

The Intracellular Localization of Enzymes in White-Adipose-Tissue Fat-Cells and Permeability Properties of Fat-Cell Mitochondria

TRANSFER OF ACETYL UNITS AND REDUCING POWER BETWEEN MITOCHONDRIA AND CYTOPLASM

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1. A method is described for extracting separately mitochondrial and extra-mitochondrial enzymes from fat-cells prepared by collagenase digestion from rat epididymal fat-pads. The following distribution of enzymes has been observed (with the total activities of the enzymes as units/mg of fat-cell DNA at 25°C given in parenthesis). Exclusively mitochondrial enzymes: glutamate dehydrogenase (1.8), NAD-isocitrate dehydrogenase (0.5), citrate synthase (5.2), pyruvate carboxylase (3.0); exclusively extramitochondrial enzymes: glucose 6-phosphate dehydrogenase (5.8), 6-phosphogluconate dehydrogenase (5.2), NADP-malate dehydrogenase (11.0), ATP-citrate lyase (5.1); enzymes present in both mitochondrial and extramitochondrial compartments: NADP-isocitrate dehydrogenase (3.7), NAD-malate dehydrogenase (330), aconitate hydratase (1.1), carnitine acetyltransferase (0.4), acetyl-CoA synthetase (1.0), aspartate aminotransferase (1.7), alanine aminotransferase (6.1). The mean DNA content of eight preparations of fat-cells was 109 $\mu\text{g/g}$ dry weight of cells. 2. Mitochondria showing respiratory control ratios of 3-6 with pyruvate, about 3 with succinate and P/O ratios of approaching 3 and 2 respectively have been isolated from fat-cells. From studies of rates of oxygen uptake and of swelling in iso-osmotic solutions of ammonium salts, it is concluded that fat-cell mitochondria are permeable to the monocarboxylic acids, pyruvate and acetate; that in the presence of phosphate they are permeable to malate and succinate and to a lesser extent oxaloacetate but not fumarate; and that in the presence of both malate and phosphate they are permeable to citrate, isocitrate and 2-oxoglutarate. In addition, isolated fat-cell mitochondria have been found to oxidize acetyl L-carnitine and, slowly, L-glycerol 3-phosphate. 3. It is concluded that the major means of transport of acetyl units into the cytoplasm for fatty acid synthesis is as citrate. Extensive transport as glutamate, 2-oxoglutarate and isocitrate, as acetate and as acetyl L-carnitine appears to be ruled out by the low activities of mitochondrial aconitate hydratase, mitochondrial acetyl-CoA hydrolyase and carnitine acetyltransferase respectively. Pathways whereby oxaloacetate generated in the cytoplasm during fatty acid synthesis by ATP-citrate lyase may be returned to mitochondria for further citrate synthesis are discussed. 4. It is also concluded that fat-cells contain pathways that will allow the excess of reducing power formed in the cytoplasm when adipose tissue is incubated in glucose and insulin to be transferred to mitochondria as L-glycerol 3-phosphate or malate. When adipose tissue is incubated in pyruvate alone, reducing power for fatty acid, L-glycerol 3-phosphate and lactate formation may be transferred to the cytoplasm as citrate and malate.

The biosynthesis of fatty acids from glucose or pyruvate by adipose tissue involves the oxidative decarboxylation of pyruvate to acetyl-CoA within mitochondria and the synthesis of fatty acids from acetyl-CoA outside mitochondria. The biosynthesis

thus requires the production of extramitochondrial acetyl-CoA from mitochondrial acetyl-CoA. It is generally accepted that mitochondria are not directly permeable to acetyl-CoA. The indirect pathway most favoured in mammalian tissues

(including adipose tissue) involves in sequence: formation of citrate within mitochondria from acetyl-CoA and oxaloacetate by citrate synthase (EC 4.1.3.7), transfer of citrate across the mitochondrial membrane and the production of extra-mitochondrial acetyl-CoA and oxaloacetate from citrate by citrate lyase (EC 4.1.3.6) (Spencer & Lowenstein, 1962; Srere & Bhaduri, 1962). The oxaloacetate so formed may return to mitochondria directly or, more likely since most mammalian mitochondria seem to be relatively impermeable to oxaloacetate, indirectly either as pyruvate (Kornacker & Ball, 1965; Rognstad & Katz, 1966) or as malate or aspartate. Other pathways for the transfer of acetyl units that have been envisaged include transfer as acetate or acetyl L-carnitine (Srere, 1965; Lowenstein, 1968) or as 2-oxoglutarate or glutamate (D'Adamo & Haft, 1965). When adipose tissue synthesizes fatty acids from glucose in the presence of insulin it has been calculated that in the cytoplasm production of reduced coenzymes slightly exceeds utilization (Flatt & Ball, 1964; Katz & Rognstad, 1966). Presumably excess reducing power is transferred into mitochondria; pathways have been described in other tissues in which reducing power is transferred as L-glycerol 3-phosphate or malate (see Greville, 1969). In contrast, when adipose tissue synthesizes fatty acids from pyruvate alone (Jeanrenaud & Renold, 1960; Kneer & Ball, 1968) the reducing power required for cytoplasmic synthesis of fatty acid and L-glycerol 3-phosphate must be transferred from mitochondria. This could be accomplished by one or more of a number of pathways including those in which reducing power is transferred as isocitrate or malate (Kneer & Ball, 1968).

Two approaches have been used in the present study to establish which of these various pathways for the transfer of acetyl units and reducing power may be of importance in white-adipose-tissue fat-cells. First, since enzymes in a pathway must be present in the appropriate cell compartment and in sufficient activity to satisfy observed rates of fatty acid synthesis if a pathway is to be of importance, total enzyme activities extracted from fat-cells have been measured and their distribution between extramitochondrial and mitochondrial compartments investigated by fractional extraction. Secondly, since the pathways involve transfer of metabolites across the mitochondrial membrane, the permeability properties of fat-cell mitochondria have been investigated. This was done by observing rates of oxygen uptake of isolated fat-cell mitochondria and rates of mitochondrial swelling in iso-osmotic solutions of ammonium salts (Chappell & Haarhoff, 1967; Chappell & Robinson, 1968). Fat-cells isolated from rat epididymal fat-pads by digestion with collagenase

have been employed both for the preparation of enzyme extracts and for the preparation of mitochondria. The isolated cells can be broken without the degree of mechanical force required to homogenize pads and thus with less damage to fat-cell mitochondria.

EXPERIMENTAL

Materials

Rats. Epididymal fat-pads were obtained from male Wistar rats (130–150g) fed on a stock laboratory diet 41B (Short & Parkes, 1949). In all experiments the animals were allowed free access to food and water before the time of killing (approx. 10 a.m.).

Chemicals. Except where otherwise stated below, enzymes, glycolytic intermediates, GSH, adenine nucleotides, coenzymes and triethanolamine hydrochloride were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. and other chemicals were obtained from BDH (Chemicals) Ltd., Poole, Dorset, U.K.

Collagenase (bacterial grade B) was from Calbiochem Ltd., London W.1, U.K.; oligomycin, L-malic acid, sodium DL-isocitrate, 5,5'-dithiobis-(2-dinitrobenzoic acid), calf thymus DNA and tris were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and 2-mercaptoethanol, L-carnitine and DL-carnitine were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Acetyl-L-carnitine and acetyl-DL-carnitine were prepared by the method of Fraenkel & Freidman (1957). Sodium DL-isocitrate and sodium *threo-D*₃-isocitrate were from Fluka A.-G., Buchs, Switzerland; malonate, hydroxymalonate and rotenone were from Emmanuel Co. Ltd., Alperton, Middx., U.K., and monensin was from Eli Lilly Laboratories, Indianapolis, Ind., U.S.A. Acetyl-CoA was prepared as described by Simon & Shemin (1953) and L-glycerol 3-phosphate as described by Denton & Randle (1967). Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., and freed of fatty acids, citrate and other impurities as described in Denton & Halperin (1968).

Isolated fat-cells. These were prepared as described by Rodbell (1964) by vigorously shaking rat epididymal fat-pads in bicarbonate buffered medium (about 3ml/g of fat-pad) containing albumin (40mg/ml) and collagenase (3mg/ml). The bicarbonate buffered medium was that described by Krebs & Henseleit (1932) but with half the stated concentration of Ca²⁺. Medium containing albumin was dialysed before use for 18h at 4°C against 20 vol. of medium buffer.

Extraction and assay of enzymes from isolated fat-cells

Preparation of extracts containing total cell enzyme activities. Fat-cells (approximately 1g dry weight) prepared from the pads of three rats were suspended in 6ml of bicarbonate buffered medium (Krebs & Henseleit, 1932) containing albumin (40mg/ml) and divided into two equal samples with a siliconized glass pipette. One sample was used for the determination of the DNA content of fat-cells (see below). The other sample was used for the preparation of an extract containing the total enzyme activities in fat-cells. For most enzymes the cells were

resuspended in 1.5 ml of 100 mM-potassium phosphate buffer, pH 7.3, and extracted by homogenizing the suspension with a motor-driven all-glass tissue grinder (Kontes Glass Co., Vineland, N.J., U.S.A.) for 5 min at 0°C. The homogenate was freed of fat by centrifuging for 1 min at 500g. For the extraction of NAD-isocitrate dehydrogenase the phosphate buffer was supplemented with GSH (10 mM) and ADP (1 mM). For the extraction of acetyl-CoA synthetase fat-cells were suspended in 0.25 M-sucrose containing 20 mM-tris-HCl buffer, 2 mM EGTA* and 2% defatted bovine serum albumin, final pH 7.4, and extracted by sonication for eight 10 s periods over 5 min at 0°C.

Preparation of extracts containing extramitochondrial and mitochondrial enzyme activities. The extramitochondrial and mitochondrial enzyme activities in fat-cells have been separated by using a fractional extraction procedure based on that described by Pette (1966). In this procedure, the same sample of tissue is subjected to repeated extractions of increasing ionic strength and mechanical force. In this way, complete extraction of extramitochondrial enzymes should be achieved before releasing appreciable activities of mitochondrial enzymes. A flow chart of the procedure used for most enzymes is shown in Scheme 1. Details were as follows.

Fat-cells (approx. 2g dry weight) prepared from the pads of six rats were broken by treatment on a vortex mixer in 5 ml of sucrose medium (0.25 M-sucrose containing 2 mM-EGTA, 20 mM-tris-HCl, 2% albumin, adjusted to pH 7.4) at 0°C in a stoppered glass tube. As judged by inspection with an optical microscope about 10–30% of the original cells remained intact after 1 min of treatment. The resulting suspension was freed of fat and any unbroken cells by centrifugation at 400g for 1 min and was then used for the preparation of five successive extracts (I–V). Extract I was the supernatant after centrifuging at 38000g for 30 min. The pellet was shaken with 1 ml of sucrose medium in a flask shaker for 10 min at 4°C and extract II was the supernatant after centrifuging the resulting suspension again at 38000g for 30 min. Extract III was obtained by repeating the procedure for extract II and extract IV by a further repetition with 1 ml of 0.1 M-potassium phosphate buffer, pH 7.3, in place of sucrose medium. Finally, the pellet was homogenized with 1 ml of the phosphate buffer in a Kontes tissue grinder for 5 min to give extract V.

No isolated mitochondria appeared to be associated with the fat-cells and unbroken cells removed by centrifugation and discarded at the beginning of the procedure. The ratios of mitochondrial and extramitochondrial enzymes released upon extraction of the fat and fat-cells in phosphate buffer were the same as in whole fat-cells.

For NAD-isocitrate dehydrogenase, extracts were prepared as above but both sucrose medium and phosphate buffer contained GSH (10 mM) and ADP (1 mM).

For acetyl-CoA synthetase, extracts IV and V were replaced by a single extract obtained by suspending the pellet of extract III in sucrose medium and treating the suspension ultrasonically at 0°C for eight 10 s periods over 5 min.

Enzyme assays. Enzymes in the above extracts of fat-cells were assayed at 25°C by using, with one exception, a Hilger spectrophotometer with a Gilford Kinetic Recording Attachment. Assay methods were essentially similar to those of the references cited with minor modifications in some cases. Preliminary experiments indicated that rates of reaction were proportional to the volume of extract used and that the concentrations of substrates and pH values of the buffers used gave maximum activities. Rates were constant over the measured period (3–7 min). Simultaneous blanks were carried out in all cases by omission of a substrate.

The following enzymes were assayed by monitoring the production or disappearance of NADH or the production of NADPH at 340 nm.

(a) Glutamate dehydrogenase (EC 1.4.1.2) was assayed in 50 mM triethanolamine-HCl buffer, pH 8.0, containing EDTA (3 mM), 2-oxoglutarate (10 mM), NADH (0.1 mM), ADP (1.6 mM) and ammonium acetate (120 mM) (Delbrück, Zebe & Bücher, 1959; Frieden, 1959).

(b) NAD-malate dehydrogenase (EC 1.1.1.37) was assayed in 25 mM tris-HCl buffer, pH 7.4, containing NADH (0.1 mM) and oxaloacetate (0.25 mM) (Ochoa, 1955c).

(c) NAD-isocitrate dehydrogenase (EC 1.1.1.41) was assayed in 0.1 M-potassium phosphate buffer, pH 7.6, containing MgCl₂ (8 mM), NAD⁺ (2 mM), ADP (2 mM) and DL-isocitrate (1.6 mM) (Goebell & Klingenberg, 1964; Chen & Plaut, 1963).

(d) Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was assayed in 50 mM-tris-HCl buffer, pH 7.4, containing glucose 6-phosphate (1.5 mM), NADP⁺ (0.2 mM) and MgCl₂ (10 mM). There appeared to be no contribution to the rate of NADP reduction by 6-phosphogluconate dehydrogenase under these conditions since rates were linear and doubled on the addition of 1 unit of yeast 6-phosphogluconate dehydrogenase/ml.

(e) 6-phosphogluconate dehydrogenase (EC 1.1.1.44) was assayed in 50 mM-tris-HCl buffer containing 6-phosphogluconate (2 mM), NADP⁺ (0.5 mM) and MgCl₂ (10 mM) (Kornberg & Horecker, 1955).

(f) NADP-malate dehydrogenase (EC 1.1.1.40) was assayed in 50 mM-tris-HCl buffer containing MnCl₂ (3 mM), NADP⁺ (0.3 mM) and L-malate (1 mM) (Ochoa, 1955d).

(g) NADP-isocitrate dehydrogenase (EC 1.1.1.42) was assayed in 50 mM-tris-HCl buffer, pH 7.4, containing MgCl₂ (5 mM), NADP⁺ (0.5 mM) and DL-isocitrate (5 mM) (Ochoa, 1955b).

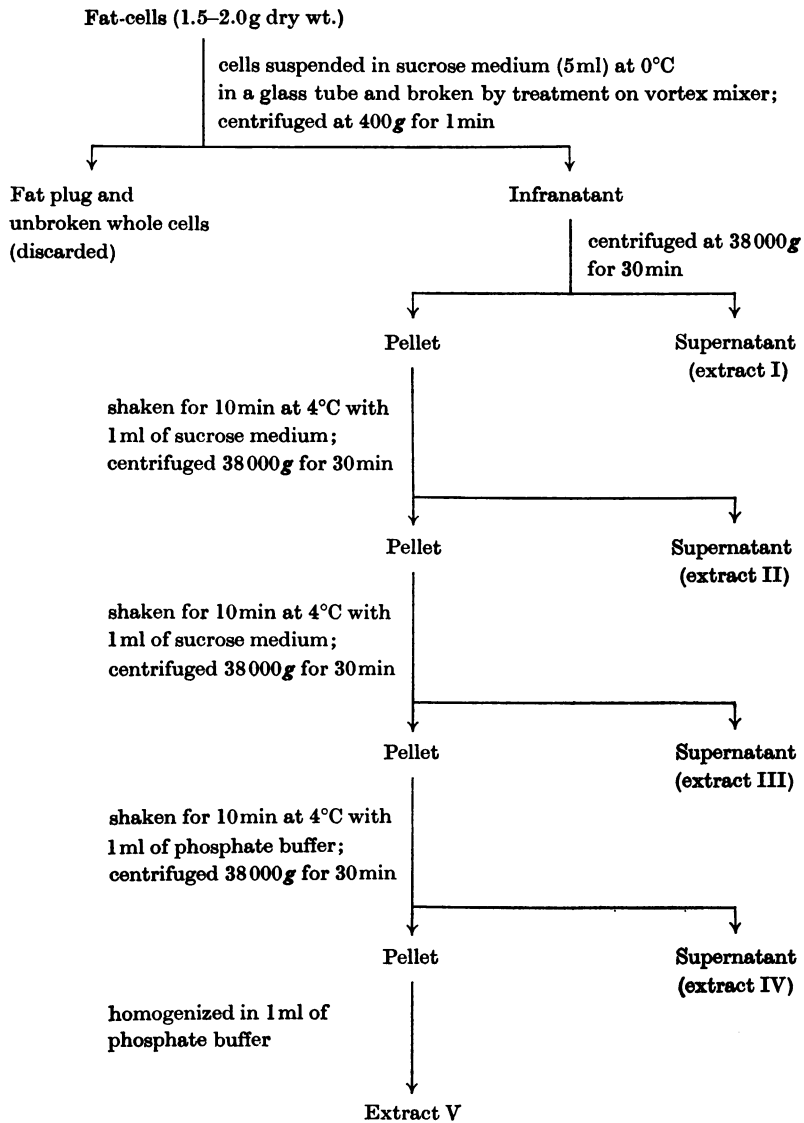
(h) Aconitate hydratase (EC 4.2.1.3) was assayed in 50 mM-tris-HCl buffer, pH 7.4, containing MgCl₂ (10 mM), potassium citrate (10 mM), NADP⁺ (0.5 mM) and NADP-isocitrate dehydrogenase (1 unit/ml) (Ochoa, 1948).

(i) Citrate synthase (EC 4.1.3.7) was assayed in 50 mM-triethanolamine buffer, pH 8.0, containing L-malate (7 mM), NAD⁺ (3 mM), acetyl-CoA (0.2 mM) and NAD-malate dehydrogenase (1 unit/ml) (Ochoa, 1955a).

(j) Acetyl-CoA synthetase (EC 6.2.1.1) was assayed in 0.1 M-tris-HCl buffer containing ATP (5 mM), NAD⁺ (3 mM), CoA (0.2 mM), MgCl₂ (4 mM), potassium acetate (10 mM), L-malate (7 mM), GSH (10 mM), NAD-malate dehydrogenase (1 unit/ml) and citrate synthase (1 unit/ml).

(k) Aspartate aminotransferase (EC 2.6.1.1) was assayed in 0.1 M-potassium phosphate buffer, pH 7.4,

* Abbreviations: EGTA, ethanedioxybis(ethylamine)-tetra-acetate; DTNB, 5,5'-dithiobis-2-dinitrobenzoic acid.



Scheme 1. Flow chart of procedure for the preparation of extracts containing extramitochondrial and mitochondrial enzyme activities. The sucrose medium was 0.25M-sucrose containing 2mM-EGTA, 20mM-tris-HCl, 2% albumin and adjusted to pH7.4; the phosphate buffer was 0.1M potassium phosphate, pH7.3. Extracts I to III were assumed to contain extramitochondrial enzyme activities since these were the only extracts containing detectable activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, ATP-citrate lyase and NADP-malate dehydrogenase. Extracts IV and V were assumed to contain mitochondrial enzyme activities since only these two extracts contained citrate synthase, glutamate dehydrogenase and NAD-isocitrate dehydrogenase (Table 2). For the extraction of NAD-isocitrate dehydrogenase (Table 2, Expt. 7) sucrose medium and phosphate buffer also contained GSH (10mM) and ADP (1mM). For the extraction of acetyl-CoA synthetase (Table 2, Expt. 8) extracts IV and V were replaced by a single extract (IV) obtained by suspending the pellet from extract III and treating the suspension ultrasonically at 0°C for eight 10s periods over 5 min.

containing aspartate (30mM), NADH (0.1mM), 2-oxoglutarate (6mM) and NAD-malate dehydrogenase (1 unit/ml) (Karmen, Wróblewski & La Due, 1955).

(l) Alanine aminotransferase (EC 2.6.1.2) was assayed in 0.1M-potassium phosphate buffer, pH 7.4, containing alanine (30mM), NADH (0.1mM), 2-oxoglutarate (6mM) and lactate dehydrogenase (Wróblewski & La Due, 1956).

(m) ATP-citrate lyase (EC 4.1.3.8) was assayed in 50mM-triethanolamine-HCl buffer, pH 7.7, containing MgATP (7mM), potassium citrate (10mM), NADH (0.1mM), mercaptoethanol (10mM), CoA (0.24mM) and NAD-malate dehydrogenase (1 unit/ml) (Srere, 1959).

The following enzymes were assayed by following the increase in extinction at 412nm due to the reaction of DTNB with free thiol. In the blanks for these assays, it was noticeable that appreciable acetyl-CoA deacylating activity was present in extracts containing total cell enzyme activities and those containing only extramitochondrial enzyme activities. In contrast, extracts containing only mitochondrial enzyme activities did not hydrolyse acetyl-CoA at any appreciable rate.

(a) Pyruvate carboxylase (EC 6.4.1.1) was assayed in 0.1M-tris-HCl buffer, pH 7.4, containing sodium pyruvate (10mM), ATP (2.5mM), acetyl-CoA (0.75mM), NaHCO₃ (50mM), MgCl₂ (5mM), DTNB (0.2mM) and citrate synthase (1 unit/ml). This assay method was found more convenient than the assay based on [¹⁴C]bicarbonate incorporation into oxaloacetate or citrate (Utter & Keech, 1960; Ballard & Hanson, 1967).

(b) Carnitine acetyltransferase (EC 2.3.1.7) was assayed in 0.1M-tris-HCl buffer, pH 7.8, containing acetyl-CoA (0.34mM), DTNB (0.2mM) and L-carnitine chloride (5mM) (Chase & Tubbs, 1965). This enzyme was also assayed in the opposite direction by the method of Fritz, Schutz & Srere (1963). In this case, the enzyme was assayed in 0.1M-triethanolamine-HCl buffer, pH 7.8, containing NAD⁺ (3mM), L-malate (7mM), CoA (0.5mM), GSH (10mM), acetyl L-carnitine (7mM), NAD-malate dehydrogenase (1 unit/ml) and citrate synthase (1 unit/ml). The production of NADH was followed fluorimetrically. No activity was detected if acetyl DL-carnitine was used as substrate instead of acetyl L-carnitine.

DNA assay. Fat cells suspended in 3ml of bicarbonate-buffered medium were acidified by the addition of 70% (w/v) HClO₄ to give a final concentration of 2% (w/v) HClO₄. Diethyl ether (10ml) was then added and the suspension was homogenized in a motor-driven Kontes tissue grinder and centrifuged at 2000g for 3–5 min. The ether was aspirated off and most of the remaining fat was removed by a further extraction with 10ml of ether. Traces of ether in the aqueous phase were removed under reduced pressure before the precipitate was compacted by centrifugation. The supernatant was discarded and the precipitate was analysed for DNA content by the method of Burton (1956).

Expression of total enzyme activities. In all experiments enzyme activities in total extracts of fat-cells have been expressed as unit/mg of fat-cell DNA where 1 unit of enzyme converts 1 μmol of substrate in 1 min at 25°C. DNA was chosen as a convenient measure of cell number; expression in terms of cell protein was precluded by the presence of albumin in the cell preparation. The DNA content of different fat-cell preparations varied between

90 and 130 μg DNA/g dry wt. of cells; the mean ± s.e.m. of determinations on eight separate preparations of fat-cells was 109 ± 9 μg DNA/g dry wt. of cells.

Mitochondrial studies

Preparation of isolated mitochondria from fat-cells. Isolated fat-cells (approx. 2g dry wt.) were suspended in 7ml of sucrose medium (0.5M-sucrose containing 20mM-tris-HCl, 2mM EGTA, 10mM GSH and 2% defatted bovine serum albumin, final pH 7.4) and broken in a glass tube by treatment on a vortex mixer for up to 1 min. The sucrose medium used contained twice the EGTA concentration normally employed for mitochondrial preparations to ensure that all the Ca²⁺ from the bicarbonate buffered medium (Krebs & Henseleit, 1932) was chelated. It was also found essential to have 2% defatted bovine serum albumin in the sucrose medium and the inclusion of 10mM-GSH was found to increase the time during which the mitochondria remained well coupled from less than 2 to about 4 h. The mitochondria were then isolated by a rapid centrifugation procedure similar to that used in the isolation of brain mitochondria (Chappell & Hansford, 1969). Unbroken cells, fat, nuclei and cell debris were removed by centrifuging at 3000g for 1 min and the mitochondria were sedimented by centrifuging at 20000g for 1 min. The mitochondria were then resuspended in sucrose medium by stirring with a small polythene tube packed with ice and sedimented again at 20000g for 1 min. All centrifugations were performed with an MSE High Speed 18 set to accelerate at its maximum rate until the desired speed was attained. This speed was held for the stated time and the centrifuge was then slowed down with maximum braking. The final mitochondrial pellet was taken up in sucrose medium to give a final concentration of 5–10mg of mitochondrial protein/ml and stored on ice. Mitochondrial protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) after suspending samples (50 μl) of mitochondria in 6ml of KCl medium (0.13M-KCl containing 2mM-MgCl₂, 1mM-EGTA and 5mM tris-HCl, adjusted to pH 7.4) and centrifuging at 38000g for 15 min to remove albumin. The yield of fat-cell mitochondria was 1–2mg of mitochondrial protein/g dry weight of fat-cells.

Measurement of oxygen uptake. Rates of respiration of mitochondria were recorded by the polarographic technique with a Clark-type oxygen electrode at 30°C. Mitochondria (0.1–0.2mg of protein) were incubated in 0.6–0.8ml of KCl medium (0.13M-KCl containing 2mM MgCl₂, 2mM-EGTA, 2% defatted bovine serum albumin and 5mM tris-HCl, adjusted to pH 7.4, and plus other additions as indicated in text, figures and tables). Calibration of the electrode was achieved with medium gassed with air and with medium treated with dithionite.

Mitochondrial swelling. Mitochondria were prepared as described above but the sucrose medium contained 0.25M sucrose rather than 0.5M sucrose. Swelling was observed in suspending medium (1.5ml) similar to those described by Chappell & Haarhoff (1967) by recording changes in extinction at 578nm of the mitochondrial suspension in a 4 cm light-path cell using an Eppendorf photometer with a recording attachment (Chappell & Crofts, 1966; Chappell & Haarhoff, 1967).

RESULTS

Activities and location of enzymes

Total activities of enzymes. The procedure used for measuring the total activities appeared to give complete extraction of the enzymes assayed. Thus homogenization for longer periods (up to 10 min) did not increase the yield of enzyme activity and the proportion of enzyme activity which could be extracted from the fat plug (less than 10%) could be attributed to extract sequestered into the plug. The total activities of enzymes in fat-cells are given in Table 1 in terms of the DNA content. As will be seen from the standard errors in the table the variation between different preparations of cells was small. The total activities of enzymes in fat-cells are also given in Table 1 as a percentage of the glutamate dehydrogenase activity. This enzyme activity has been used to check the recovery of other enzymes in the fractional extraction procedure.

NAD-malate dehydrogenase activity was very much greater than that of any other enzyme assayed. Certain enzymes which gave low activities or have been reported to be cold-labile have been subjected to the following investigations. L-Carnitine acetyltransferase was assayed by two separate methods that measured the enzymic activity in opposite directions (see the Experimental section); no significant difference was found and the value given in Table 1 is a mean value. Aconitate hydratase was also assayed after extraction with medium containing 10 mM-tricarballoylate (which

stabilizes heart muscle aconitase), and following treatment of the extract with mercaptosuccinate and ferrous ammonium sulphate (to replace any Fe^{2+} that may have been lost during extraction) as described in the purification of heart-muscle aconitase by Fansler & Lowenstein (1969). No significant increases in activity were detected with these methods. Pyruvate carboxylase from adipose tissue has been reported to be cold-labile (Ballard & Hanson, 1967) like the enzyme from kidney (Henning, Stumpf, Ohly & Seubert, 1966) and mammary gland (Gul & Dils, 1968). Under our conditions no increase in activity of this enzyme was detected when extracts prepared at 0°C were allowed to stand at 20°C for up to 2 h. In some instances this procedure led to substantial losses in activity whereas the activity appeared constant in extracts stored at 0°C for up to 2 h. The enzyme from chicken liver also shows no loss of activity when stored at 0°C in whole cell homogenates but becomes cold-labile on purification (Scrutton & Utter, 1965).

NAD-isocitrate dehydrogenase from heart and other mammalian tissues rapidly loses activity in the absence of ADP (Chen & Plaut, 1963; Goebell & Klingenberg, 1964) and this was also found to be true for the enzyme from isolated fat-cells. The activity of this enzyme in fat-cells was therefore assayed after extraction in phosphate buffer containing 1 mM-ADP and 10 mM-GSH. The liver enzyme is cold-labile in crude extracts (Plaut & Aogaichi, 1967) but no increase in activity of the

Table 1. *Enzyme activities in total extracts of isolated fat-cells*

Enzymes were extracted as described in the Experimental section. Results are expressed either as units/mg of fat-cell DNA where one unit converts 1 μ mol of substrate in 1 min at 25°C or as percentage of glutamate dehydrogenase activity assayed in the same extracts, and are given as means \pm S.E.M. for the number of observations on separate preparations of fat-cells given in parenthesis.

Enzyme	Activity expressed as	
	units/mg of DNA	percentage of glutamate dehydrogenase activity
NADP-malate dehydrogenase	11.0 \pm 1.0	710 \pm 83 (12)
NAD-malate dehydrogenase	329 \pm 41	20 900 \pm 3 300 (8)
Citrate synthase	5.16 \pm 0.24	316 \pm 9 (8)
ATP-citrate lyase	5.13 \pm 0.24	265 \pm 9 (8)
Aconitate hydratase	1.14 \pm 0.09	59 \pm 4 (8)
Glucose 6-phosphate dehydrogenase	5.80 \pm 0.73	297 \pm 33 (12)
6-Phosphogluconate dehydrogenase	5.19 \pm 0.55	267 \pm 23 (8)
NADP-isocitrate dehydrogenase	3.74 \pm 0.34	196 \pm 18 (12)
Aspartate aminotransferase	1.70 \pm 0.13	99 \pm 9 (12)
Alanine aminotransferase	6.13 \pm 0.50	373 \pm 42 (12)
Carnitine acetyltransferase	0.44 \pm 0.08	22 \pm 5 (4)
Pyruvate carboxylase	3.04 \pm 0.37	170 \pm 15 (8)
NAD-isocitrate dehydrogenase	0.47 \pm 0.05	32 \pm 3 (4)
Acetyl-CoA synthetase	0.98 \pm 0.08	41 \pm 2 (4)
Glutamate dehydrogenase	1.78 \pm 0.09	- (20)

fat-cell enzyme was found if extracts were stored at room temperature. Acetyl-CoA synthetase from fat-cells was found to be inactivated by phosphate as had previously been noted for the enzyme from heart muscle (Beinert *et al.* 1953). This enzyme was therefore assayed in extracts prepared by subjecting fat-cells suspended in a medium of tris-HCl-buffered sucrose to ultra-sonic treatment. The activity of glutamate dehydrogenase (taken for reference purposes) in extracts prepared in this way was in agreement with the activity found in extracts prepared with phosphate buffer.

Extramitochondrial and mitochondrial activities of enzymes. The extramitochondrial and mitochondrial enzyme activities in fat-cells have been separated by using a procedure based on that described by Pette (1966), which allows complete extraction of extramitochondrial enzymes (in extracts I and II) before releasing appreciable activities of mitochondrial enzymes (in extracts IV and V). As shown in Table 2 only extracts I and II contained appreciable activities of NADP-malate dehydrogenase, ATP-citrate lyase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and only extracts IV and V contained citrate synthase and glutamate dehydrogenase. It was found that this good separation could only be accomplished if EGTA and defatted bovine serum albumin were present in the sucrose medium used for the preparation of extracts I, II and III.

In the experiments reported in Table 2, the activities of glutamate dehydrogenase and up to three other enzymes were assayed in each experiment. The assay of glutamate dehydrogenase was used to check that there was no release of mitochondrial enzymes into extracts I, II and III. Also it was a means of checking the recovery of enzymes other than glutamate dehydrogenase through the fractional extraction procedure. In all cases, with the possible exception of glucose 6-phosphate dehydrogenase, the total of the activities found in the fractional extracts expressed in terms of glutamate dehydrogenase agreed well with the activities found in total cell extracts expressed in similar terms. The procedure appeared to be satisfactory for the study of cell location since there was no indication that serious loss, inactivation or activation of any enzyme was occurring during the fractional extraction procedure. Table 3 shows the extramitochondrial and mitochondrial activities of enzymes in fat-cells calculated from the total cell activities given in Table 1 and the distribution between extramitochondrial and mitochondrial compartments given in Table 2.

In addition to the enzymes already mentioned that appeared in the procedure to be either exclusively extramitochondrial or mitochondrial a number of enzymes appeared in appreciable

activities in both compartments. NAD-malate dehydrogenase was largely extramitochondrial with only about 9% of the total activity appearing to be mitochondrial. Both aconitate hydratase and NADP-isocitrate dehydrogenase were also largely extramitochondrial with about 15 and 24% of their respective total activities being mitochondrial. Both the aminotransferases studied were present in both cell compartments but the distribution of the enzymes between the compartments was markedly different. Aspartate aminotransferase was largely mitochondrial with about 30% of its activity being extramitochondrial whereas 93% of the activity of alanine aminotransferase was extramitochondrial. Most of the activity of carnitine acetyltransferase was associated with the mitochondria but about 17% was extramitochondrial.

Pyruvate carboxylase appeared to be exclusively mitochondrial (Table 2, Expt. 6). Extracts were prepared at about 0°C and stored at that temperature for not more than 30 min before assay. Pyruvate carboxylase activity could only be detected in extracts IV and V. Seubert, Henning, Schoner & L'Age (1967) have suggested that part of the pyruvate carboxylase in rat liver is bound to the outside of the mitochondria and have presented evidence indicating that this bound pyruvate carboxylase may be released on addition of 20 mM-triethanolamine, 0.5 mM-acetyl-CoA or 40 mM-sodium acetate to the sucrose medium. However, the supplementation of the sucrose medium with any of these additions in the fractional extraction of fat-cells did not result in any detectable extramitochondrial activity of pyruvate carboxylase.

In the fractional extraction procedure for NAD-isocitrate dehydrogenase (Table 2, Expt. 7) ADP and GSH were added to all extraction media to stabilize the enzyme. The enzyme was only detected in extract V and thus appears to be exclusively mitochondrial.

Extraction with phosphate buffer could not be used in the study of the location of acetyl-CoA synthetase because of the inactivating effects of phosphate on this enzyme. The extracts previously prepared in phosphate buffer (extracts IV and V) were therefore replaced by a single extract (IV) obtained by suspending the pellet from the previous extract (extract III) in sucrose medium and by ultrasonically treating the suspension at 0°C. Activities of acetyl-CoA synthetase, together with those of aspartate aminotransferase, alanine aminotransferase and glutamate dehydrogenase found in the fractions of this procedure are given in Table 2 (Expt. 8). The distribution of the aminotransferases and glutamate dehydrogenase is in close agreement with that obtained in Expt. 4. Acetyl-CoA synthetase appeared to be mainly present in the

Table 2. *Fractional extraction of enzymes from isolated fat-cells*

Procedures were as indicated in Scheme I and in the Experimental section. Results are given as means \pm s.e.m. of observations on four or five separate fractional extractions. —, Activity was below the limit of detection (i.e. less than 5% of the total extracted activity for NAD-isocitrate dehydrogenase and carnitine acetyltransferase, less than 2% for glutamate dehydrogenase, pyruvate carboxylase, aconitate hydratase and aspartate aminotransferase and less than 1% for other enzymes). In Expt. 8, extracts IV and V were replaced by a single extract prepared by ultrasonic treatment of the pellet of extract III in sucrose medium (see legend to Scheme I and the Experimental section). Enzyme activities in this extract are shown under extract IV.

Expt. no.	Enzyme	Activity as percentage of total extracted activity (I+II+III+IV+V)										Total extracted activity as percentage of that of glutamate dehydrogenase
		Extract no.					Total extramitochondrial (I+II+III)	Total mitochondrial (IV+V)	Total (IV+V)	Total extracted activity as percentage of that of glutamate dehydrogenase		
		I	II	III	IV	V						
1	NADP-malate dehydrogenase	95.0 \pm 0.9	5.0 \pm 0.9	—	—	—	100	—	—	—	906 \pm 49	
	NAD-malate dehydrogenase	85.5 \pm 2.1	4.3 \pm 1.2	1.4 \pm 0.3	1.4 \pm 0.3	7.4 \pm 1.2	91.2 \pm 1.5	8.8 \pm 1.5	8.8 \pm 1.5	17000 \pm 1320		
	Citrate synthase	—	—	—	35.5 \pm 1.8	64.5 \pm 1.9	—	100	100	272 \pm 18		
2	Glutamate dehydrogenase	—	—	—	23.7 \pm 3.0	76.3 \pm 3.0	—	—	—	100	—	
	ATP-citrate lyase	95.0 \pm 0.9	5.0 \pm 0.9	—	—	—	100	—	—	152 \pm 27		
	Aconitate hydratase	85.2 \pm 1.7	—	—	15.8 \pm 2.4	84.2 \pm 2.4	85.2 \pm 1.7	14.8 \pm 1.7	14.8 \pm 1.7	74 \pm 7		
3	Glutamate dehydrogenase	—	—	—	—	—	—	—	—	100	—	
	Glucose 6-phosphate dehydrogenase	89.1 \pm 3.0	10.9 \pm 3.0	—	—	—	100	—	—	549 \pm 62		
	6-Phosphogluconate dehydrogenase	96.3 \pm 0.3	3.7 \pm 0.3	—	—	—	100	—	—	244 \pm 13		
4	NADP-isocitrate dehydrogenase	72.5 \pm 1.7	3.9 \pm 0.8	—	—	—	76.4 \pm 1.0	23.6 \pm 1.0	23.6 \pm 1.0	221 \pm 10		
	Glutamate dehydrogenase	—	—	—	3.0 \pm 0.7	20.6 \pm 0.5	—	100	100	92.5 \pm 11.6		
	Aspartate aminotransferase	28.9 \pm 1.8	1.6 \pm 1.1	—	10.3 \pm 0.6	89.7 \pm 0.6	30.5 \pm 2.5	69.5 \pm 2.5	6.4 \pm 0.7	44.9 \pm 43		
5	Alanine aminotransferase	90.4 \pm 1.0	3.2 \pm 0.8	—	2.4 \pm 1.4	67.1 \pm 2.2	93.6 \pm 0.7	100	100	30.4 \pm 2.9		
	Glutamate dehydrogenase	—	—	—	—	6.4 \pm 0.7	—	—	—	155 \pm 15		
	Carnitine acetyltransferase	17.1 \pm 3.0	—	—	5.2 \pm 1.2	77.7 \pm 3.9	17.1 \pm 3.0	82.9 \pm 3.0	100	33 \pm 3.2		
6	Glutamate dehydrogenase	—	—	—	—	100	—	—	—	26 \pm 1.7		
	Pyruvate carboxylase	—	—	—	4.4 \pm 1.7	95.6 \pm 1.7	—	100	100	74.7 \pm 7.1		
	Glutamate dehydrogenase	—	—	—	4.5 \pm 0.2	95.5 \pm 0.2	—	100	100	223 \pm 22		
7	NAD-isocitrate dehydrogenase	—	—	—	—	100	—	—	—	—		
	Glutamate dehydrogenase	—	—	—	—	100	—	—	—	—		
	Acetyl-CoA synthetase	65.4 \pm 0.7	14.2 \pm 0.9	—	20.4 \pm 0.6	*	79.6 \pm 0.6	20.4 \pm 0.6	82.2 \pm 1.5	9.8 \pm 1.1		
8	Aspartate aminotransferase	15.6 \pm 1.7	2.2 \pm 0.1	—	82.2 \pm 1.5	*	17.8 \pm 1.5	90.2 \pm 0.1	—	—		
	Alanine aminotransferase	88.9 \pm 1.2	1.3 \pm 0.1	—	9.8 \pm 1.1	*	—	—	—	—		
	Glutamate dehydrogenase	—	—	—	—	100	—	—	—	—		

Table 3. *Extramitochondrial and mitochondrial enzyme activities in isolated fat-cells*

Activities have been calculated from the total enzyme activities in fat-cells given in Table 1, and the distribution of the enzymes between extramitochondrial and mitochondrial compartments given in Table 2.

Enzyme	Activity as units/mg of fat-cell DNA measured at 25°C	
	Extramitochondrial	Mitochondrial
Glucose 6-phosphate dehydrogenase	5.80	0
6-Phosphogluconate dehydrogenase	5.19	0
NADP-malate dehydrogenase	11.0	0
NADP-isocitrate dehydrogenase	2.86	0.88
NAD-malate dehydrogenase	300	29
Glutamate dehydrogenase	0	1.76
NAD-isocitrate dehydrogenase	0	0.47
Citrate synthase	0	5.16
Aconitate hydratase	0.97	0.17
ATP-citrate lyase	5.13	0
Carnitine acetyltransferase	0.08	0.36
Acetyl-CoA synthetase	0.78	0.20
Pyruvate carboxylase	0	3.04
Aspartate aminotransferase	0.41	1.29
Alanine aminotransferase	5.61	0.52

extramitochondrial compartment but about 20% of its total activity was intramitochondrial.

Studies on the permeability properties of isolated fat-cell mitochondria

Oxygen uptake of fat-cell mitochondria. For the measurement of oxygen uptakes, mitochondria were prepared with 0.5M-sucrose medium and were suspended in KCl medium containing EGTA, MgCl₂ and defatted bovine serum albumin. Under these conditions, fat-cell mitochondria showed P/O ratios of 2.6–3.1 and respiratory control ratios of 3–6 with NAD-linked substrates (pyruvate alone, pyruvate plus malate, citrate plus malate) and P/O ratios of 1.6–2.0 and respiratory control ratios of about 3 with succinate. The mitochondria were uncoupled by 2,4-dinitrophenol (0.2mM) and trifluoromethoxycarbonyl cyanide phenylhydrazine (0.01mM), and oligomycin (0.2µg/ml) inhibited coupled respiration.

Table 4 shows the rates of oxygen uptake of fat-cell mitochondria incubated at 30°C with various substrates in the absence or presence of malate, hydroxymalonate or malonate in medium containing 2mM-phosphate and 0.5mM-ADP. The rate on addition of 2.5mM-succinate was 192ng-atoms/min per mg of mitochondrial protein.

On addition of citrate, isocitrate, 2-oxoglutarate or malate to the medium, the rate of oxygen consumption was less than 10% of that with succinate. However, on addition of citrate, isocitrate or 2-oxoglutarate in the presence of 0.5mM-malate the rate of oxygen consumption was greatly

stimulated and was 40–60% of that obtained with succinate. This effect of malate on the oxidation of citrate and isocitrate was also observed with hydroxymalonate but not with malonate. The effect of malate on the oxidation of 2-oxoglutarate could be partially mimicked by malonate but not by hydroxymalonate. Neither malonate nor hydroxymalonate were oxidized by fat-cell mitochondria.

In the presence of pyruvate the rate of oxygen uptake was about one third of that observed with succinate and was increased on addition of malate to about half that with succinate. Acetyl DL-carnitine was not apparently oxidized in the presence or absence of malate but acetyl L-carnitine slightly stimulated oxygen uptake in the absence of malate and markedly stimulated in the presence of malate. The rate of oxygen consumption in the absence or presence of malate was not increased on addition of glutamate, acetate or fumarate or on addition of NADH or NADPH. In the presence of DL-glycerol 3-phosphate (5mM) or L-glycerol 3-phosphate (2.5mM) the rate of oxygen uptake was about 10% of the rate obtained with succinate.

The stimulating effects of malate on the oxidation of citrate, isocitrate and 2-oxoglutarate have also been described by Halperin & Robinson (see Halperin, Robinson, Martin & Denton, 1969).

Mitochondrial swelling. For these experiments, the mitochondria were prepared in 0.25M-sucrose and swelling was observed in iso-osmotic solutions of ammonium (or in some cases potassium) salts. Rotenone and antimycin A were added to prevent any oxidations. When fat-cell mitochondria were

Table 4. Rates of oxygen uptake of fat-cell mitochondria incubated with various substrates in the absence and presence of malate, hydroxymalonate and malonate

Fat-cell mitochondria (0.2 mg of protein) were incubated at 30°C in 0.6–0.8 ml of air-saturated medium (0.13 M-KCl with 2 mM-EGTA, 2 mM-MgCl₂, 20 mM-tris-HCl, 2% defatted bovine serum albumin, adjusted to pH 7.4) containing 2.0 mM-P_i and 0.5 mM-ADP. Rates of oxygen uptake were measured polarographically during one of four procedures: 1, in the presence of substrate and then after addition of malate (0.5 mM); 2, in the presence of malate (0.5 mM) and then after addition of substrate; 3 and 4, in the presence of either hydroxymalonate (0.5 mM) or malonate (0.5 mM) respectively and then after addition of substrate. Rates are the mean of two or three observations on separate preparations of mitochondria except the rate seen with succinate which is expressed as the mean ± s.e.m. of nine determinations.

Procedure ...	Rate of oxygen uptake ng-atoms/min per mg of protein							
	1		2		3		4	
Substrate	Substrate alone	Substrate plus malate	Malate alone	Malate plus substrate	Hydroxymalonate alone	Hydroxymalonate plus substrate	Malonate alone	Malonate plus substrate
Succinate (2.5 mM)	192±10	160						
Citrate (2.5 mM)	11	70	17	69	0	60	0	16
<i>Arco</i> -D ₃ -Isocitrate (2.5 mM)	21	102	14	94	0	98	0	14
2-Oxoglutarate (2.5 mM)	11	71	18	67	0	9	0	48
Pyruvate (2.5 mM)	66	89	10	100				
Acetyl-DL-carnitine (5 mM)	0	17	17	21				
Acetyl-L-carnitine (2.5 mM)	10	40	17	49				
Glutamate (2.5 mM)	0	17	17	17				
Acetate (2.5 mM)	0	15	20	20				
Fumarate (2.5 mM)	0							
DL-Glycerol 3-phosphate (5 mM)	22							
L-Glycerol 3-phosphate (2.5 mM)	14							
NADH (1 mM)	0							
NADPH (1 mM)	0							

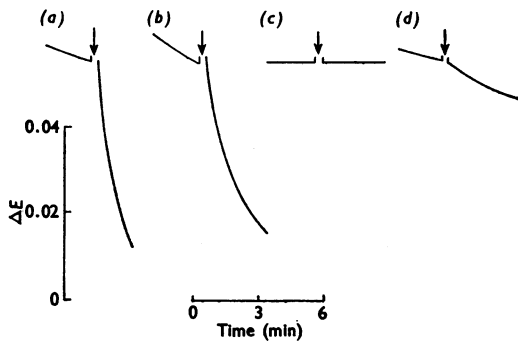


Fig. 1. Rates of swelling of mitochondria (0.2mg of protein) suspended in 1.5 ml of medium (pH 7.4) containing tris-HCl (10mM), EGTA (1mM), rotenone (0.5 μ g/ml), antimycin A (3 μ g/ml) and in (a) ammonium malate (90mM); (b) ammonium succinate (90mM); (c) ammonium fumarate (90mM); (d) ammonium oxaloacetate (90mM). A downward deflection in the spectrophotometric traces is taken as representing swelling. Addition of phosphate (3 μ mol) is indicated by an arrow.

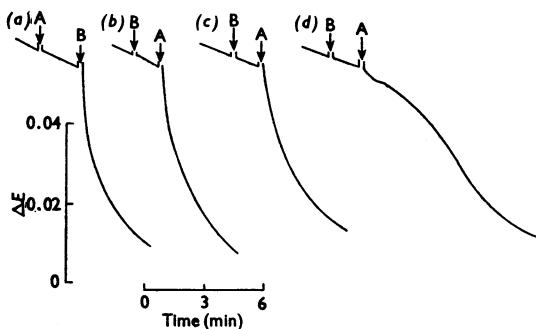


Fig. 2. Rates of swelling of mitochondria (0.2mg of protein) suspended in 1.5 ml of medium (pH 7.4) containing tris-HCl (10mM), EGTA (1mM), rotenone (0.5 μ g/ml), antimycin A (3 μ g/ml) and in (a) and (b) 70mM-ammonium citrate; (c) 70mM-ammonium DL-isocitrate; (d) 90mM-ammonium 2-oxoglutarate. Additions were made as follows: A, phosphate (3 μ mol); B, malate (3 μ mol).

placed in iso-osmotic solutions of ammonium acetate or ammonium pyruvate, the extinction of the mitochondrial suspension rapidly decreased indicating that the mitochondria were swelling because of the entry of acetate or pyruvate (Chappell & Haarhoff, 1967). The mitochondria did not swell in iso-osmotic solutions of potassium acetate or potassium pyruvate until the antibiotic monensin, which renders the mitochondrial membrane permeable to K^+ , (Henderson, McGivan & Chappell, 1969) was added.

In solutions of the ammonium salts of the dicarboxylic acids malate, succinate, oxaloacetate and fumarate there was little or no swelling. If 2mM-phosphate was added rapid and extensive swelling was observed with the mitochondria suspended in ammonium malate or ammonium succinate. Addition of 2mM-phosphate to mitochondria suspended in ammonium oxaloacetate caused a slower and less extensive swelling; addition to mitochondria in ammonium fumarate caused no swelling (Fig. 1).

In solutions of the ammonium salts of citrate, isocitrate and 2-oxoglutarate there was no swelling unless both malate (2mM) and phosphate (2mM) were present (Fig. 2). The rate of swelling was rapid and extensive in the presence of ammonium citrate and ammonium isocitrate. With ammonium 2-oxoglutarate the swelling, though extensive, was slower and its onset seemed to be subject to some lag period.

DISCUSSION

Activities and location of enzymes in fat-cells.

Estimates of the total activities in whole rat epididymal fat pads or isolated fat-cells of some of the enzymes studied in this paper have been reported previously. Comparisons between these estimates and those given in Table 1 can be made if a number of conversion factors are employed. These are given in the legend to Table 5. On this basis the agreement with published total activities is in general good. The activity of pyruvate carboxylase was close to the activities reported by Gorin, Tal-Or & Shafrir (1969) and Reshef, Hanson & Ballard (1969) but considerably greater than those given by Wise & Ball (1964) and Ballard & Hanson (1967). The activities of the four NAD-linked dehydrogenases and ATP-citrate lyase agreed or were slightly lower than those given by McLean, Brown & Greenbaum (1968) and the activity of NAD-malate dehydrogenase was very near to that reported by Wise & Ball (1964). However, the activities of the two aminotransferases were only 15–20% of those reported by Gorin *et al.* (1969) in extracts of whole pads. This might be a reflection of the different strains of rats used since repetition of these authors' procedure with whole pads from the Wistar strain used in this study yielded activities consistent with those extracted from the isolated fat-cells. The activities of the other enzymes studied (carnitine acetyltransferase, acetyl-CoA synthetase, glutamate dehydrogenase, aconitate hydratase and NAD-isocitrate dehydrogenase) do not appear to have been previously reported.

No extensive study of the location of enzymes in adipose tissue or fat-cells appears to have been published before. The extramitochondrial location

of NADP-malate dehydrogenase and the small proportion of the total NAD-malate dehydrogenase activity that is mitochondrial has been reported by Wise & Ball (1964). In our studies, no extramitochondrial activity of pyruvate carboxylase was detected even under conditions suggested to promote the release of pyruvate carboxylase from the outside of rat liver mitochondria (Seubert *et al.* 1967). In contrast, according to Ballard & Hanson (1967) and Reshef *et al.* (1969), about one-third of the total activity of pyruvate carboxylase in fat-cells is extramitochondrial. However, these authors extracted fat-cells in the absence of albumin and did not use any mitochondrial marker enzymes. In the absence of albumin, we found that an appreciable proportion of mitochondrial enzymes such as glutamate dehydrogenase and citrate synthase appears in the fractions containing extramitochondrial enzymes presumably because mitochondria are damaged by the free fatty acids present in adipose tissue extracts.

Permeability properties of isolated fat-cell mitochondria. Fat-cell mitochondria appear to have permeability properties very similar to those previously found in rat liver mitochondria (for review see Greville, 1969). On the basis of the observations on swelling in iso-osmotic solutions of ammonium salts, fat-cell mitochondria appear to be permeable to the monocarboxylate anions acetate and pyruvate; in the presence of phosphate, fat-cell mitochondria appear to be permeable to succinate and malate and to a lesser extent oxaloacetate but not fumarate; and, in the presence of both malate and phosphate, they appear to be permeable to citrate, isocitrate and 2-oxoglutarate. These observations can be correlated in most instances with the observations of rates of oxygen uptake (Table 4). Thus the oxygen uptake of fat-cell mitochondria incubated with ADP and phosphate is stimulated on addition of pyruvate, succinate or malate (but not fumarate) and in the presence of malate on addition of citrate, isocitrate or 2-oxoglutarate.

In liver mitochondria, it has been proposed that there is a dicarboxylate carrier catalysing counter-transport of malate/phosphate and succinate/phosphate; a tricarboxylate carrier catalysing counter-transport of citrate/malate and isocitrate/malate and a separate 2-oxoglutarate carrier catalysing exchange of 2-oxoglutarate for malate (Chappell & Haarhoff, 1967; Chappell, Henderson, McGivan & Robinson, 1968; Chappell & Robinson, 1968; Meijer & Tager, 1966; De Haan & Tager, 1968). Fat-cell mitochondria appear to contain all three carriers; the existence of two separate carriers for transport of tricarboxylate ions and 2-oxoglutarate is indicated by differences in the analogues of malate stimulating uptake of these anions. As

found with liver mitochondria, hydroxymalonnate but not malonnate stimulated citrate or isocitrate oxidation whereas malonnate but not hydroxymalonnate stimulated 2-oxoglutarate oxidation.

The lack of detectable stimulation of oxygen uptake by acetate even in the presence of malate (acting as a potential source of oxaloacetate) may simply be due to the very small activity of acetyl-CoA synthetase present in fat-cell mitochondria (Table 3). The reason for the lack of stimulation of oxygen uptake by glutamate is not clear since fat-cell mitochondria appear to contain appreciable activities of both glutamate dehydrogenase and aspartate aminotransferase (Table 3).

A small stimulation of oxygen uptake was also noted in the presence of L-glycerol 3-phosphate (or DL-glycerol 3-phosphate) presumably due to oxidation through the cytochrome-linked L-glycerol 3-phosphate dehydrogenase present in particulate fractions prepared from rat epididymal adipose tissue (Lee & Lardy, 1965).

The oxygen uptake of fat-cell mitochondria was also increased markedly by acetyl L-carnitine in the presence of malate. Malate presumably acts as a source of intramitochondrial oxaloacetate. No increase was seen if acetyl DL-carnitine was used; this correlates with the observation that no carnitine acetyltransferase activity could be detected if acetyl DL-carnitine rather than acetyl L-carnitine was used as substrate.

Transfer of acetyl units and reducing power between fat-cell mitochondria and cytoplasm

Estimates of rates of fatty acid synthesis and tricarboxylic acid cycle turnover and of cytoplasmic utilization and production of reduced coenzymes in epididymal fat pads from normal fed rats incubated with glucose and insulin have been made by Flatt & Ball (1964) and Katz & Rognstad (1966) and in pads incubated with pyruvate by Kneer & Ball (1968). Table 5 shows these rates expressed as $\mu\text{mol}/\text{min}$ per mg of fat-cell DNA at 25°C. Scheme 2 summarizes the pathways which would appear to be available for the transfer of acetyl units and reducing power on the basis of the observations made in the present study.

Only the entry of pyruvate, malate, 2-oxoglutarate, isocitrate and citrate into mitochondria has been shown in this study; however, it is assumed in the following discussion that the requirements for transport of these metabolites is the same in both directions. The tentative assumption is also made that fat-cell mitochondria are permeable to aspartate and glutamate, although no evidence has been obtained on this point.

Transfer of acetyl units. Because of the striking parallelism between the activity of ATP-citrate

Table 5. *Rates of fatty acid synthesis and tricarboxylic acid cycle turnover and of cytoplasmic utilization and production of reduced coenzymes in epididymal fat pads from normal fed rats incubated with glucose and insulin or pyruvate*

Values from the indicated source have been converted into the units used in the present paper ($\mu\text{mol}/\text{min}$ per mg of fat-cell DNA at 25°C) assuming the following conversion factors: fat-cells contain $109 \mu\text{g}$ of DNA/g of dry cells (see the Experimental section); 1 g of dry fat-cells is equivalent to 1.25 g of wet whole fat pad; 1 g of wet whole fat pad yields 15 mg of homogenate protein (C. I. Pogson & R. M. Denton, unpublished work; Wise & Ball, 1964; Ballard & Hanson, 1967; Reshef, Hanson & Ballard, 1969); enzyme activities double with each 10°C rise in temperature.

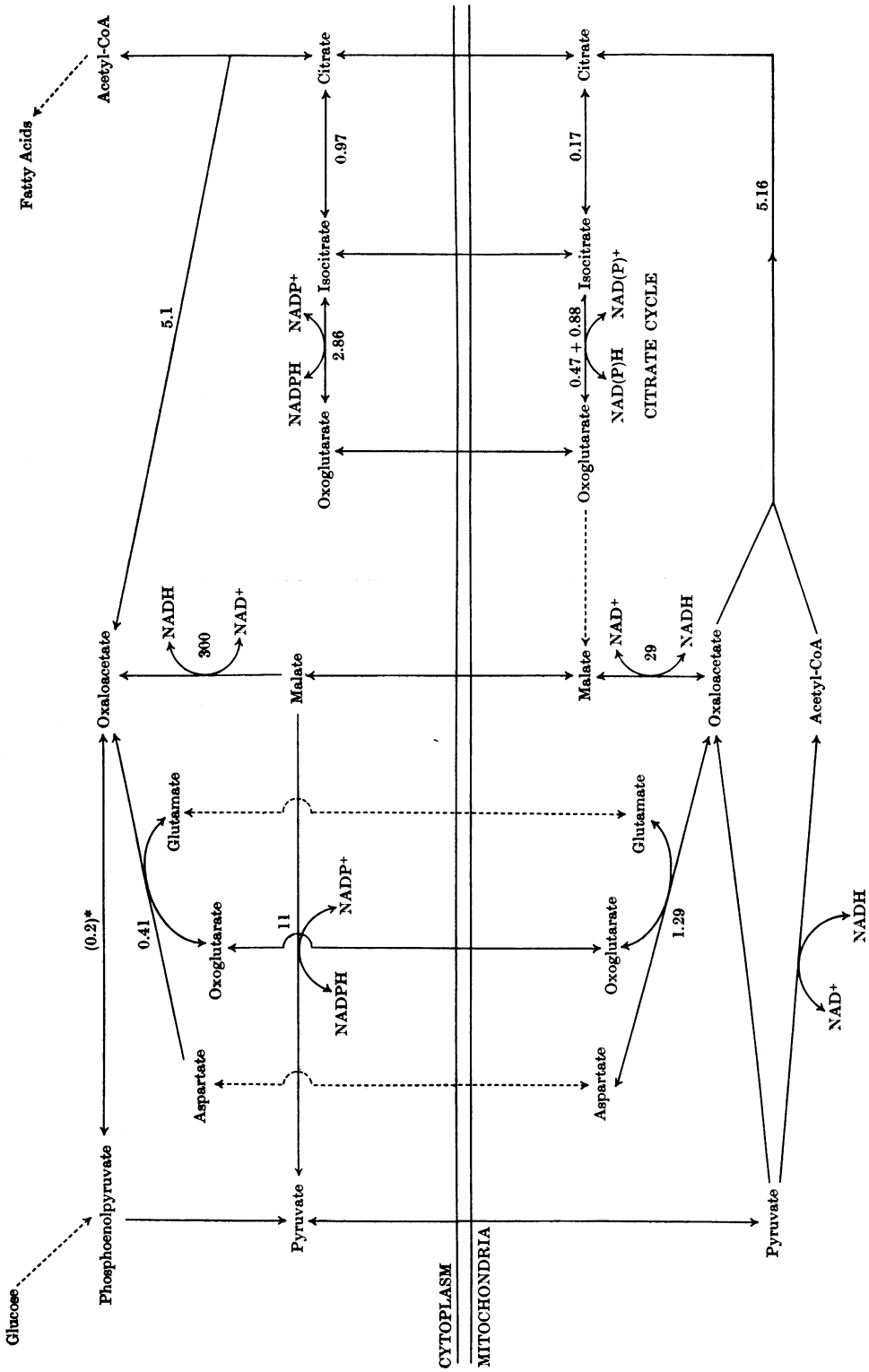
	Glucose and insulin		Pyruvate
	Flatt & Ball (1964)	Katz & Rognstad (1966)	Kneer & Ball (1968)
Rate ($\mu\text{mol}/\text{min}$ per mg of fat-cell DNA at 25°C)			
Fatty acid synthesis (as acetyl units)	1.94	0.96	0.81
Tricarboxylic acid cycle turnover (as acetyl units)	0.11	0.11	1.01
Utilization of reduced coenzymes in cytoplasm			
Fatty acid synthesis	3.24	1.59	1.35
Lactate and L-glycerol 3-phosphate dehydrogenases	0.37	0.55	0.28
Production of reduced coenzymes in cytoplasm			
Glyceraldehyde 3-phosphate dehydrogenase	2.25	1.53	-0.14
Pentose phosphate cycle	2.04	1.24	0
(Utilization-production) of reduced coenzymes in cytoplasm	-0.58	-0.37	+1.77

lyase and the rates of fatty acid synthesis in many mammalian tissues, it has been proposed that the major pathway for the production of extra-mitochondrial acetyl-CoA for fatty acid synthesis involves the sequence: acetyl CoA_{mit.} → citrate_{mit.} → citrate_{cyt.} → acetyl-CoA_{cyt.} (Kornacker & Lowenstein, 1965a,b; Kornacker & Ball, 1965). Further evidence in support of this pathway has been obtained from studying fatty acid synthesis from citrate in liver and other tissue homogenates (Spencer, Corman & Lowenstein, 1964; Leveille & Hanson, 1966) and from studies in whole tissue preparations on the incorporation of specifically labelled substrates into fatty acids (Bartley, Abraham & Chaikoff, 1965; Rognstad & Katz, 1968). The results of the present study are also in full agreement with the view that this is the major pathway whereby acetyl units for fatty acid synthesis are transferred to the cytoplasm in adipose tissue.

Rates of fatty acid synthesis observed in fat pads from normal fed rats incubated in the presence of glucose and insulin can be as high as 2.0 acetyl units/mg of fat-cell DNA per min at 25°C (Table 5; Denton & Halperin, 1968). Rates of fatty acid synthesis in pads incubated with pyruvate alone are about half this (Table 5; Schmidt & Katz, 1969). The maximum activities of both ATP-citrate lyase and citrate synthase are sufficient to account for these rates. Moreover, citrate synthase was found to be wholly mitochondrial and ATP-citrate lyase wholly extramitochondrial. In

addition, citrate was found to enter fat-cell mitochondria rapidly but only in the presence of malate (and phosphate). It seems likely that fat-cell mitochondria contain a system for the rapid counter-transport of citrate and malate similar to that present in liver mitochondria and the possibility arises that the rate of citrate transport may be affected by the availability of L-malate.

Appreciable transfer of acetyl units as isocitrate, 2-oxoglutarate, glutamate, acetate or acetyl L-carnitine seems unlikely because of the low activities of certain enzymes that would be necessary. Thus the activity of aconitate hydratase in fat-cell mitochondria appears to be equivalent to only about 10% of the rates of fatty acid synthesis from acetyl units in pads incubated with glucose and insulin (Table 5 and Scheme 2). Unless some additional precaution is necessary to avoid inactivation of mitochondrial aconitate hydratase during extraction, this would seem to rule out the possibility of the transfer of acetyl groups as isocitrate, 2-oxoglutarate or glutamate being important in fat cells. Transfer of acetyl units as acetate requires mitochondrial acetyl-CoA hydrolyase and acetyl-CoA synthetase in the cytoplasm. Fat-cells do contain some cytoplasmic acetyl-CoA synthetase activity (0.78 units/mg of fat-cell DNA at 25°C), but the amount of acetyl-CoA-hydrolysing activity found in mitochondrial fractions was barely detectable (less than 0.1 unit/mg of fat-cell DNA; see the Experimental section). Some acetyl units may be transported as acetyl carnitine since fat-cells have

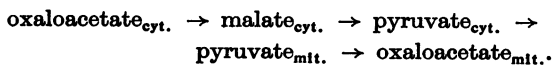


Scheme 2. Pathways possibly involved in the transport of acetyl units and reducing power between fat-cell mitochondria and cytoplasm. The figures denote maximum activity of the enzyme as $\mu\text{mol}/\text{min}$ per mg of fat-cell DNA at 25°C. *Activity reported by Reshef *et al.* (1969) and Gorin *et al.* (1969); other activities are taken from Table 3.

been found to contain a small amount of carnitine acetyltransferase activity (0.44 unit/mg of fat-cell DNA at 25°C). About 80% of this activity was associated with the mitochondrial fraction as found in liver (Norum & Bremer, 1967). If this enzyme is vectorially mounted in the mitochondrial membrane as suggested by Tubbs & Garland (1968) then up to 50% of the acetyl units required for fatty acid synthesis in pads incubated in glucose and insulin could be transferred through carnitine acetyltransferase.

In the following discussion it is assumed that, in adipose tissue incubated with glucose and insulin or pyruvate, all acetyl units for fatty acid synthesis are transported across the mitochondrial membrane as citrate. It follows from this that oxaloacetate will be formed in the cytoplasm by ATP-citrate lyase at the rate at which acetyl-CoA is incorporated into fatty acid. Regeneration of mitochondrial oxaloacetate for further citrate synthesis is unlikely to occur by direct entry of oxaloacetate into mitochondria because of the poor permeability of mitochondria to oxaloacetate; more likely pathways are discussed below.

Transfer of reducing power in adipose tissue incubated with glucose and insulin. When adipose tissue is incubated with glucose and insulin it appears that in the cytoplasm production of reduced coenzymes slightly exceeds utilization (Table 5). The excess reducing power may be transferred into fat cell-mitochondria as L-glycerol 3-phosphate since fat-cells contain considerable activity of the cytoplasmic enzyme NAD-L-glycerol 3-phosphate dehydrogenase and isolated fat-cell mitochondria oxidize L-glycerol 3-phosphate, though rather slowly. Alternatively, the excess may be transferred as malate. Malate appears to be transported in exchange for citrate and therefore one malate molecule would be expected to be transferred from the cytoplasm for each acetyl unit incorporated into fatty acid. However, the rate of fatty acid synthesis (in acetyl units) is about three times the excess reducing power needed to be transferred from the cytoplasm so it would appear that metabolism of malate through mitochondrial NAD-malate dehydrogenase may be restricted and that a large fraction of the malate entering mitochondria in exchange for citrate returns to the cytoplasm via the separate dicarboxylate carrier. Thus regeneration of mitochondrial oxaloacetate formed by ATP-citrate lyase in the cytoplasm may be in part by the pathway: oxaloacetate_{cyt.} → malate_{cyt.} → malate_{mit.} → oxaloacetate_{mit.}. The most important pathway would appear to be as suggested by Kornacker & Ball (1965), i.e.



This pathway transfers hydrogen from cytoplasmic NADH to cytoplasmic NADP⁺ which is necessary because the rate of production of NADPH by the pentose phosphate pathway is not sufficient to account for the observed rate of fatty acid synthesis.

Transfer of reducing power in adipose tissue incubated with pyruvate. When adipose tissue is incubated with pyruvate alone, all reducing power for fatty acid and L-glycerol 3-phosphate synthesis and lactate formation must be transferred from mitochondria to the cytoplasm. The total amount required can be calculated from the data of Kneer & Ball (1968) and is 1.8 μmol/min per mg of fat-cell DNA at 25°C (Table 5). Although most of the reducing power is required in the form of NADPH for fatty acid synthesis about 20% appears to be required as NADH for L-glycerol 3-phosphate synthesis and lactate formation. Pathways are thus necessary to form both NADH and NADPH from mitochondrial reducing power.

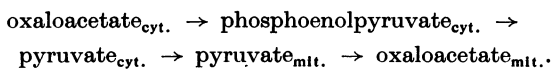
Pathways appear to be present in fat-cells which would allow this reducing power to be transferred out of fat-cell mitochondria as citrate and malate. Citrate may give rise to cytoplasmic NADPH through extramitochondrial NADP-isocitrate dehydrogenase and 2-oxoglutarate so formed may then enter mitochondria. In fat pads incubated with pyruvate the rate of oxidation of acetyl-CoA by the citrate cycle appears to be some six times greater than the maximal activity of aconitate hydratase found in fat-cell mitochondria (Table 5, Scheme 2). This and the high activity of extramitochondrial NADP-isocitrate dehydrogenase compared with that of mitochondrial isocitrate dehydrogenase may indicate that under these conditions an appreciable proportion of flow in the span citrate to 2-oxoglutarate in the tricarboxylic acid cycle occurs outside mitochondria, forming NADPH.

If malate moves from mitochondria it may give rise to either NADPH or NADH in the cytoplasm. Pyruvate formed extramitochondrially by NADP-malate dehydrogenase may enter mitochondria and regenerate mitochondrial oxaloacetate through pyruvate carboxylase. Pathways whereby oxaloacetate formed extramitochondrially by NADH-malate dehydrogenase may return to mitochondria are discussed below.

The requirement for cytoplasmic reducing power in fat pads incubated with pyruvate would be met if about 90% of the flow through the citrate cycle in the two spans, citrate to 2-oxoglutarate and malate to oxaloacetate, occurred outside mitochondria. This would require restriction of the metabolism of citrate and malate within fat-cell mitochondria by aconitate hydratase and/or isocitrate dehydrogenases and malate dehydrogenase respectively. One possible mechanism for the latter

could be end-product inhibition by oxaloacetate (Delbrück *et al.* 1959).

Two possible pathways can be envisaged whereby oxaloacetate formed extramitochondrially by ATP-citrate lyase and NAD-malate dehydrogenase may return to mitochondria in fat-cells incubated with pyruvate. It could be transferred as aspartate using cytoplasmic and mitochondrial aspartate aminotransferases. This pathway requires the transfer of aspartate, glutamate and 2-oxoglutarate but only transfer of 2-oxoglutarate has been demonstrated in this study. Moreover, the maximum activity of aspartate aminotransferase in the cytoplasm (0.41 $\mu\text{mol}/\text{min}$ per mg of fat-cell DNA at 25°C) appears to be less than the rate at which oxaloacetate is required to be transferred into mitochondria (1.09 $\mu\text{mol}/\text{min}$ per mg of fat-cell DNA at 25°C; calculated from the rates of fatty acid synthesis, cytoplasmic NADH requirement and L-glycerol 3-phosphate synthesis given in Table 5). Alternatively, or in addition, oxaloacetate could return to mitochondria by the pathway:



Reported activities of phosphoenolpyruvate carboxykinase in fat-cells are rather low (about 0.2 $\mu\text{mol}/\text{min}$ per mg of fat-cell DNA at 25°C; Reshef *et al.* 1969). However, in this report activity was measured in the direction phosphoenolpyruvate \rightarrow oxaloacetate; activity in the opposite direction is probably considerably greater. With the liver enzyme, Chang & Lane (1966) found that activity in this direction was about seven times greater.

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