishi, T., Ragan, C. I. & Wong, D. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 1598.
- Chase, J. F. A., Pearson, D. J. & Tubbs, P. K. (1965). *Biochim. biophys. Acta*, **96**, 162.
- French, C. S. & Lawrence, M. (1968). *Yb. Carnegie Instn Wash.* **66**, 175.
- Fritz, I. B. & Yue, K. T. N. (1963). *J. Lipid Res.* **4**, 279.
- Galzigna, L., Rossi, C. R., Sartorelli, L. & Gibson, D. M. (1967). *J. biol. Chem.* **242**, 2111.
- Garland, P. B., Chance, B., Ernster, L., Lee, C. P. & Wong, D. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 1696.
- Garland, P. B., Haddock, B. A. & Yates, D. W. (1969). In *FEBS Symp. 17: Mitochondria Structure and Function*, p. 111. Ed. by Ernster, L. & Drahota, Z. London & New York: Academic Press.
- Garland, P. B., Shepherd, D. & Yates, D. W. (1965). *Biochem. J.* **97**, 587.
- Garland, P. B. & Yates, D. W. (1967). In *Mitochondrial Structure and Compartmentation*, p. 385. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.
- Garland, P. B., Yates, D. W. & Haddock, B. A. (1970). *Biochem. J.* **119**, 553.
- Goodman, D. S. (1958). *J. Am. chem. Soc.* **80**, 3887.
- Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Hilton, M. A. & Dallam, R. D. (1969). *Archs Biochem. Biophys.* **130**, 347.
- Jones, M. S. & Jones, O. T. G. (1969). *Biochem. J.* **113**, 507.
- Klingenberg, M. (1958). *Archs Biochem. Biophys.* **75**, 376.
- Klingenberg, M. & Pfaff, E. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 180. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Kulka, R. G. (1956). *Biochem. J.* **63**, 542.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Lynen, F. & Wieland, O. (1955). In *Methods in Enzymology*, vol. 1, p. 566. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.
- Malamed, S. & Recknagel, P. O. (1959). *J. biol. Chem.* **234**, 3027.
- Norum, K., Farstad, M. & Bremer, J. (1966). *Biochem. biophys. Res. Commun.* **24**, 797.
- Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.
- Parsons, D. F., Williams, G. R., Thompson, W., Wilson, D. & Chance, B. (1967). In *Round Table Discussion on Mitochondrial Structure and Compartmentation*, p. 29. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.

- Pearson, D. J. & Tubbs, P. K. (1964a). *Biochem. J.* **91**, 2c.
- Pearson, D. J. & Tubbs, P. K. (1964b). *Nature, Lond.*, **202**, 91.
- Pfaff, E. (1965). Diplomarbeit: Philipps-Universität, MarburgLahn. p. 11.
- Pfaff, E. (1967). In *Mitochondrial Structure and Compartmentation*, 165. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.
- Schnaitman, C., Erwin, V. G. & Greenawalt, J. W. (1967). *J. Cell Biol.* **32**, 719.
- Seubert, W. (1960). *Biochem. Prep.* **7**, 80.
- Simon, E. J. & Shemin, D. (1953). *J. Am. chem. Soc.* **75**, 2520.
- Sottocasa, G. L., Kuylenskierna, B., Ernster, L. & Bergstrand, A. (1967). *J. Cell Biol.* **32**, 415.
- Srere, P. A., Brazil, H. & Gonen, L. (1963). *Acta chem. scand.* **17**, 5129.
- Stern, J. R. (1955). In *Methods in Enzymology*, vol. 1, p. 581. Ed. by Colowick, S. P. & Kaplan, N. O. New York and London: Academic Press.
- Tedeschi, H. & Harris, D. L. (1955). *Archs Biochem. Biophys.* **58**, 52.
- Tubbs, P. K. & Garland, P. B. (1968). In *Membranes and Lipid Metabolism*, p. 158. Ed. by Northcote, D. H. British Medical Bulletin, vol. 24. London: The British Council.
- van den Bergh, S. G. (1967). In *Mitochondrial Structure and Compartmentation*, p. 400. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.
- van den Bergh, S. G., Modder, C. P., Souverijn, J. H. M. & Pierrot, H. C. J. M. (1969). In *FEBS Symp.* 17: *Mitochondria Structure and Function*, p. 137. Ed. by Ernster, L. & Drahota, Z. London and New York: Academic Press.
- Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
- Wieland, T. & Rueff, L. (1953). *Angew. Chem.* **65**, 186.
- Yang, C. C. (1954). *Rev. scient. Instrum.* **25**, 807.
- Yates, D. W. & Garland, P. B. (1966). *Biochem. biophys. Res. Commun.* **23**, 460.
- Yates, D. W. & Garland, P. B. (1967a). *Abstr. 4th FEBS Meet.* p. 127. Oslo: Universitetsforlaget.
- Yates, D. W. & Garland, P. B. (1967b). *Biochem. J.* **102**, 40P.
- Yates, D. W. & Garland, P. B. (1970). *Biochem. J.* **119**, 547.
- Yates, D. W., Shepherd, D. & Garland, P. B. (1966). *Nature, Lond.*, **209**, 1213.