Some Aspects of Fatty Acid Oxidation in Isolated Fat-Cell Mitochondria from Rat

By RAYMOND D. HARPER* and E. DAVID SAGGERSON
Department of Biochemistry, University College London, Gower Street,
London WC1E 6BT, U.K.

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1. Mitochondria were prepared from fat-cells isolated from rat epididymal adipose tissues of fed and 48 h-starved rats to study some aspects of fatty acid oxidation in this tissue. The data were compared with values obtained in parallel experiments with liver mitochondria that were prepared and incubated under identical conditions. 2. In the presence of malonate, fluorocitrate and arsenite, malate, but not pyruvate + bicarbonate, facilitated palmitoyl-group oxidation in both types of mitochondria. In the presence of malate, fat-cell mitochondria exhibited slightly higher rates of palmitoylcarnitine oxidation than liver. Rates of octanoylcarnitine oxidation were similar in liver and fat-cell mitochondria. Uncoupling stimulated acylcarnitine oxidation in liver, but not in fat-cell mitochondria. Oxidation of palmitoyl- and octanoyl-carnitine was partially additive in fat-cell but not in liver mitochondria. Starvation for 48 h significantly decreased both palmitoylcarnitine oxidation and latent carnitine palmitoyltransferase activity in fat-cell mitochondria. Starvation increased latent carnitine palmitoyltransferase activity in liver mitochondria but did not alter palmitoylcarnitine oxidation. These results suggested that palmitoylcarnitine oxidation in fat-cell but not in liver mitochondria may be limited by carnitine palmitoyltransferase 2 activity. 3. Fat-cell mitochondria also differed from liver mitochondria in exhibiting considerably lower rates of carnitine-dependent oxidation of palmitoyl-CoA or palmitate, suggesting that carnitine palmitoyltransferase 1 activity may severely rate-limit palmitoyl-CoA oxidation in adipose tissue.

The importance of white adipose tissues in the overall fatty acid metabolism of the mammalian body is well established. Most investigations have concentrated on the main role of the tissue, the esterification and mobilization of fatty acids. Little attention has been paid to the oxidation of fatty acids, presumably because observed rates of fatty acid oxidation are low compared with rates of fatty acid esterification. This disparity in the two processes, however, in part reflects the extraordinarily high capacity of adipose tissue to synthesize glycerides and does not necessarily suggest that fatty acid oxidation is a process of no consequence. Study of fatty acid oxidation in white adipose tissue has been discouraged both in isolated tissue, where it is difficult to assess the size of the fatty acid oxidation substrate pool (Vaughan, 1961; Vaughan *et al.*, 1964), and at the mitochondrial level, owing to the difficulty of obtaining suitable quantities of mitochondria.

Early studies established the ability of rat white adipose tissues to oxidize [1-¹⁴C]stearate,

[1-¹⁴C]palmitate and [1-¹⁴C]octanoate to ¹⁴CO₂ (Shapiro *et al.*, 1957; Perry & Bowen, 1957; Milstein & Driscoll, 1958; Bally *et al.*, 1960). Milstein & Driscoll (1958) in fact suggested that, expressed on a tissue nitrogen or protein basis, adipose tissue was at least as active as liver in fatty acid oxidation. The relatively large amount of adipose tissue in the body could therefore contribute significantly to the total fatty acid oxidation by an animal. There is also evidence that fatty acid oxidation in adipose tissue responds to changes in the physiological state. The RQ (respiratory quotient: vol. of CO₂ formed/vol. of O₂ consumed) of adipose tissue *in vitro* is significantly decreased by prior starvation (Wertheimer & Shapiro, 1948) and the proportion of CO₂ that arises through oxidation of endogenous substrates (presumed to be fatty acids) decreases with administration of insulin (Flatt & Ball, 1964) and increases with prior starvation (Flatt, 1970). Flatt (1970) has in fact proposed that changes in rates of endogenous fatty acid oxidation may have profound effects on lipogenesis from carbohydrate precursors in rat adipose tissue.

It is generally agreed that the rate of fatty acid β -oxidation in several non-adipose tissues such

* Present address: Department of Science, Luton College of Technology, Park Square, Luton, Beds., U.K.

as liver and muscle is largely regulated by the availability of free fatty acids to the tissues (Fritz, 1961), reflecting the rate of mobilization from adipose tissue. In addition there have been various suggestions as to the nature of subsequent rate-limiting steps in β -oxidation within these tissues (Bode & Klingenberg, 1965; Bunyan & Greenbaum, 1965; Shepherd *et al.*, 1966; Pande, 1971). Adipose tissue would be expected always to contain a plentiful supply of β -oxidation substrate and therefore enzymic or compartmental control must be expected to modulate fatty acid oxidation to suit the requirements of the tissue.

In the present study mitochondria have been isolated from rat fat-cells in order to compare their ability to oxidize fatty acids and other substrates and to attempt to locate sites that may regulate fatty acid oxidation. Identically prepared and treated liver mitochondria were used for comparison.

Materials and Methods

Chemicals

Triethanolamine hydrochloride, Tris, sodium pyruvate, oxaloacetic acid, 2-oxoglutaric acid, NADH, ADP, CoA, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, yeast hexokinase and collagenase (from *Clostridium histolyticum*) were obtained from Boehringer Corp. (London) Ltd. (London W.5, U.K.), and L-malic acid, GSH, 5,5'-dithiobis-(2-nitrobenzoic acid), phenazine methosulphate, Triton X-100, EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], sodium DL-3-hydroxybutyrate, palmitoyl-CoA and palmitoyl-DL-carnitine chloride were obtained from Sigma (London) Chemical Co. (London S.W.6, U.K.). Octanoyl-DL-carnitine chloride and palmitoyl-L-carnitine chloride were obtained from P-L Biochemicals (Milwaukee, Wis., U.S.A.). L-Carnitine chloride was obtained from P-L Biochemicals or from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Sodium octanoate and sodium palmitate were obtained from Nu Chek Prep (Elysian, Minn., U.S.A.), and the palmitate was bound to fatty acid-poor bovine serum albumin (Evans & Mueller, 1963). The albumin (fraction V) from Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.) was defatted as described by Saggerson (1972). DL-Fluorocitrate (barium salt) from Calbiochem (Los Angeles, Calif., U.S.A.) was converted into the potassium salt by the addition of a slight excess of K_2SO_4 . Acetyl-CoA was prepared by the method of Simon & Shemin (1953) and standardized with phosphotransacetylase (Stadtman, 1957). Sodium D-3-hydroxybutyrate, resolved from the racemic mixture by the method of Lehninger & Greville (1953), was a gift from Professor A. L. Greenbaum. Radioactively labelled palmitic acid from The Radiochemical Centre (Amersham, Bucks., U.K.)

was supplied dissolved in organic solvents and was normally subjected to the following procedure, which resulted in appreciable decrease in background radioactivity counts. The labelled palmitate was extracted into 50mM-NaHCO₃ in ethanol-water (1:1, v/v). The pH was then adjusted to 3.0 by the addition of 2.5M-HCl and palmitic acid was extracted into hexane, which was evaporated under N₂ at 50–60°C. The palmitic acid was finally converted into the sodium salt and associated with fatty acid-poor albumin as described above. 2,5-Bis-(5-*t*-butylbenzoxazol-2-yl)thiophen was from CIBA (A.R.L.) Ltd. (Duxford, Cambridge, U.K.). All other chemicals were of the highest purity available from BDH Chemicals Ltd. (Poole, Dorset, U.K.) or Fisons Scientific Apparatus Ltd. (Loughborough, Leics., U.K.).

Animals

These were either male Wistar rats obtained from A. Tuck and Son Ltd. (Rayleigh, Essex, U.K.), male Sprague-Dawley rats from Bantin and Kingman (Hull, U.K.) or were Wistar or Sprague-Dawley rats bred in the animal colony at University College London. Fed animals were maintained on cube diet 41B (Bruce & Parkes, 1949) and weighed 140–200g. Starved animals were within this weight range at the time of withdrawal of food. Animals were supplied with water at all times. The differences in source and strain of animals did not appear to affect any of the results.

Methods

Preparation of fat-cells. The technique of Rodbell (1964) was used as described by Saggerson & Tomassi (1971).

Preparation of fat-cell mitochondria. These were prepared as described by Martin & Denton (1970), with minor modifications. Centrifugations were performed at 2°C on a Sorval Superspeed RC2-B centrifuge fitted with an SS-34 rotor ($r_{av.} = 10.8$ cm). Fat-cells prepared from the epididymal fat-pads of 12–20 rats were suspended in 12–18ml of ice-cold 0.25M-sucrose medium containing 20mM-Tris, 2mM-EGTA, 10mM-GSH and 20mg of fatty acid-poor albumin/ml adjusted to pH 7.4 with KOH. The fat-cells were then broken in a glass tube by treatment on a vortex mixer for 1min. Centrifugation of this broken-cell preparation was achieved by acceleration of the centrifuge to 3000g_{av.}, holding at that field for 1min and then decelerating the centrifuge with the brake on (integrated field-time = 4500g-min). The infranatant below the fat pellet was removed and re-centrifuged by accelerating to 20000g_{av.} holding at that field for 1min and then decelerating with the brake on (integrated field-time = 30200g-min). The mitochondrial pellet was resuspended in 10ml of 0.3M-sucrose containing additions as for the 0.25M-

sucrose medium and sedimented again at 20000g_{av}. for 1 min as described above. The mitochondrial pellet was then resuspended in sufficient 0.3M-sucrose medium (0.5–1.0 ml) to give a final concentration of 2–6 mg of mitochondrial protein/ml and was stored on ice.

Preparation of liver mitochondria. The sucrose media were identical with those used for fat-cell mitochondria. Liver (2–3 g) from a single rat was placed in ice-cold 0.25M-sucrose medium, rapidly cut into small pieces and the sucrose medium drained off. A fresh addition of 12 ml of 0.25M-sucrose medium was made and the mixture homogenized in a glass Potter–Elvehjem homogenizer with a motor-driven Teflon pestle of 0.2 mm radial clearance. Details of centrifugation were identical with those for fat-cell mitochondria. The final mitochondrial pellet was resuspended in sufficient 0.3M-sucrose medium (5–10 ml) to give a final concentration of 4–8 mg of mitochondrial protein/ml.

Measurement of mitochondrial respiration. O₂ uptake was measured polarographically by using an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) in conjunction with a multirange potentiometric pen recorder and back-off circuit which permitted expansion of the scale when low rates of O₂ uptake were measured. The oxygen electrode was standardized by determination of the recorder deflexion when approx. 100 nmol of spectrophotometrically standardized NADH was added to 1.9 ml of 'basal KCl medium' (see below) containing 100 µg of phenazine methosulphate/ml.

Normally 0.1 ml samples of mitochondrial preparations were incubated at 30°C in final volumes of 2.0 ml in the electrode chamber in 0.13M-KCl, 2 mM-EGTA, 2 mM-MgCl₂, 20 mM-Tris, 2 mM-KH₂PO₄, 0.25 mM-ADP and 20 mg of fatty acid-poor albumin/ml adjusted to pH 7.4 with HCl. This is referred to as 'basal KCl medium' in the legends to the Figures and Tables. In some experiments 2 mM-glucose and 10 µg of hexokinase/ml were also included. Further additions to this basal medium and the final concentrations of mitochondrial protein in the electrode chamber are indicated in the legends.

Measurement of conversion of [1-¹⁴C]palmitate into water-soluble products. Samples (0.2 ml) of mitochondrial preparations were incubated in duplicate for 15 min at 30°C in shaken 25 ml Erlenmeyer flasks (65 cycles/min). The final volume was 4.0 ml and consisted of 0.1 mM-sodium [1-¹⁴C]-palmitate (0.25 µCi/ml) in the basal KCl medium described above, except that fatty acid-poor albumin was present at 5.4 mg/ml. Further additions are indicated in the legend to Table 3. After 15 min 1.0 ml of 16.5% (v/v) HClO₄ was added. The flask contents were chilled in ice, centrifuged to remove precipitated protein and the supernatants extracted with 3 × 8 ml of water-saturated light petroleum (b.p.

40–60°C). Acid samples (3 ml) of the aqueous layers were counted for radioactivity with 2 ml of methanol and 10 ml of a 4 g/litre solution of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen in toluene–Triton X-100 (2:1, v/v). Suitable blanks were performed in quadruplicate with each experiment.

Determination of mitochondrial protein. Samples (25 and 50 µl) of mitochondria suspended in 0.3M-sucrose medium were washed free of albumin as described by Martin & Denton (1970). The protein was determined by the method of Lowry *et al.* (1951), with fatty acid-poor albumin as a standard.

Measurement of enzyme activities in mitochondria. Extracts were prepared by exposure of suspensions of mitochondria in 0.3M-sucrose medium to ultrasound for 3 × 20 s periods at 0°C. Citrate synthase (EC 4.1.3.7) and glutamate dehydrogenase (EC 1.4.1.2) were assayed by methods described by Saggerson & Tomassi (1971) and Martin & Denton (1970) respectively. Carnitine palmitoyltransferase (EC 2.3.1.21) was assayed in whole mitochondria and in mitochondrial sonicates as described by Harano *et al.* (1972). The overt (type 1) carnitine palmitoyltransferase activity was determined in intact mitochondria and the latent (type 2) activity determined by subtracting the type 1 activity from the activity observed in sonicates (types 1+2).

Determination of liver DNA. Pieces of liver (approx. 300 mg) were homogenized in 10 ml of ice-cold 5% (v/v) HClO₄. After brief centrifugation the pellet was washed once with 10 ml of 5% HClO₄ and resuspended in a further 10 ml of 5% HClO₄. The suspension was heated at 70°C for 20 min, cooled and centrifuged and 1 ml of the supernatant was assayed by the method of Burton (1956), with hydrolysed calf thymus DNA as a standard.

Expression of results

In every case where several determinations of a particular parameter are reported each was made on a separate preparation of mitochondria. Statistical significance of results was determined by Student's *t* test.

Results and Discussion

General considerations

Both liver and fat-cell mitochondria prepared by the methods described appeared to be intact and coupled as judged by several criteria. There was no increment in O₂ consumption on the addition of NADH or when palmitoyl-CoA was added unless carnitine was also present. In addition there was no detectable leakage of the matrix enzymes glutamate dehydrogenase and citrate synthase into the final 0.3M-sucrose suspension medium. The degree of respiratory stimulation obtained on ADP addition

was measured in all mitochondrial preparations. This was performed as a routine in 'basal KCl medium' lacking ADP, with further additions of 2.5 mM-succinate or 2.5 mM-2-oxoglutarate as respiratory substrate, and finally of ADP (250 μ M). With succinate as substrate, ADP addition increased the rate of respiration by an average of 5.2-fold in liver and 8.0-fold in fat-cell mitochondria. The corresponding values for liver and fat-cell mitochondria were 6.6 and 5.1 respectively when 2-oxoglutarate was the respiratory substrate. These values were not significantly altered by the dietary status of the animals.

To measure the O_2 uptake due to the oxidation of a particular substrate, mitochondria were first incubated in the 'basal KCl medium' with ADP present and the increment in respiratory rate was determined on the addition of the substrate. To minimize respiration owing to endogenous substrates and to prevent tricarboxylic acid-cycle metabolism of acetyl-CoA derived from β -oxidation, malonate (Quastel & Wooldridge, 1928) and fluorocitrate (Morrison & Peters, 1954) were included. Since it was intended to make some measurements of palmitoyl-group oxidation in the presence of pyruvate, arsenite (Searls & Sanadi, 1960) was also included. In preliminary experiments it was established that inclusion of these inhibitors considerably decreased O_2 uptake in the presence of 2.5 mM-malate and that the chosen concentration of arsenite completely abolished oxidation of 2.5 mM-pyruvate in the presence of 2.5 mM-malate.

The inclusion of albumin in the mitochondrial incubation medium was absolutely necessary, since in its absence convenient experimental concen-

trations of palmitoyl-CoA and palmitoylcarnitine inhibited respiration. Other preliminary experiments with both liver and fat-cell mitochondria established that under the conditions used there was no increment in O_2 uptake on the addition of these substrates unless ADP was previously added; the increases in respiration cannot therefore be attributed to uncoupling effects. When liver or fat-cell mitochondria were both prepared and incubated in the electrode chamber in the presence of 20 mg of albumin/ml, maximum rates of respiration were obtained with each of the chosen experimental concentrations of 100 μ M-DL- or 50 μ M-L-carnitine esters and of 50 μ M-palmitoyl-CoA.

Comparison of substrate oxidation by liver and fat-cell mitochondria from fed rats

In the experiment summarized in Table 1, the efficacy of malate or pyruvate + bicarbonate as sources of mitochondrial oxaloacetate was examined with respect to the palmitoylcarnitine-dependent respiration observed. Malate (2.5 mM) was very effective in promoting palmitoylcarnitine-dependent respiration in fat-cell mitochondria and also gave an appreciable promotion in liver mitochondria. At 0.5 mM, malate was less effective in this respect in fat-cell mitochondria and was ineffective in liver mitochondria. Therefore 2.5 mM-malate was used in all subsequent experiments. Pyruvate and bicarbonate did not promote palmitoylcarnitine-dependent respiration in fat-cell mitochondria under the conditions used and were in fact inhibitory in liver mitochondria. These results were unexpected, since citrate and malate are readily formed by fat-cell mitochondria

Table 1. *Effect of malate or pyruvate + bicarbonate on palmitoylcarnitine oxidation by liver and fat-cell mitochondria from fed rats*

Mitochondria were incubated at 30°C in the electrode chamber in 'basal KCl medium' containing in addition 0.75 mM-sodium arsenite, 2.5 mM-potassium malonate and 10 μ M-potassium fluorocitrate. In addition 2 mM-glucose and 10 μ g of hexokinase/ml were present in Expt. B but were omitted from Expt. A. Tris-malate or sodium pyruvate + $KHCO_3$ were added as indicated. A steady rate of respiration was obtained, 100 μ M-palmitoyl-DL-carnitine was added and the increment in respiratory rate determined. The results, which are means $\pm$ S.E.M., are expressed as nmol of O_2 /min per mg of mitochondrial protein.

	Substrate additions to basal medium	Liver mitochondria			Fat-cell mitochondria		
		Palmitoyl-carnitine-dependent O_2 uptake	No. of determinations	Mitochondrial protein (mg/ml)	Palmitoyl-carnitine-dependent O_2 uptake	No. of determinations	Mitochondrial protein (mg/ml)
Expt. A	None	29.2 $\pm$ 3.6	4	0.27 $\pm$ 0.06	12.7 $\pm$ 1.5	5	0.18 $\pm$ 0.03
	Malate (0.5 mM)	31.0 $\pm$ 2.6			30.5 $\pm$ 3.5		
	Pyruvate (0.5 mM)	18.3 $\pm$ 3.4			15.8 $\pm$ 2.3		
	+ $KHCO_3$ (12.5 mM)						
Expt. B	None	25.0 $\pm$ 4.5	3	0.26 $\pm$ 0.02	19.6 $\pm$ 2.4	3	0.19 $\pm$ 0.02
	Malate (2.5 mM)	40.5 $\pm$ 2.5			56.6 $\pm$ 2.4		

incubated under state-3 conditions with pyruvate and bicarbonate (Martin & Denton, 1971), suggesting oxaloacetate formation. It is possible that the use of malonate, fluorocitrate and arsenite led to a decrease in the mitochondrial matrix ATP/ADP ratio sufficient to inactivate pyruvate carboxylation (Stucki *et al.*, 1972). For the liver mitochondria, however, the use of these inhibitors should not have completely depleted ATP, since fluoride- and uncoupler-sensitive oxidation of octanoate and palmitate could be demonstrated. Alternatively it is possible that, as shown for liver and heart mitochondria, palmitoylcarnitine may both displace mitochondrial pyruvate and inhibit a postulated mitochondrial monocarboxylate carrier (Mowbray, 1975), thereby decreasing this intramitochondrial source of oxaloacetate. In this second case it may be envisaged that an elevation in adipose-tissue long-chain acylcarnitine concentration such as is encountered in certain physiological states (Böhmer, 1967) could interact with the postulated 'malate-pyruvate' cycle (Rognstad & Katz, 1966) by decreasing pyruvate carboxylation and increasing utilization of malate as a mitochondrial oxaloacetate source. This in turn would decrease the provision of NADPH for lipogenesis by NADP-malate dehydrogenase and lead to increased mitochondrial oxidation of cytosolic reducing equivalents. The consequences of such changes on adipose tissue lipogenesis have been discussed by Flatt (1970).

The experiments summarized in Table 1 were performed with freshly prepared mitochondria. When fat-cell mitochondria were aged by leaving the

preparation in ice for 3–4 h, thereby depleting endogenous substrates, palmitoylcarnitine-dependent respiration was then almost zero unless malate was added. The addition of carnitine did not promote palmitoylcarnitine respiration in aged fat-cell mitochondria in the absence of malate, suggesting that acetylcarnitine formation is unable to act as an 'acetate sink' under these conditions. This was an unexpected observation since, although the mitochondrial activity of carnitine acetyltransferase is low in rat fat-cells (Martin & Denton, 1970; Saggerson, 1974), Martin & Denton (1971) have observed appreciable acetylcarnitine formation on the addition of pyruvate and carnitine to fat-cell mitochondria.

The stoichiometry of palmitoylcarnitine oxidation by fat-cell mitochondria was established by determination of O₂ uptake in a low-albumin (2.0 mg/ml) variation of the basal KCl medium which contained the normal concentrations of glucose, hexokinase, fluorocitrate, arsenite and malonate. The basal rate of respiration was determined, 10–15 nmol of palmitoyl-L-carnitine added to stimulate respiration, and, after the basal respiratory rate had been re-established, the amount of palmitoylcarnitine-dependent O₂ consumption determined. The values observed for liver and fat-cell mitochondria in the presence of 2.5 mM-malate were 18.1 and 21.2 nequiv. of O/nmol of palmitoylcarnitine respectively. These values are in reasonable agreement with a value of 22 that would be expected if citrate were the product of oxidation (Shepherd *et al.*, 1965).

Table 2 shows that fat-cell mitochondria showed

Table 2. Oxidation of substrates by liver and fat-cell mitochondria from fed rats

Mitochondria were incubated at 30°C in the electrode chamber in 'basal KCl medium' containing in addition 0.75 mM-sodium arsenite, 2.5 mM-potassium malonate, 10 μ M-potassium fluorocitrate, 2 mM-glucose and 10 μ g of hexokinase/ml. Tris-malate was added where indicated. A steady rate of respiration was obtained, a final addition of a substrate was made as indicated, and the increment in respiratory rate determined. The results, which are means $\pm$ S.E.M., are expressed as nmol of O₂/min per mg of mitochondrial protein. n.d. indicates that no increment in respiratory rate was detectable.

Substrate additions to basal medium	Last addition	Liver mitochondria			Fat-cell mitochondria		
		O ₂ uptake	No. of determinations	Mitochondrial protein (mg/ml)	O ₂ uptake	No. of determinations	Mitochondrial protein (mg/ml)
None	—	2.2 $\pm$ 1.1	7	0.20 $\pm$ 0.03	1.0 $\pm$ 0.7	3	0.24 $\pm$ 0.03
	DL-3-Hydroxybutyrate (2.5 mM)	23.7 $\pm$ 2.5	7	0.20 $\pm$ 0.03	n.d.	3	0.24 $\pm$ 0.03
Malate (2.5 mM)	—	5.7 $\pm$ 0.6	7	0.20 $\pm$ 0.03	7.7 $\pm$ 2.1	3	0.24 $\pm$ 0.03
	Palmitoyl-L-carnitine (50 μ M) or palmitoyl-DL-carnitine (100 μ M)	38.3 $\pm$ 4.4	10	0.25 $\pm$ 0.03	54.1 $\pm$ 7.8	6	0.20 $\pm$ 0.03
	Palmitoyl-CoA (50 μ M) + L-carnitine (1 mM)	n.d.			n.d.		
		17.1 $\pm$ 1.9	10	0.25 $\pm$ 0.03	4.7 $\pm$ 0.6	7	0.27 $\pm$ 0.02
	Sodium octanoate (50 μ M)	13.5 $\pm$ 2.3	7	0.20 $\pm$ 0.03	n.d.	3	0.24 $\pm$ 0.03
	Sodium palmitate (50 μ M)	n.d.	5	0.21 $\pm$ 0.02	n.d.	3	0.24 $\pm$ 0.03
	Sodium pyruvate (2.5 mM)	24.3 $\pm$ 1.7	3	0.19 $\pm$ 0.02	98.0 $\pm$ 6.3	3	0.15 $\pm$ 0.01

negligible oxidation of 3-hydroxybutyrate (D- or DL-) or of octanoate, although these substrates were oxidized by liver mitochondria under the chosen conditions. Oxidation of palmitate was not detectable in either type of mitochondria with the oxygen electrode. Fat-cell mitochondria showed far higher rates of respiration with pyruvate than did liver mitochondria. This may not, however, represent a difference pertaining to the state *in vivo*, but possibly reflects the far higher Ca^{2+} requirement of the liver pyruvate dehydrogenase phosphate phosphatase compared with the fat-cell enzyme reported by Denton *et al.* (1972). The use of EGTA in the preparation and incubation of the mitochondria may well have depleted Ca^{2+} to a suboptimum concentration for the liver enzyme.

Fat-cell mitochondria oxidized palmitoylcarnitine slightly faster than those from liver. Rates of respiration with 50 μM -palmitoyl-L-carnitine were not detectably different from rates observed with 100 μM -palmitoyl-DL-carnitine. In fat-cell mitochondria 36.5% of the observed O_2 consumption was presumed to be due to the malate present. The percentage contribution due to malate may be slightly less in the liver mitochondria owing to ketogenesis. In accord with the observations of Shepherd *et al.* (1966) liver mitochondria showed a carnitine-dependent respiration with palmitoyl-CoA which was 45% of that observed with palmitoylcarnitine. In fat-cell mitochondria, respiration due to palmitoyl-CoA+carnitine was low, being only 8% of that observed with palmitoylcarnitine. Shepherd *et al.* (1966) concluded that the conversion of extramitochondrially generated palmitoyl-CoA into palmitoylcarnitine was the rate-limiting step in liver β -oxidation of palmitate or palmitoyl-CoA. The present data indicate that the same may apply to adipose tissue, although in the fat-cells the rate limitation would appear to be far more severe. This is of physiological

relevance since it may contribute to a difference in the partitioning of fatty acids between oxidation and esterification in the two tissues. The conclusions of Shepherd *et al.* (1966) have been challenged by Pande (1971), who observed identical oxidation rates with palmitoylcarnitine or palmitoyl-CoA+carnitine in mitochondria from several rat tissues. We consider that the applicability of the observations of Pande (1971) may be questioned, since the experiments were performed in the absence of albumin and therefore at free concentrations of palmitoyl-CoA similar to or greater than the critical micelle concentration (Zahler *et al.*, 1968). Excessive concentrations of palmitoyl-CoA appear to render mitochondria 'leaky' and the inner carnitine palmitoyltransferase 2 activity becomes overt under such conditions (Harano *et al.*, 1972). Also since palmitoyl-CoA was added after carnitine it is not possible to deduce from the data of Pande (1971) what proportion of the palmitoyl-CoA oxidation was carnitine-dependent.

The possibilities that the low oxidation of palmitoyl-CoA by fat-cell mitochondria could be due to inhibition of carnitine acyltransferase by palmitoyl-CoA (Bremer & Norum, 1967) or resulted from palmitoyl-CoA inhibition of adenine nucleotide translocation (Pande & Blanchaer, 1971; Harris *et al.*, 1972) were considered and discounted. Raising the concentrations of L-carnitine from 1 to 3 mM (competitive with respect to fatty acyl-CoA for carnitine acyltransferase) and of ADP from 250 to 750 μM had no significant effect on the carnitine-dependent oxidation of palmitoyl-CoA. The high concentration of albumin used should anyway preclude such inhibitory effects of palmitoyl-CoA.

Although respiration owing to addition of palmitate alone could not be detected with the oxygen electrode at the low mitochondrial concentrations used (Table 2), palmitate oxidation could be measured by conversion of [$1\text{-}^{14}\text{C}$]palmitate into

Table 3. Oxidation of [$1\text{-}^{14}\text{C}$]palmitate to water-soluble products by liver and fat-cell mitochondria from fed rats

Mitochondria were incubated with shaking at 30°C for 15 min in a variation of the 'basal KCl medium' which contained 5.4 mg of albumin/ml. In addition the medium contained 0.75 mM-sodium arsenite, 2.5 mM-potassium malonate, 10 μM -potassium fluorocitrate, 2 mM-glucose, 10 μg of hexokinase/ml, 2.5 mM-Tris-malate, 100 μM -sodium [$1\text{-}^{14}\text{C}$]palmitate and further additions where appropriate. The results are means $\pm$ S.E.M. of three determinations for liver mitochondria and of four determinations for fat-cell mitochondria, and are expressed as ng-atoms of palmitate C-1 converted into water-soluble products/15 min per mg of mitochondrial protein. The concentrations of mitochondrial protein/ml of incubation were 0.20 ± 0.01 and 0.12 ± 0.01 mg for liver and fat-cell mitochondria respectively. * $P < 0.05$, ** $P < 0.01$ versus the appropriate controls.

Additions to incubation medium	Liver mitochondria	Fat-cell mitochondria
None	2.47 ± 0.32	0.58 ± 0.08
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone (6 μM)	$1.38 \pm 0.11^*$	—
Sodium octanoate (100 μM)	$0.88 \pm 0.11^{**}$	0.56 ± 0.12
ATP (0.61 mM), L-carnitine (0.66 mM), CoA (0.13 mM)	15.28 ± 0.66	3.78 ± 0.49
ATP (0.61 mM), L-carnitine (0.66 mM), CoA (0.13 mM) + sodium octanoate (100 μM)	$11.78 \pm 0.48^*$	4.80 ± 0.60

water-soluble/light-petroleum-insoluble products (Table 3). Preliminary experiments established that conversion into $^{14}\text{CO}_2$ was negligible, incidentally indicating that the inhibitors malonate, arsenite and fluorocitrate were effective in suppressing tricarboxylic acid-cycle activity. A lower albumin concentration was used in these experiments, since the radioactivity recovered in water-soluble products appeared to be largely a function of the free palmitate concentration. When supplied together with ATP, carnitine and CoA, palmitate was oxidized by fat-cell mitochondria at 25% of the rate observed in liver mitochondria. This correlated with the observation that the rate of carnitine-dependent oxidation of palmitoyl-CoA in fat-cell mitochondria was 28% of that observed in liver mitochondria (Table 2). Oxidation of palmitate by liver, but not that by fat-cell mitochondria, was inhibited by octanoate. Groot *et al.* (1974) have shown that ATP-dependent octanoyl-CoA synthetase and palmitoyl-CoA synthetase activities are most likely to be due to the same enzyme in the rat liver mitochondrial matrix, octanoate and palmitate being competing substrates. Extrapolating this finding to fat-cell mitochondria and considering the absence of octanoate oxidation in these mitochondria, it appears reasonable to propose that fat-cell mitochondria lack matrix ATP-dependent palmitoyl-CoA synthetase activity. Considering also the observation of Lippel *et al.* (1971) that fat-cell mitochondria contain negligible GTP-dependent palmitoyl-CoA synthetase, fatty acid oxidation in adipose tissue would appear to be essentially an entirely carnitine-dependent process.

Measurements of carnitine palmitoyltransferase activity were made in fat-cell mitochondria and compared with those in liver mitochondria (Table 4). In liver mitochondria from fed rats the ratio of

latent to overt carnitine palmitoyltransferase activities correlated well with the relative rates of respiration observed with palmitoylcarnitine and palmitoyl-CoA+carnitine (Table 2). However, in fat-cell mitochondria from fed rats, although the ratio of latent to overt carnitine palmitoyltransferase activities was higher than in liver, the low carnitine-dependent oxidation of palmitoyl-CoA was not matched by a correspondingly low overt activity of carnitine palmitoyltransferase. If carnitine palmitoyltransferase activity limits the rate of palmitoyl-CoA oxidation in adipose tissue as is proposed for liver, it must be proposed that some of the measured overt activity in the mitochondrial preparations is ineffective in generating palmitoylcarnitine that is readily available for oxidation.

Pande (1971) has proposed that some segment of the respiratory chain coupled to oxidative phosphorylation may limit palmitoyl-group oxidation in liver. Results shown in Table 5 support this contention or an alternative interpretation that the capacity of the adenine nucleotide translocase may impose rate limitation. Liver carnitine palmitoyltransferase 2 activity or the activity of a component of the β -oxidation process would appear unlikely to limit the rate of respiration under state-3 conditions. This is shown by the following observations. Octanoylcarnitine, which may be metabolized via a medium-chain-length carnitine acyltransferase (Kopec & Fritz, 1971; Solberg, 1971) is oxidized at the same rate as palmitoylcarnitine. The oxidation of palmitoyl- and octanoylcarnitine is not additive but carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone stimulates the oxidation of both of these carnitine esters. Fat-cell mitochondria, on the other hand, showed increased respiration when octanoylcarnitine was added in addition to palmitoylcarnitine (Table 5), but no

Table 4. Activities of carnitine palmitoyltransferase, citrate synthase and glutamate dehydrogenase in liver and fat-cell mitochondria from fed and starved rats

Mitochondria were prepared from fed or 48 h-starved rats as described in the Materials and Methods section except that GSH was omitted from the preparation buffers. The results are expressed as nmol of substrate used or product produced/min per mg of mitochondrial protein at 25°C, and are means $\pm$ S.E.M. of the numbers of determinations indicated in parentheses. * $P < 0.01$, ** $P < 0.001$ for comparison of activities from starved animals with those from fed controls.

	Liver mitochondria		Fat-cell mitochondria	
	From fed rats (8)	From 48 h-starved rats (8)	From fed rats (5)	From 48 h-starved rats (5)
Carnitine palmitoyltransferase 1 activity	1.85 $\pm$ 0.12	4.61 $\pm$ 0.23**	1.54 $\pm$ 0.32	1.20 $\pm$ 0.32
Carnitine palmitoyltransferase 2 activity	4.94 $\pm$ 0.29	7.54 $\pm$ 0.38**	5.96 $\pm$ 0.24	3.74 $\pm$ 0.50*
Total carnitine palmitoyltransferase activity	6.79 $\pm$ 0.27	12.14 $\pm$ 0.50**	7.50 $\pm$ 0.45	4.94 $\pm$ 0.49*
Ratio $\frac{\text{carnitine palmitoyltransferase 2}}{\text{carnitine palmitoyltransferase 1}}$ activity	2.81 $\pm$ 0.35	1.66 $\pm$ 0.09	4.90 $\pm$ 1.32	4.42 $\pm$ 1.74
Glutamate dehydrogenase activity	1801 $\pm$ 117	1791 $\pm$ 254	263 $\pm$ 27	214 $\pm$ 32
Citrate synthase activity	45.8 $\pm$ 3.2	79.8 $\pm$ 9.7	1062 $\pm$ 53	963 $\pm$ 61

Table 5. Oxidation of palmitoylcarnitine and octanoylcarnitine by coupled and uncoupled liver or fat-cell mitochondria

Mitochondria from fed animals were incubated at 30°C in the electrode chamber in the 'basal KCl medium' containing in addition 0.75 mM-sodium arsenite, 2.5 mM-potassium malonate, 10 μ M-potassium fluorocitrate, 2 mM-glucose, 10 μ g of hexokinase/ml and 2.5 mM-Tris-malate. A steady rate of respiration was obtained, additions of palmitoyl-DL-carnitine (100 μ M) or octanoyl-DL-carnitine (100 μ M) were made where appropriate and the increment in respiratory rate was determined. Where appropriate carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (6 μ M) was then added and the total increment in respiratory rate determined. The results, which are means $\pm$ S.E.M., where appropriate, are expressed as nmol of O₂/min per mg of mitochondrial protein. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the appropriate control, which in each experiment is the top line of data. — indicates that no measurements were made.

Expt.	Additions	Liver mitochondria			Fat-cell mitochondria		
		O ₂ uptake rate	No. of determinations	Mitochondrial protein (mg/ml)	O ₂ uptake rate	No. of determinations	Mitochondrial protein (mg/ml)
A	Palmitoylcarnitine	40.0 $\pm$ 3.2	5	0.19 $\pm$ 0.04	51.8 $\pm$ 5.2	4	0.19 $\pm$ 0.02
	Octanoylcarnitine	37.7 $\pm$ 4.2			36.8 $\pm$ 3.0*		
B	Palmitoylcarnitine	34.2 $\pm$ 1.9	3	0.19 $\pm$ 0.05	51.6 $\pm$ 4.7	3	0.17 $\pm$ 0.01
	Palmitoylcarnitine + octanoylcarnitine	34.2 $\pm$ 1.9			73.5 $\pm$ 2.9*		
C	Palmitoylcarnitine	35.2 $\pm$ 3.9	4	0.28 $\pm$ 0.04	53.5 $\pm$ 3.8	3	0.17 $\pm$ 0.01
	Palmitoylcarnitine + carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone	51.7 $\pm$ 1.9**			55.6 $\pm$ 2.4		
D	Octanoylcarnitine	34.6	2	0.25	32.3	1	0.19
	Octanoylcarnitine + carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone	57.2			32.3		
E	Palmitoylcarnitine + octanoylcarnitine	34.3 $\pm$ 2.7	6	0.30 $\pm$ 0.03	—	—	—
	Palmitoylcarnitine + octanoylcarnitine + carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone	57.2 $\pm$ 2.9***			—		

enhancement of acylcarnitine oxidation on uncoupling, suggesting that carnitine palmitoyltransferase 2 activity may limit long-chain acylcarnitine oxidation in adipose tissue.

Effect of starvation on palmitoyl-group oxidation by liver and fat-cell mitochondria

Table 6 shows that the oxidation of palmitoylcarnitine by fat-cell mitochondria under state-3 conditions was significantly decreased by starvation, although the change of dietary status produced no change in carnitine-dependent palmitoyl-CoA oxidation in these mitochondria (results not shown). Starvation also resulted in a significant decrease in the latent carnitine palmitoyltransferase activity in fat-cell mitochondria (Table 4). This is further evidence suggesting that carnitine palmitoyltransferase 2 activity may limit the rate of palmitoylcarnitine oxidation in these mitochondria. The data of Tables 4 and 6 also further support the contention that palmitoylcarnitine oxidation in liver mitochondria is not rate-limited by carnitine palmitoyltransferase 2 activity. In starvation, latent carnitine palmitoyl-

transferase activity in liver was significantly increased, whereas palmitoylcarnitine oxidation was virtually unchanged. The increase in overt carnitine palmitoyltransferase activity observed in liver mitochondria after starvation (Table 4) could not be correlated with a parallel change in carnitine-dependent palmitoyl-CoA oxidation. This result again suggests that measurement of overt carnitine palmitoyltransferase activity does not necessarily yield a reliable estimate of the capability of a mitochondrial preparation to oxidize palmitoyl-CoA in a carnitine-dependent manner. An increase in liver carnitine palmitoyltransferase activity owing to starvation has been reported by Norum (1965). Aas & Daae (1971) measuring total (overt+latent) activity, however, found no significant effect of starvation on the adipose-tissue activity. The results of Table 4 suggest a fundamental difference between liver and adipose tissue, particularly when the starvation-induced changes in the latent activities are considered. The relevance of these changes to regulation of processes in the intact cell is, however, difficult to assess, since the working environment of the enzymes cannot as yet be defined.

Table 6. *Effect of starvation on palmitoylcarnitine oxidation by liver and fat-cell mitochondria*

Mitochondria from fed or 48 h-starved rats were incubated at 30°C in the electrode chamber in 'basal KCl medium' containing in addition 0.75 mM-sodium arsenite, 2.5 mM-potassium malonate, 10 μ M-potassium fluorocitrate and 2.5 mM-Tris-malate. A steady rate of respiration was obtained, the appropriate concentration of palmitoylcarnitine added and the increment in respiratory rate was determined. The results, which are means $\pm$ S.E.M. of three determinations, are expressed as nmol of O₂/min per mg of mitochondrial protein. * $P < 0.01$, ** $P < 0.001$ for comparison of mitochondria from starved animals with those from fed animals.

Concn. of palmitoyl- DL-carnitine	Liver mitochondria		Fat-cell mitochondria	
	From fed rats	From 48 h-starved rats	From fed rats	From 48 h-starved rats
5	16.0 $\pm$ 1.8	13.5 $\pm$ 2.1	14.9 $\pm$ 2.5	13.3 $\pm$ 0.4
20	35.4 $\pm$ 3.0	33.6 $\pm$ 4.8	40.2 $\pm$ 0.6	23.5 $\pm$ 0.4**
100	38.3 $\pm$ 4.2	32.1 $\pm$ 4.3	49.4 $\pm$ 2.7	34.5 $\pm$ 0.6*
Mitochondrial protein (mg/ml)	0.35 $\pm$ 0.05	0.37 $\pm$ 0.03	0.16 $\pm$ 0.05	0.17 $\pm$ 0.02

In the course of these studies with isolated mitochondria an additional difference between liver and adipose tissue became apparent during the course of routine determination of the degree of mitochondrial coupling. The state-3 oxidation rate per min for 2-oxoglutarate (2.5 mM) oxidation was 34.0 $\pm$ 3.1 nmol of O₂/mg of protein in liver mitochondria of fed rats and 39.8 $\pm$ 11.1 nmol of O₂/mg of protein in liver mitochondria of starved rats. On the other hand, 2-oxoglutarate oxidation was decreased from 55.2 $\pm$ 6.0 nmol of O₂/mg of protein in fat-cell mitochondria of fed rats to 29.5 $\pm$ 6.6 nmol of O₂/mg of protein in fat-cell mitochondria of starved rats (four determinations in each case). It is at present unclear whether this reflects a specific modification or adaptation of the activity of 2-oxoglutarate dehydrogenase or reflects a general decrease in tricarboxylic acid-cycle capacity in adipose tissue from starved animals.

General conclusions

Fat-cell mitochondria are at least as competent as liver mitochondria in acylcarnitine oxidation. The physiological significance of this relatively high capacity for acylcarnitine oxidation is unclear because the intact fat-cell mitochondria have a low capacity for carnitine-dependent palmitoyl-CoA oxidation. This limitation presumably diverts acyl groups towards esterification.

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