Biochem. J. (1976) **154**, 209–223
Printed in Great Britain

Exchangeable and Total Calcium Pools in Mitochondria of Rat Epididymal Fat-Pads and Isolated Fat-Cells

ROLE IN THE REGULATION OF PYRUVATE DEHYDROGENASE ACTIVITY

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(*Received* 22 *July* 1975)

1. Isolated fat-cells and intact epididymal fat-pads were incubated in medium containing $^{45}Ca^{2+}$ and the incorporation of ^{45}Ca into mitochondrial and extramitochondrial fractions was studied. Redistribution of ^{45}Ca between these fractions was essentially prevented by the addition of EGTA [ethanedioxybis(ethylamine)tetra-acetate] and Ruthenium Red to the sucrose-based extraction medium. 2. Incorporation of ^{45}Ca into mitochondrial fractions of both fat-cells and fat-pads was found to be complete within 2–5 min, suggesting that mitochondria contain a pool of calcium in rapid isotopic exchange with extracellular Ca^{2+}. This pool was about 20 times larger in mitochondria within fat-cells than within fat-pads. In fat-cells, ^{45}Ca incorporation into the mitochondrial fraction accounted for about 34% of the total ^{45}Ca incorporation into cells after 20 min and about 50% of the total mitochondrial calcium content measured by atomic absorption; values in fat-pads were about 7 and 20% respectively. 3. Total ^{45}Ca incorporation into both fat-cells and fat-pads continued to increase after the first few minutes, when there was no further increase into mitochondrial fractions, suggesting the presence of an extramitochondrial pool of calcium into which ^{45}Ca is only slowly incorporated. 4. Parallel changes in ^{45}Ca incorporation into mitochondrial and extramitochondrial fractions were not observed in general. With fat-cells, Ruthenium Red, $NiCl_2$, $MnCl_2$, ionophore A23187, high P_i concentration and Li^+ replacement all markedly increased ^{45}Ca incorporation into the extramitochondrial fraction but caused modest or no increase into the mitochondrial fraction. Uncoupler (dinitrophenol) decreased and respiratory substrates increased incorporation into the mitochondrial fraction, with little change into the extramitochondrial fraction. 5. No changes in ^{45}Ca incorporation into the mitochondrial fraction of fat-pads were found with insulin under conditions where pyruvate dehydrogenase activity was increased. However, addition of adrenaline to fat-pads incubated with insulin lowered ^{45}Ca incorporation into the mitochondrial fraction and also decreased pyruvate dehydrogenase activity. Incubation of fat-pads in Ca^{2+}-free medium containing EGTA after preincubation with medium containing $^{45}Ca^{2+}$ caused an equally marked lowering of ^{45}Ca incorporation into the mitochondrial fraction without altering the pyruvate dehydrogenase activity. In view of these findings, it is concluded that a rise in the mitochondrial concentration of Ca^{2+} is unlikely to be important in the increase in pyruvate dehydrogenase activity seen with insulin, but the possibility remains that the effects of adrenaline may involve a decrease in mitochondrial Ca^{2+} concentration.

Pyruvate dehydrogenase exists in active (dephosphorylated) and inactive (phosphorylated) forms in mitochondria of mammalian tissues. Interconversion of these forms of pyruvate dehydrogenase is catalysed by a phosphatase (requiring Mg^{2+}) and by a kinase utilizing $ATPMg^{2-}$ (Linn *et al.*, 1969*a*,*b*). Mitochondrial Ca^{2+} concentration could be an important factor regulating the relative concentrations of active and inactive forms. Pyruvate dehydrogenase phosphate phosphatases from heart, kidney and adipose tissue are activated by Ca^{2+} at saturating concentrations of Mg^{2+} (Denton *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974), whereas heart muscle pyruvate dehydrogenase kinase is inhibited by Ca^{2+}

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at least at low Mg^{2+} concentration (Cooper *et al.*, 1974). Use of Ca^{2+}/EGTA* buffers has suggested that the kinase and the phosphatase are sensitive to concentrations of Ca^{2+} of 0.01–10μM. Experiments with the bivalent-metal ionophore A23187 described by Reed & Lardy (1972) have shown that the phosphatase reaction within fat-cell mitochondria is sensitive to changes in the intramitochondrial concentrations of Mg^{2+} and of Ca^{2+} (Severson *et al.*, 1974).

Exposure of adipose tissue to insulin leads within 5–10min to an increase in the proportion of pyruvate dehydrogenase in its active form (Jungas, 1971; Coore *et al.*, 1971; Weiss *et al.*, 1971). This action of insulin on adipose tissue is inhibited by Ruthenium Red, by $NiCl_2$ and by $MnCl_2$, which are inhibitors of cellular and/or mitochondrial uptake of Ca^{2+} (Randle & Denton, 1973; Severson *et al.*, 1974). Further indirect evidence that Ca^{2+} may be involved in the regulation of pyruvate dehydrogenase was provided by the observation that exposure of fat-pads to ouabain led to activation of pyruvate dehydrogenase (Martin *et al.*, 1972; Clausen *et al.*, 1974); it has been suggested that ouabain might lead to increased intracellular Ca^{2+} concentrations (Baker, 1970). These findings, which appeared to indicate that increased cellular and/or mitochondrial uptake of Ca^{2+} might be involved in the action of insulin, have led us to investigate effects of insulin and other agents on concentrations of calcium in mitochondrial and extramitochondrial fractions of fat-cells. Martin *et al.* (1973) have reported that insulin may increase the rate of ^{45}Ca release from pre-loaded fat-cells.

In the present studies, fat-cells isolated from epididymal adipose tissue or intact epididymal fat-pads were incubated with $^{45}Ca^{2+}$ and the incorporation of ^{45}Ca into mitochondrial fractions was studied. Methods were devised for the rapid preparation of mitochondrial fractions under conditions where little or no redistribution of ^{45}Ca occurred during the procedure. Incorporation of extracellular ^{45}Ca into mitochondrial fractions was found to be essentially complete within a few minutes and much greater into those isolated from fat-cells than from fat-pads. No convincing effect of insulin on ^{45}Ca incorporation was observed, but adrenaline in the presence of insulin diminished the incorporation of ^{45}Ca into the mitochondrial fraction of fat-pads.

Experimental

Materials

Rats. Epididymal fat-pads were obtained from male albino Wistar rats (body wt. 150–200g) with free access to water and stock laboratory diet (modified 41B; Oxoid, London S.E.1, U.K.). The animals were killed by decapitation and in any one experiment were closely matched for age and weight.

Chemicals. These were as given in Severson *et al.* (1974), with the following changes. Collagenase (type II, *Clostridium histolyticum*) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Adrenaline and dinonyl phthalate were from BDH Chemicals, Poole, Dorset, U.K. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ficoll was from Pharmacia, Uppsala, Sweden.

Isolated fat-cells. These were prepared by collagenase digestion of epididymal fat-pads as described by Severson *et al.* (1974).

Media. Fat-pads and fat-cells were incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 118mM-NaCl, 4.7mM-KCl, 1.9mM-KH_2PO_4, 1.9mM-$MgSO_4$, 24.9mM-$NaHCO_3$ and 1.25mM-$CaCl_2$ (i.e. one-half the recommended concentration) and gassed with O_2+CO_2 (95:5). Defatted bovine serum albumin (1%, w/v) was included in all incubation media for fat-cells. Media containing albumin were dialysed overnight before use against 20vol. of appropriate media without albumin.

Methods

Determination of ^{45}Ca incorporation into mitochondrial and supernatant fractions of isolated fat-cells. Isolated fat-cells (200–300mg dry wt.) were pre-incubated for 30min at 37°C in 10ml of bicarbonate-buffered medium containing glucose (0.2mg/ml) in a polythene bottle. A syringe was used to remove 9ml of the medium from below the cells, and an equal volume of fresh medium containing [^{3}H]inulin (2μCi/ml; 0.1mg/ml), and $^{45}CaCl_2$ (0.2μCi/ml; 1.25mM) was added; the cells were incubated with gentle shaking for a further period and with additions as indicated in the text and Tables. Incubations were terminated by rapidly transferring the contents of the incubation bottle to a polythene or polycarbonate centrifuge tube containing dinonyl phthalate (5ml) and immediately centrifuging for 15s at room temperature in a MSE Super Medium centrifuge set at maximum acceleration. The medium below the dinonyl phthalate phase and much of the dinonyl phthalate was removed quickly by syringe. The floating fat-cells and adhering dinonyl phthalate were transferred immediately to a glass-stoppered tube at 0°C containing 5ml of extraction medium (0.25M-sucrose, 20mM-Tris/HCl, 5μg of Ruthenium Red/ml, 7.5mM-glutathione, 20mg of defatted albumin/ml, 2mM-EGTA, pH7.4). Cells were broken by vigorous agitation on a vortex mixer for two periods of 30s. The fat-plug was separated by centrifugation at 1000*g* for 1min at 4°C, and samples

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

(1ml) of the infranatant were then centrifuged at 16000*g* for 1min in an Eppendorf 3200 centrifuge at 4°C to give mitochondrial and supernatant fractions. Mitochondrial pellets were frozen in liquid N_2 and then suspended in 250μl of potassium phosphate buffer, pH7.0, containing 10mM-EDTA at 0°C. Assays of ^{45}Ca and ^{3}H in appropriate samples (10–100μl) of the incubation media, infranatants (total cell extracts), supernatant and mitochondrial fractions, and of the glutamate dehydrogenase and pyruvate dehydrogenase content of the mitochondrial fraction, were made as given below.

Determination of ^{45}Ca incorporation into the mitochondrial fraction of intact fat-pads. Groups of fat-pads (four to six) were preincubated for 30–60min at 37°C in bicarbonate-buffered medium (10–15ml) containing fructose (2mg/ml). The pads were lightly blotted and transferred to fresh media containing fructose and ^{45}Ca (1.5μCi/ml) and other additions as indicated in the text and Tables. After incubation (usually 30min) the pads and medium were transferred to a polycarbonate centrifuge tube containing dinonyl phthalate (5ml), and the pads were separated from the medium by centrifugation for 15s at room temperature in a MSE Super Medium centrifuge set at maximum acceleration. The floating pads were immediately picked off the surface with forceps and rapidly disrupted in ice-cold sucrose extraction medium (approx. 1ml/pad) by homogenization in a Polytron PT-20 tissue homogenizer set at about one-quarter full speed for 3–5s (the sucrose extraction medium was as used above for fat-cells but contained 30mg of defatted albumin/ml and [^{3}H]sucrose, 1.5μCi/ml). The fat-plug was separated by centrifugation at 1000*g* for 90s at 4°C, and three samples (1ml) of the infranatant were then centrifuged at 16000*g* for 1min in an Eppendorf 3200 centrifuge at 4°C to give mitochondrial and supernatant fractions. The three mitochondrial pellets were combined, and washed by resuspending in a total of 1ml of sucrose extraction medium containing no [^{3}H]sucrose and centrifuging at 16000*g* for 1min to give a single washed mitochondrial pellet, which was frozen in liquid N_2 before being resuspended in 250μl of 100mM-potassium phosphate buffer, pH7.0, containing 2mM-EDTA at 0°C. Care was taken that fat was excluded from the mitochondrial pellet. Assays of ^{45}Ca and ^{3}H in appropriate samples (20–100μl) of incubation medium, supernatant fraction, wash supernatant and washed mitochondrial fraction, and of glutamate dehydrogenase and pyruvate dehydrogenase in mitochondrial fraction, were made as described below.

Determination of total calcium content of mitochondrial fractions. Mitochondrial pellets from fat-cells or fat-pads were prepared as described above, but no $^{45}CaCl_2$ was present in the incubation media. The pellets were dissolved in conc. HNO_3 and diluted into 2.5mM-EDTA disodium salt before determination of calcium content with an EEL 240 atomic absorption spectrophotometer. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Separation of plasma membranes and microsomal particles from mitochondrial fractions. This was accomplished by a modification of the procedure of McKeel & Jarrett (1970). Mitochondrial fractions suspended in sucrose extraction medium were layered on a discontinuous Ficoll gradient consisting of 20ml of 9% Ficoll in 0.25M-sucrose, 10mM-Tris/HCl, pH7.5, and 20ml of 15% Ficoll in the same buffer. After centrifugation at 4°C for 1h at 24000rev./min in an SW25.2 rotor in a Beckman L2-HV ultracentrifuge, plasma membranes were associated with the sucrose/9% Ficoll interphase and the mitochondria were present as a pellet in the bottom of the gradient.

Simultaneous assay of ^{45}Ca and ^{3}H. Samples were added to 10ml of scintillation fluid [naphthalene (80g/l) and 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6g/l) in toluene/methoxyethanol (3:2, v/v)]. Radioactivity was determined with a Nuclear-Chicago Isocap liquid-scintillation counter set up for the simultaneous assay of ^{14}C and ^{3}H. On this setting, counting efficiency for ^{45}Ca in the ^{14}C channel in an unquenched sample was about 76% and spill-over of ^{45}Ca into the ^{3}H channel about 15% of the total ^{45}Ca d.p.m. Correction for quenching calculated by external standardization was similar for all samples.

Extraction and assay of enzymes from fat-pads, fat-cells and mitochondrial fractions. Methods were essentially those given by Coore *et al.* (1971), Martin *et al.* (1972) and Severson *et al.* (1974). Fat-cells and fat-pads were frozen in liquid N_2 and then extracted with about 4ml of 100mM-potassium phosphate buffer, pH7.0, containing 2–5mM-EDTA and 1mM-dithiothreitol/g at 0°C in a Polytron PT-10 tissue homogenizer at one-half maximum speed for 30–60s. Extracts were then centrifuged for approx. 1min at 16000*g* in an Eppendorf 3200 centrifuge before assay. Mitochondrial pellets were frozen in liquid N_2, then suspended in 250μl of the phosphate buffer containing EDTA, and frozen and thawed twice before assay. Enzymes in the extracts were assayed as described by Coore *et al.* (1971). Initial pyruvate dehydrogenase activity is the activity observed immediately after extraction; total pyruvate dehydrogenase activity is the activity after incubation with pig heart pyruvate dehydrogenase phosphate phosphatase in the presence of Mg^{2+} and Ca^{2+} (Severson *et al.*, 1974).

Glycerol output and conversion of glucose into CO_2 and fatty acids in fat-cells. Glycerol was measured spectrophotometrically in neutralized $HClO_4$ extracts of incubation media (Garland & Randle, 1962). Conversion of [1-^{14}C]glucose into CO_2 was measured

by incubation in flasks with serum caps and centre wells. At the end of the incubation, flasks were acidified by injection of $HClO_4$ (final concn. 2%, w/v) and CO_2 was collected in 0.5ml of 2-phenethylamine/methanol (1:1, v/v) in the centre well.

ATP content, O_2 uptake and calcium uptake by mitochondrial fractions. These were measured as given in Severson *et al.* (1974).

Expression of results. Enzyme activities are given in units (1 μmol of substrate converted/min) measured at 30°C. Glutamate dehydrogenase is a mitochondrial enzyme; the activity of this enzyme has been used as a convenient index of recovery of fat-cell and fat-pad mitochondria throughout this study. Fat-pads contain 0.8–1 unit of glutamate dehydrogenase/g wet wt.; fat-cells contain 0.6–0.8 unit/g dry wt. Mitochondrial fractions from both fat-pads and fat-cells contain about 0.25 unit of glutamate dehydrogenase/mg of protein.

Results

Determination of extracellular ^{45}Ca incorporation into mitochondrial fractions prepared from isolated fat-cells and intact fat-pads

The intracellular space of incubation of fat-cells and fat-pads is small compared with the extracellular space. To measure ^{45}Ca incorporation into mitochondria in fat-cells it was necessary to separate fat-cells or fat-pads from incubation medium as completely as possible and then to prepare a mitochondrial fraction under conditions where transfer of ^{45}Ca across the mitochondrial membrane is essentially completely inhibited. Fat-cells and fat-pads were separated from the medium by centrifugation through dinonyl phthalate (Gliemann *et al.*, 1972): redistribution of ^{45}Ca between mitochondrial and extramitochondrial fractions during homogenization was prevented by rapidly breaking cells into a sucrose medium containing EGTA (to chelate extramitochondrial calcium) and Ruthenium Red (a very potent inhibitor of calcium transport in fat-cell mitochondria; Severson *et al.*, 1974).

Detailed results of a typical determination of ^{45}Ca incorporation into fat-cells and fat-cell fractions are given in Table 1(a). After preincubation, cells were incubated with ^{45}Ca (in this instance for 20min) and as an extracellular marker [^{3}H]inulin. The remarkable efficiency of the dinonyl phthalate procedure is shown by the small amount of [^{3}H]inulin carried over with the cells. In this case, this was equivalent to 11.9 μl of extracellular space; the mean of all determinations was 7.1 μl, or less than 0.1% of the volume of the original incubation medium.

Table 1. *Incorporation of extracellular ^{45}Ca into mitochondrial and supernatant fractions of fat-cells*

Fat-cells (approx. 250mg dry wt.) were preincubated for 30min at 37°C in medium containing glucose (0.2mg/ml) and then incubated for the indicated period in fresh medium containing glucose (0.2mg/ml), $^{45}Ca^{2+}$ (0.2 μCi/ml) and, as an extracellular marker, [^{3}H]inulin (2 μCi/ml). After separation of the cells from the medium by centrifugation through dinonyl phthalate, the cells were rapidly extracted in sucrose medium containing EGTA and Ruthenium Red. Fat was removed by low-speed centrifugation, and the total cell extracts were then separated by further centrifugation into supernatant and mitochondrial fractions. Details are given in the Experimental section. The radioactivity content of the fractions was determined and calculated as the equivalent volume of extracellular medium. After allowance for extracellular contamination of the fractions (given by the [^{3}H]inulin content), ^{45}Ca incorporation was calculated as ng-atoms/unit of glutamate dehydrogenase in the mitochondrial fraction. Detailed results of a typical experiment are given in section (*a*); the mitochondrial fraction in this experiment contained 60.7munits of glutamate dehydrogenase. Sections (*b*) and (*c*) show the time-course of ^{45}Ca incorporation into fat-cell fractions. The results in section (*b*) are the means of duplicate determinations in samples of a single preparation of cells. The results in section (*c*) are the means ± S.E.M. of determinations on the numbers of separate preparations of cells given in parentheses.

Expt.	Measurement	Incubation time with $^{45}Ca^{2+}$ (min)	Fraction: Total cell extract	Fraction: Supernatant	Fraction: Mitochondrial
(*a*)	Space (as μl of extracellular medium/fraction)				
	[^{3}H]Inulin	20	11.9	10.0	0.3
	^{45}Ca	20	26.3	18.5	6.5
	^{45}Ca – [^{3}H]inulin	20	14.4	8.5	6.2
	^{45}Ca incorporation (ng-atoms/unit of glutamate dehydrogenase)	20	297	175	128
(*b*)	^{45}Ca incorporation (ng-atoms/unit of glutamate dehydrogenase)	2	485	247	177
		8	479	309	146
		26	604	328	193
		50	505	290	191
(*c*)	^{45}Ca incorporation (ng-atoms/unit of glutamate dehydrogenase)	2	292 ± 63 (5)	172 ± 37 (5)	111 ± 19 (5)
		20	359 ± 33 (11)	216 ± 22 (11)	122 ± 10 (11)

The cells were then broken in sucrose-based extraction medium containing EGTA and Ruthenium Red, and the total cell extract was separated into supernatant and mitochondrial fractions by a procedure based on that devised for the preparation of fat-cell mitochondria (Martin & Denton, 1970, 1971). Separation was satisfactory as over 90% of the [^{3}H]inulin content of the total cell extract was present in the supernatant fraction and over 90% of the glutamate dehydrogenase content of the total cell extract was found in the mitochondrial fraction. Thus little or no breakage of mitochondria appears to occur during cell disruption. However, comparison of glutamate dehydrogenase activity in the mitochondrial fraction or total cell extract with total glutamate dehydrogenase activity extractable from separate samples of cells indicated that the recovery of glutamate dehydrogenase through the procedure was 40–60%. This incomplete recovery was taken to reflect both the proportion of unbroken cells (which are removed by the low-speed centrifugation) and the loss of cell extracts during the various transfers and other manipulations in the procedure. Incorporation of ^{45}Ca into cell fractions was therefore calculated in terms of ng-atoms of medium calcium/unit of glutamate dehydrogenase to correct for this variability. In the typical determination given in Table 1(*a*), of the ^{45}Ca in total cell extract 45% could be accounted for as extracellular on the basis of [^{3}H]inulin, giving a net total cell ^{45}Ca content of 297 ng-atoms/unit of glutamate dehydrogenase. About 40% of this was present in the mitochondrial fraction and the remainder was associated with the supernatant fraction.

The procedure used for the determination of ^{45}Ca incorporation into the mitochondrial fraction of fat-pads was based on that used for fat-cells but contained a number of modifications. These were necessary because more vigorous extraction conditions were required to release mitochondria from the fat-cells within fat-pads, and because the incorporation of ^{45}Ca into the mitochondrial fraction was found to be very much less in fat-pads than fat-cells. Moreover, the dinonyl phthalate procedure was far less effective, since the volume of medium retained by the pads was about 20 times greater than that associated with isolated fat-cells. The specific radioactivity of ^{45}Ca in the medium was increased from 0.2 μCi to 1.5 μCi/ml, and [^{3}H]sucrose was added as an extramitochondrial marker to the sucrose-based extraction media rather than [^{3}H]inulin to the incubation medium in order that a favourable ^{3}H/^{45}Ca ratio could be achieved in the subsequent fractionation. Extraction of mitochondria was achieved by rapid homogenization (3–5 s) in ice-cold sucrose-based extraction media containing EGTA and Ruthenium Red with a Polytron tissue homogenizer followed by separation into supernatant and mitochondrial fractions by centrifugation. Preliminary experiments showed that a single centrifugation was not satisfactory because of contamination of the mitochondrial fraction with extracellular ^{45}Ca (shown with [^{3}H]sucrose). The proportion of ^{45}Ca in the mitochondrial fraction that was extramitochondrial on the basis of [^{3}H]sucrose content was decreased from over 70% to about 10% by inclusion of an additional wash in sucrose medium. Recovery of glutamate dehydrogenase through the procedure was 15–25%. This low recovery was probably largely due to incomplete cell breakage during the short homogenization. However, longer periods of homogenization were found to lead to breakage of mitochondria and increased appearance of glutamate dehydrogenase in the supernatant fraction. With homogenization for only 3–5 s the supernatant fraction contained 10–20% of the glutamate dehydrogenase and citrate synthase activities found in the mitochondrial fraction. Detailed results of a typical determination of ^{45}Ca incorporation into the mitochondrial fraction of fat-pads incubated with ^{45}Ca for 30 min is given in Table 2(a). The ^{45}Ca content in the wash supernatant was equivalent to a slightly greater volume of supernatant fraction than the [^{3}H]sucrose content, but this difference was small compared with the difference observed in the mitochondrial fraction. The mean net ^{45}Ca content of the wash supernatant in 28 separate experiments was 8.4±2.6% of the net ^{45}Ca content of the washed mitochondrial fraction. The ^{45}Ca incorporation was calculated as 5.47 ng-atoms of medium Ca^{2+}/unit of glutamate dehydrogenase, from the glutamate dehydrogenase content of the mitochondrial fraction and from the values of the ^{45}Ca content of the original medium and the supernatant.

Further studies were aimed at demonstrating that negligible amounts of ^{45}Ca were taken up or lost by the mitochondrial fractions during preparation. Previous studies with isolated fat-cell mitochondria have shown that they have the capacity for rapid energy-dependent accumulation of more than 400 ng-atoms of calcium/unit of glutamate dehydrogenase (Severson *et al.*, 1974); thus there is a real risk of very appreciable uptake of ^{45}Ca during the preparation of mitochondrial fractions, which took about 5 min for both fat-cells and intact fat-pads. However, addition of Ruthenium Red and EGTA to the sucrose-based extraction medium appeared to be most effective in inhibiting uptake during this time. If fat-pads or fat-cells were incubated without ^{45}Ca and then mitochondrial fractions were prepared with sucrose extraction medium containing an appropriate amount of ^{45}Ca, it was found that negligible amounts of ^{45}Ca were taken up into the mitochondrial fractions if both Ruthenium Red and EGTA were present in the sucrose extraction. (Table 3

Table 2. *Incorporation of extracellular ^{45}Ca into mitochondrial fraction of fat-pads*

Sections (*a*) and (*b*); groups of four fat-pads (about 1.5g) were preincubated for 30min at 37°C in bicarbonate-buffered medium containing fructose (3mg/ml) and then incubated in fresh medium containing fructose (10mM) and $^{45}Ca^{2+}$ (1.5μCi/ml) for the time given. After separation from the medium by centrifugation through dinonyl phthalate, the fat-pads were rapidly extracted in sucrose extraction medium containing Ruthenium Red, EGTA and, as extramitochondrial marker, [6,6′(*n*)-^{3}H]sucrose (1.5μCi/ml). Fat was removed by low-speed centrifugation, and the fat-pad extracts were separated by further centrifugation into supernatant and mitochondrial fractions. The mitochondrial fraction was washed by resuspension in sucrose medium without [^{3}H]sucrose and centrifugation to yield washed supernatant and washed mitochondrial fractions. Details are given in the Experimental section. The radioactivity content of the washed supernatant and washed mitochondrial fractions was calculated as the equivalent volume (μl) of original supernatant. After allowance for extramitochondrial contamination (given by the [^{3}H]sucrose content), ^{45}Ca incorporation was calculated as ng-atoms of medium Ca/unit of glutamate dehydrogenase in the mitochondrial fraction. Detailed results of a typical experiment are given in section (*a*); in this experiment the mitochondrial fraction contained 208munits of glutamate dehydrogenase; the ^{45}Ca concentration of the supernatant was 8.0% of that of the incubation medium. Results in section (*b*) show the time-course of ^{45}Ca incorporation into fat-pad washed mitochondrial fraction and are given as means±S.E.M. of determinations of the numbers of separate groups of pads given in parentheses. Results in section (*c*) show the time-course of the total ^{45}Ca incorporation into fat-pad pieces (100mg) incubated as described above except that [^{3}H]inulin (2μCi/ml) was present as an extracellular marker in the incubation medium for 30min before the addition of ^{45}Ca (0.2μCi/ml). After the indicated time, each fat-pad piece was dispersed in scintillation fluid (10ml) with the Polytron PT-10 tissue homogenizer and the radioactivity determined. Results are the means±S.E.M. for the numbers of separate determinations given in parentheses.

Measurement	Incubation time with $^{45}Ca^{2+}$ (min)	Fraction: Total fat-pad	Fraction: Wash supernatant	Fraction: Washed mitochondria
(*a*) Space (as μl of supernatant/fraction)				
[^{3}H]sucrose	30	—	16.10	1.07
^{45}Ca	30	—	17.10	12.45
^{45}Ca−[^{3}H]sucrose	30	—	1.00	11.38
^{45}Ca incorporation (ng-atoms of medium Ca^{2+}/unit of glutamate dehydrogenase)	30	—	0.48	5.47
(*b*) ^{45}Ca incorporation (ng-atoms of medium Ca^{2+}/unit of glutamate dehydrogenase)	5	—	1.21±0.50	8.10±0.80 (2)
	15	—	1.50±0.02	7.22±0.41 (2)
	30	—	0.19±0.49	6.51±0.65 (26)
	60	—	0.21±0.04	6.62±1.18 (3)
(*c*) ^{45}Ca incorporation (ng-atoms of medium Ca^{2+}/unit of glutamate dehydrogenase)	5	80±18.2(4)	—	—
	10	108±15.9(4)	—	—
	30	116± 4.8(8)	—	—
	60	137± 6.7(12)	—	—

shows similar results for fat-pads; similar findings were obtained for fat-cells.) There was some evidence of ^{45}Ca uptake if Ruthenium Red was omitted, and massive uptake was observed if both Ruthenium Red and EGTA were omitted (Table 3, Expt. 1). In other experiments (not shown) it was found that Ruthenium Red alone in the absence of EGTA decreased this massive uptake by more than 75%. Omission of both EGTA and Ruthenium Red also resulted in a large increase in the ^{45}Ca content of mitochondrial fractions when fat-pads or fat-cells had been incubated with ^{45}Ca under standard conditions (Table 3, Expt. 2). Mitochondrial fractions prepared from fat-cells and fat-pads previously incubated with ^{45}Ca in sucrose-based extraction medium with EGTA and Ruthenium Red were also suspended in the same medium and incubated at 0°C. The loss of ^{45}Ca from the mitochondrial fractions was less than 10% after 30min. Some loss of ^{45}Ca from mitochondrial fraction from fat-cells was apparent during incubation at 30°C in KCl (125mM)/Tris/HCl (20mM), pH7.4, and this loss was markedly increased on the addition of uncoupler (0.1mM-2,6-dinitrophenol), so that 70% of ^{45}Ca was lost in 10min. Taken together, these studies indicated that little or no redistribution of ^{45}Ca occurred during the procedures used in the preparation of the mitochondrial fractions from both fat-cells and fat-pads.

The mitochondria in the fractions from both fat-cells and fat-pads appeared to be intact. Little or no glutamate dehydrogenase activity was evident unless the fractions were frozen and thawed. The mitochondria exhibited good respiratory control (Chance & Williams, 1956), with ratios of 4–6 for the oxidation of pyruvate (5mM) and malate (0.5mM), 3.5–4 for the oxidation of succinate (5mM) and

Table 3. *Effect of omission of EGTA and Ruthenium Red from the sucrose extraction medium on ^{45}Ca incorporation into the mitochondrial fraction during preparation from fat-pads*

Groups of fat-pads were incubated and washed mitochondrial fractions were prepared as described in Table 2(*a*) except that in Expt. 1 no ^{45}Ca was added to the incubation but it was added to the sucrose-based extraction medium. Where indicated EGTA and Ruthenium Red were omitted from the extraction medium. Each result is the mean of duplicate determinations on a separate group of fat-pads; allowance for extramitochondrial contamination of the washed mitochondrial fraction was made as indicated in the legend to Table 2.

Expt. no.	^{45}Ca in incubation media (μCi/ml)	Sucrose-based extraction medium	^{45}Ca content: Supernatant fraction (d.p.m./μl)	^{45}Ca content: Washed mitochondrial fraction (d.p.m./munit of glutamate dehydrogenase)
1	None	Complete plus $^{45}CaCl_2$	287	<0.5, <0.5
	None	Complete plus $^{45}CaCl_2$ minus Ruthenium Red	287	3.0, 1.3
	None	Complete plus $^{45}CaCl_2$ minus Ruthenium Red and EGTA	287	1779, 1160
2	1.5	Complete	239, 241	14, 16
	1.5	Complete minus Ruthenium Red	232, 268	21, 24
	1.5	Complete minus Ruthenium Red and EGTA	237, 247	468, 466

4–8 for the oxidation of 2-oxoglutarate (5mM) and malate (0.5mM). If prepared in the absence of Ruthenium Red the fractions readily accumulated ^{45}Ca if either ATP or a respiratory substrate was present. Preparations of mitochondrial fractions from fat-pads were examined by electron microscopy. The fraction very largely consisted of intact mitochondria, with a few lysosomes and isolated fragments of endoplasmic reticulum and plasma membrane. However, it was considered important to demonstrate that the ^{45}Ca content of the mitochondrial fractions corresponded to the ^{45}Ca content of mitochondria and not of other cell components contaminating the fractions. Mitochondrial fractions derived from fat-pads and fat-cells incubated with ^{45}Ca were separated by ultracentrifugation on a Ficoll gradient (McKeel & Jarrett, 1970) as described in the Experimental section. Over 80% of ^{45}Ca in the original mitochondrial fractions sedimented with the mitochondria and less than 10% was associated with the plasma-membrane fraction.

The ratio of activities of four mitochondrial enzymes, glutamate dehydrogenase, citrate synthase, pyruvate carboxylase and pyruvate dehydrogenase (total activity), were compared in the fat-pad mitochondrial fraction and in a total extract of frozen fat-pads. No appreciable difference was found, indicating that, although the mitochondrial fraction may contain some mitochondria from cell types other than from fat-cells, the proportion of these mitochondria present was probably no greater than that in the whole fat-pad. Moreover the ratio of the activities was also close to that found in preparations of fat-cell mitochondria (Martin & Denton, 1970; Coore *et al.*, 1971), suggesting that most of the mitochondria were derived from fat-cells.

Comparison of extracellular ^{45}Ca incorporation into mitochondrial fractions of fat-cells and fat-pads

No time-course for the incorporation of ^{45}Ca into the mitochondrial fractions was observed (Tables 1*b*, 1*c* and 2*b*). Incorporation was essentially complete within 2min for fat-cells and 5min for fat-pads, suggesting that in both fat-pads and fat-cells there is a pool of calcium within the mitochondria that is in relatively rapid isotopic exchange with extracellular calcium. The size of this pool was found to be very much larger in mitochondria within fat-cells than in mitochondria within fat-pads (Tables 1 and 2). The mean value in the fat-cell mitochondrial fraction after 20min incubation with ^{45}Ca was 122ng-atoms of Ca^{2+}/unit of glutamate dehydrogenase, which is about 20 times the value (8.6) found in the mitochondrial fraction from fat-pads after 30min exposure to $^{45}Ca^{2+}$.

In fat-cells the ^{45}Ca in the mitochondrial fraction accounted for about 34% of the total ^{45}Ca incorporation into the cells. The remainder, which appeared in the supernatant fraction, presumably includes ^{45}Ca bound to the outside and inside of the cell membrane (sequestered by EGTA) as well as ^{45}Ca in microsomal and other extramitochondrial locations. There was some evidence for increased incorporation into the supernatant fraction after the first 2min (Tables 1 and 5). In fat-pads total ^{45}Ca incorporation was measured in separate experiments (Table 2*c*). In this case, although ^{45}Ca incorporation into the mitochondrial fraction was not appreciably different after 5, 15, 30 and 60min exposure to ^{45}Ca (Table 2*b*), there was an appreciable increase in the total amount of ^{45}Ca taken up by the fat-pads over this time-period. At 30min incorporation into the mitochondrial fraction

accounted for about 6% of the total ^{45}Ca associated with the fat-pads. Similar time-courses for the accumulation of ^{45}Ca by fat-cells and fat-pads have been found by Martin *et al.* (1975).

Total calcium contents of mitochondrial fractions from fat-cells and fat-pads were measured by atomic absorption, and the results are given in Table 4 in terms of both mg of protein and unit of glutamate dehydrogenase activity. The total calcium content of the mitochondrial fraction from fat-pads was about 42 ng-atoms of calcium/unit of glutamate dehydrogenase and that of fat-cells was about 233 ng-atoms of calcium/unit of glutamate dehydrogenase, i.e. four times greater. The proportion of the mitochondrial total calcium that was exchangeable with extracellular $^{45}Ca^{2+}$ was thus about 15% in fat-pads and over 50% in fat-cells (Table 4).

Effects of dinitrophenol, cyanide, respiratory substrates, $NiCl_2$, $MnCl_2$ and Ruthenium Red on ^{45}Ca incorporation into mitochondrial and supernatant fractions of fat-cells

Results of experiments shown in Table 5 are the means of duplicate determinations, and within each experiment were obtained with a single batch of cells. Qualitatively similar results have been obtained in each case on at least two different preparations of fat-cells. Variations in the ^{45}Ca incorporation, especially into the supernatant fractions, made combining results from different experiments difficult.

Addition of uncoupler (0.1 mM-dinitrophenol) to the incubation media decreased ^{45}Ca incorporation into the mitochondrial fraction. This was discernible after 2 min and much more marked after 45 min. There were corresponding increases in the incorporation into the supernatant fraction, so that overall there was little change in total ^{45}Ca incorporation into fat-cells. Incubation with KCN (2 mM) for 10–20 min almost completely inhibited ^{45}Ca incorporation into the mitochondrial fraction, but the fraction contained very few intact mitochondria, as indicated by the very low glutamate dehydrogenase activity. This result emphasizes the need for an index of mitochondrial recovery in experiments of this nature.

Incubation of fat-cells with succinate or 2-oxoglutarate and malate more than doubled the incorporation of ^{45}Ca into the mitochondrial fraction with little or no change in the ^{45}Ca incorporation into the supernatant fraction.

Ruthenium Red, which inhibits calcium uptake into isolated mitochondria, actually increases ^{45}Ca incorporation into the mitochondrial fraction when added to the incubation medium of fat-cells (Table 5, Expt. 3). Ruthenium Red also increased incorporation into the supernatant fraction. $MnCl_2$ and $NiCl_2$ had rather similar effects to Ruthenium Red. Both increased incorporation into the supernatant fraction, and $NiCl_2$ (but not $MnCl_2$) also increased incorporation into the mitochondrial fraction. In contrast, 1 mM-$SrCl_2$ had no effect on Ca^{2+} incorporation (results not shown).

Insulin stimulated the conversion of [^{14}C]glucose (0.2 mg/ml) into CO_2 (3–7-fold), and in the presence of low concentration of adrenaline, insulin inhibited glycerol output in preparations of fat-cells used in the present studies. However, the effect of insulin on pyruvate dehydrogenase activity in these cells was only modest. The mean increase observed in five separate preparations after 30 min exposure to insulin (100 units/ml) was 30±8%, whereas in fat-pads the effect often exceeded 100% (Coore *et al.*, 1971; Severson *et al.*, 1974; Tables 8 and 9). In the isolated cell preparations the ratio of pyruvate dehydrogenase activity/glutamate dehydrogenase activity in the absence of insulin was 0.210±0.010,

Table 4. *Comparison of total calcium content and extracellular ^{45}Ca incorporation into mitochondrial fractions of fat-cells and fat-pads*

Total calcium contents of mitochondrial fractions prepared as given in Tables 1(*a*) and 2(*a*) but without addition of $^{45}Ca^{2+}$ were measured by atomic absorption (see the Experimental section). Results are given as means±S.E.M. of the numbers of separate observations given in parentheses. Values of extracellular ^{45}Ca incorporation into mitochondrial fractions of intact fat-pads and isolated fat-cells are those found after 30 or 20 min incubation respectively and are taken from Tables 2(*b*) and 1(*c*).

	Source of mitochondrial fraction	
	Intact fat-pads	Isolated fat-cells
Total calcium content of mitochondrial fraction		
(as ng-atoms of Ca/mg of protein)	11.2± 3.9 (6)	47±11.5 (5)
(as ng-atoms of Ca/unit of glutamate dehydrogenase)	42.3±10.8 (6)	233±39.0 (5)
Extracellular ^{45}Ca incorporation into mitochondrial fraction		
(as ng-atoms of Ca/unit of glutamate dehydrogenase)	6.5± 0.7 (26)	122±10 (11)
(as % of total calcium content in mitochondrial fraction)	15.4	52.4
(as % of total ^{45}Ca incorporation into whole-cell preparation)	5.6	34.0

which is considerably higher than that observed in fat-pads incubated without insulin. In preliminary experiments no effect of insulin on extracellular ^{45}Ca incorporation into the mitochondrial fraction could be detected (the mean ^{45}Ca incorporation with insulin was $98\pm8\%$ of control in five separate preparations of fat-cells). As the effect of insulin on pyruvate dehydrogenase activity was so marginal in fat-cells, studies on the effects of insulin in fat-cells were not pursued. However, a number of incubation conditions that led to changes in the extent of ^{45}Ca incorporation into the mitochondrial fraction of fat-cells were examined for their effects on fat-cell pyruvate dehydrogenase activity (Tables 6 and 7).

The effect of introducing EGTA (5mM) into the incubation medium after fat-cells had been exposed to ^{45}Ca for 10min was a very rapid decrease in ^{45}Ca content of the supernatant fraction and a somewhat slower decrease of the ^{45}Ca content of the mito-

Table 5. *Effects of dinitrophenol, respiratory substrates, Ruthenium Red $NiCl_2$ and $MnCl_2$ on ^{45}Ca incorporation into fat-cells*

Fat-cells were preincubated, incubated and fractions prepared as outlined in the legend to Table 1(*a*). Additions indicated below were made to both preincubation and incubation media, except dinitrophenol, which was added only to incubation medium. Medium containing albumin plus dinitrophenol was dialysed overnight against 10vol. of medium containing dinitrophenol but no albumin. Period of exposure to $^{45}Ca^{2+}$ was varied as indicated. All results are the means of duplicate determinations, which agreed within 15%. A different cell preparation was used in each experiment. Qualitatively similar results have been obtained on at least two different preparations of fat-cells in all cases.

Expt. no.	Additions to media	Incubation time with $^{45}Ca^{2+}$ (min)	^{45}Ca incorporated (ng-atoms/unit of glutamate dehydrogenase) into: Total cell extract	Supernatant fraction	Mitochondrial fraction
1	None	2	197	93	116
	Dinitrophenol (0.1mM)	2	218	124	85
	None	45	294	164	105
	Dinitrophenol (0.1mM)	45	250	199	46
2	None	20	282	206	80
	Succinate (5mM)	20	394	214	174
	2-Oxoglutarate (5mM)+L-malate (0.5mM)	20	448	267	186
3	None	2	234	128	101
	Ruthenium Red (5μg/ml)	2	308	201	126
	None	45	358	234	106
	Ruthenium Red (5μg/ml)	45	520	294	234
4	None	20	397	301	133
	$NiCl_2$ (1mM)	20	679	414	217
	$MnCl_2$ (0.5mM)	20	666	539	118

Table 6. *Effect of EGTA on ^{45}Ca incorporation and pyruvate dehydrogenase activity in fat-cells*

Fat-cells were preincubated, incubated and fractions prepared as described in Table 1(*a*) with the following changes. Incubation with ^{45}Ca and [^{3}H]inulin was for 10min before addition of EGTA (5mM). Incorporation of ^{45}Ca into fractions of samples of cells was determined just before the addition of EGTA and after 0.25, 2 and 10min exposure to EGTA. Results are the means of duplicate determinations on samples from a single batch of cells. Further samples were incubated under identical conditions in the absence of ^{45}Ca and [^{3}H]inulin, and extracted and assayed for initial pyruvate dehydrogenase activity and glutamate dehydrogenase activity.

Addition to incubation medium	Time after addition of EGTA (min)	^{45}Ca incorporated (as ng-atoms/unit of glutamate dehydrogenase) into: Total cell extract	Supernatant fraction	Mitochondrial fraction	Fat-cell pyruvate dehydrogenase/glutamate dehydrogenase activity ratio
None	—	332	197	97	0.200
EGTA	0.25	104	46	57	0.184
EGTA	2	77	38	52	0.243
EGTA	10	—*	—*	27	0.226

* The net ^{45}Ca space in these fractions was less than 20% of the extracellular space (i.e. [^{3}H]inulin space) and could not be reliably measured.

chondrial fraction (Table 6). After 10min the ^{45}Ca content of the mitochondrial fraction was decreased by more than 70%. This was shown with three separate fat-cell preparations (mean decrease $83\pm7\%$), but no significant change in fat-cell pyruvate dehydrogenase activity could be detected. Since more than 50% of the total calcium content of the mitochondrial fraction of fat cells is exchangeable with extracellular $^{45}Ca^{2+}$ (Table 4), incubation of fat-cells with EGTA for 10min should at least halve the total calcium content of the mitochondrial fraction. This was confirmed by atomic absorption spectroscopy; the mean decrease was $48\pm5\%$ (three observations).

The omission of phosphate from incubation media had little or no effect on the incorporation of ^{45}Ca or on the initial activity of pyruvate dehydrogenase (Table 7). However, increasing the phosphate concentration to 5mM greatly increased the ^{45}Ca incorporation into the supernatant fraction and doubled the incorporation of ^{45}Ca into the mitochondrial fraction. Although this would suggest that the calcium content of mitochondria increased under these conditions, no appreciable change in pyruvate dehydrogenase activity was observed. Addition of the bivalent-metal ionophore A23187 and replacement of NaCl in the medium by LiCl also increased incorporation into supernatant and mitochondrial fractions without any appreciable change in pyruvate dehydrogenase activity.

Effects of insulin, Ruthenium Red, phosphate and EGTA on pyruvate dehydrogenase activity and extracellular ^{45}Ca incorporation into the mitochondrial fraction in fat-pads

The effect of insulin on pyruvate dehydrogenase activity in fat-pads persists during the preparation of mitochondria from fat-pads. If the mitochondrial fraction was frozen in liquid N_2 immediately on preparation and assayed for pyruvate dehydrogenase and glutamate dehydrogenase activity, it was found that there was little alteration in the insulin effect expressed in percentage terms. Table 8 gives results obtained when the mitochondrial fraction was prepared as described in the Experimental section but without the final wash. Under these conditions the pyruvate dehydrogenase/glutamate dehydrogenase activity ratios were very similar in the mitochondrial fraction and intact fat-pads. If the fraction was washed (as in the experiments reported in Table 9), then there was a tendency for the ratio in the mitochondrial fraction to be higher than that in the fat-pads, probably because mitochondrial ATP content was declining.

No significant change in ^{45}Ca incorporation into the mitochondrial fraction was observed with insulin, although there was a clear increase in pyruvate dehydrogenase activity (Table 9, Expt. 1). Ruthenium Red, which diminished the effect of insulin on pyruvate dehydrogenase activity, as found in

Table 7. *Effects of phosphate, ionophore* A23187 *and Li^+ replacement on ^{45}Ca incorporation, pyruvate dehydrogenase activity and ATP content of fat-cells*

In Expt. 1, fat-cells were prepared in medium without phosphate and then preincubated for 30min in medium with glucose (1.0mM) and phosphate (0, 1 or 5mM, as indicated). In Expts. 2 and 3, fat-cells were prepared and preincubated in medium containing glucose (1.0mM) as usual. Incorporation of ^{45}Ca was determined in all experiments after 20min incubation in media containing glucose (1.0mM), ^{45}Ca and [^{3}H]inulin as outlined in Table 1, but with the indicated changes. In Expt. 3, cells were incubated in medium in which NaCl was replaced by LiCl. Each experiment was performed in duplicate with a separate preparation of fat-cells and the results are the means of the duplicate determinations. Samples of each fat-cell preparation were also incubated under identical conditions in medium without ^{45}Ca or [^{3}H]inulin, and extracted and assayed for initial pyruvate dehydrogenase activity, glutamate dehydrogenase activity and ATP content.

Expt. no.	Changes in incubation media	^{45}Ca incorporation (as ng-atoms/unit of glutamate dehydrogenase) into: Total cell extract	Supernatant fraction	Mitochondrial fraction	Fat-cell pyruvate dehydrogenase/glutamate dehydrogenase activity ratio	Fat-cell ATP content (nmol/unit of glutamate dehydrogenase)
1	Omission of phosphate	269	125	124	0.221	170
	None	332	171	126	0.245	167
	Phosphate (5mM)	1904	1530	242	0.211	187
2	None	317	189	81	0.248	244
	Ionophore A23187 (10μM)	667	505	122	0.239	264
	Ionophore A23187 (50μM)	1009	802	120	0.187	258
3	None	378	187	144	0.250	225
	Li^+ replacement	481	246	214	0.262	210

Table 8. *Pyruvate dehydrogenase activity in the mitochondrial fraction prepared from fat-pads incubated in the presence and in the absence of insulin*

Fat-pads (in groups of four to six) were preincubated for 30min at 37°C in bicarbonate-buffered medium containing fructose (10mM) and then incubated in fresh medium (10ml) containing fructose (10mM) and, where indicated, insulin. Fat-pads (three or four) from each group were used for the preparation of mitochondrial fractions as described in the Experimental section except that the fractions were not washed. Immediately on preparation the mitochondrial fractions were frozen in liquid N_2. The remaining fat-pads (one or two) were frozen intact with liquid N_2. Fat-pads and mitochondria were extracted and assayed for initial pyruvate dehydrogenase and glutamate dehydrogenase activity (see the Experimental section for details). Results are given as means±S.E.M. of observations on six separate groups of pads.

	Pyruvate dehydrogenase activity (given as a ratio to glutamate dehydrogenase activity) in	
Addition to incubation media	Intact pads	Mitochondrial fraction
None (control)	0.071±0.008	0.080±0.012
Insulin (10munits/ml)	0.182±0.019	0.169±0.023
Effect of insulin (as % of control)	264±26	218±19

Table 9. *Effects of insulin, Ruthenium Red, phosphate, adrenaline and alloxan-diabetes on pyruvate dehydrogenase activity and on extracellular ^{45}Ca incorporation into mitochondrial fraction of fat-pads*

Fat-pads (in groups of four or six) were preincubated for 30min at 37°C in bicarbonate-buffered medium (10ml) containing fructose (2mg/ml), then incubated in fresh media with fructose (2mg/ml) plus $^{45}CaCl_2$ (1.5μCi/ml) and additions as indicated for 30min. In all experiments four fat-pads were used for the preparation of washed mitochondrial fraction (as Table 2*a*), which was assayed for ^{45}Ca incorporation, glutamate dehydrogenase and pyruvate dehydrogenase activity as described in the Experimental section. In Expts. 2–5 two fat-pads were frozen in liquid N_2 and extracted for determination of the pyruvate dehydrogenase/glutamate dehydrogenase activity ratio in intact pads. In Expt. 5, alloxan-diabetes was induced by the intravenous injection of 60mg of alloxan/kg 48h before the experiment. Results are given as means±S.E.M. for the numbers of separate observations given in parentheses.

Expt. no.	Source of pads	Additions to incubation media	Activity ratio pyruvate dehydrogenase/glutamate dehydrogenase in: Intact fat-pad	Activity ratio pyruvate dehydrogenase/glutamate dehydrogenase in: Washed mitochondrial fraction	^{45}Ca incorporation into washed mitochondrial fraction (ng-atoms of medium Ca/unit of glutamate dehydrogenase)
1	Normal rats	None	—	0.15±0.020	9.03±1.03 (6)
		Insulin (5munits/ml)	—	0.30±0.016*	7.83±0.58 (6)
2	Normal rats	Insulin (5munits/ml)	0.19±0.012	0.42±0.033	8.35±1.38 (4)
		Insulin plus Ruthenium Red (5μg/ml)	0.11±0.009*	0.35±0.043	8.80±1.16 (4)
3	Normal rats	Insulin plus albumin (20mg/ml)	0.27±0.027	0.42±0.048	4.81±0.45 (6)
		Insulin plus albumin plus adrenaline (1μg/ml)	0.18±0.013*	0.20±0.012*	2.77±0.52*(6)
4	Normal rats	None	0.049±0.006	0.23±0.020	4.12±0.62 (4)
	Alloxan-diabetic	None	0.020±0.005*	0.11±0.022*	4.40±1.38 (4)
5	Normal rats	None	0.16±0.010	0.29±0.031	8.46±1.29 (8)
		Phosphate (4mM)	0.18±0.015	0.27±0.022	9.99±1.16 (8)

* $P<0.02$ versus appropriate control.

previous studies (Severson *et al.*, 1974), also had no appreciable effect on ^{45}Ca incorporation into the mitochondrial fraction (Table 9, Expt. 2). On the other hand, addition of adrenaline, which also diminishes the effect of insulin on pyruvate dehydrogenase activity (Coore *et al.*, 1971), led to a significant decrease in ^{45}Ca incorporation into the mitochondrial fraction (Table 9, Expt. 3). The possibility was explored that this decrease might be the result of mitochondria in the fraction being less well coupled, since it was found that incubation of fat-pads with uncoupler (2mM-dinitrophenol) for 30min decreased the incorporation of ^{45}Ca into the mitochondrial fraction of fat-pads by over 70% (results not shown).

The respiratory control ratios (Chance & Williams, 1956) of mitochondria in the fractions prepared from pads incubated and extracted as in Table 9 (Expt. 3) were compared; ratios with pyruvate plus malate or oxoglutarate plus malate as substrate ranged from 3.0 to 4.1 for mitochondria from tissue previously exposed to adrenaline; this was somewhat less than the range (4.4–7.0) obtained with mitochondria from tissue incubated in the absence of adrenaline. Measurements were also made of the ATP content of the mitochondrial fractions after incubation for 4min in KCl (125mM)/Tris/HCl (10mM), pH7.4, containing potassium phosphate (7mM), oxoglutarate (5mM) and malate (0.5mM); the content of mitochondria from fat-pads previously exposed to adrenaline and insulin was 23±7% (four observations) lower in mitochondria from fat-pads exposed to insulin alone.

Alloxan-diabetes diminishes the activity of pyruvate dehydrogenase in adipose tissue largely by increasing the proportion of the complex in the inactive phosphorylated form (Stansbie *et al.*, 1976). This effect persists during preparation of mitochondria, but no change in ^{45}Ca incorporation into the mitochondrial fraction was observed (Table 9, Expt. 4).

Increasing the concentration of phosphate in the incubation medium from 1 to 5mM doubled the incorporation of ^{45}Ca into the mitochondrial fraction of fat-cells, but little effect was observed on incorporation into the mitochondrial fraction of fat-pads. The activity of pyruvate dehydrogenase was also unchanged (Table 9, Expt. 5).

Transfer of pads previously incubated with ^{45}Ca for 30min to Ca^{2+}-free medium containing EGTA (5mM) resulted in the loss of about 45% of the ^{45}Ca incorporated in the mitochondrial fraction over 30min; however, this loss was not accompanied by any appreciable alteration in pyruvate dehydrogenase activity (Table 10).

Discussion

General comments

The approach used in this study to determine the size of total and exchangeable calcium pools in the mitochondrial fraction derived from intact cell preparations would appear to be one that could be applied to other tissues. The important features include: the fast separation of cells or tissue from the medium (by using, in this case, dinonyl phthalate); the use of ^{3}H-labelled markers to monitor for extracellular and extramitochondrial ^{45}Ca contamination in the mitochondrial fractions; the homogenization in sucrose medium containing high concentrations of EGTA and Ruthenium Red. In the present studies no appreciable uptake or loss of ^{45}Ca from the mitochondrial fractions during preparation could be detected. The use of the Polytron PT20 homogenizer in very short bursts as a speedy means of preparing intact mitochondria has also proved useful in the preparation of mitochondria from rat heart muscle (Kerbey *et al.*, 1976) and may have general applicability.

The importance of mitochondria in regulating cellular calcium metabolism has been examined, but by rather indirect techniques. The net uptake of Ca^{2+} by rat hepatocytes and Ehrlich ascites-tumour cells required the addition to the medium of substrates for mitochondrial oxidation (Kleineke & Stratman, 1974; Cittadini *et al.*, 1973). The time-course of ^{45}Ca uptake into kidney cells and kinetic analyses of ^{45}Ca-depletion curves have been interpreted in terms of two intracellular pools, a rapidly exchanging pool assumed to be cytoplasmic and a slowly exchanging pool that has been identified as intramitochondrial (Borle, 1972, 1973). The results of the present study on fat-cells and fat-pads are not compatible with such a model. The incorporation of ^{45}Ca into the mitochondrial fractions was extremely rapid and complete within a few minutes; thus there

Table 10. *Effects of incubation with EGTA on pyruvate dehydrogenase activity and on ^{45}Ca content of mitochondrial fraction of fat-pads in the presence of insulin*

Details were as given in Table 9 except that all fat-pads were incubated with $^{45}CaCl_2$ in the presence of insulin (5munits/ml) for 30 or 60min. Some groups of fat-pads were transferred after 30min to fresh medium containing no $CaCl_2$ and EGTA (5mM) and insulin (5munits/ml). Results are means±S.E.M. for six separate observations.

Incubation conditions	Activity ratio pyruvate dehydrogenase/glutamate dehydrogenase: Intact fat-pad	Activity ratio pyruvate dehydrogenase/glutamate dehydrogenase: Washed mitochondrial fraction	^{45}Ca incorporation into washed mitochondrial fraction (ng-atoms of medium Ca/unit of glutamate dehydrogenase)
$^{45}CaCl_2$ for 30min	0.17±0.016	0.32±0.037	3.87±0.226
$^{45}CaCl_2$ for 60min	0.26±0.022	0.37±0.051	3.96±0.412
$^{45}CaCl_2$ for 30min then Ca^{2+}-free medium+EGTA for 30min	0.20±0.023	0.31±0.038	2.21±0.315*

* $P<0.01$ versus control values.

appears to be a pool of calcium associated with mitochondria that is in very rapid isotopic exchange with the Ca^{2+} in the medium. This pool accounts for only a small fraction (about 20%) of the total calcium in the mitochondrial fraction from fat-pads and for about 50% of that in the mitochondrial fraction from fat-cells. Mitochondria in these studies appear to have two distinct pools of calcium, one that is in rapid isotopic exchange with the extracellular calcium and one that does not become labelled in the time-course of the present experiments and is perhaps insoluble forms of calcium phosphate. The addition of respiratory substrates, 2-oxoglutarate and malate, or succinate, increased incorporation into the mitochondrial fraction without appreciable alteration in incorporation into the supernatant fraction. Also, the addition of an uncoupler (dinitrophenol) produced the expected decrease in ^{45}Ca incorporation into the mitochondrial fraction in both fat-pads and fat-cells. The marked loss of ^{45}Ca from mitochondrial fractions after incubation of fat-pads or fat-cells in Ca^{2+}-free medium containing EGTA demonstrates that net transfer of calcium from this pool out of mitochondria within cells can occur.

Total ^{45}Ca incorporation into both fat-cells and fat-pads continued to increase when there was little or no increase in ^{45}Ca incorporation into the mitochondria. There thus seems to be an extramitochondrial pool of calcium into which ^{45}Ca is only slowly incorporated. This probably represents binding to sites in the cell membrane and endoplasmic reticulum and perhaps to extracellular sites in fat-pads. Hales *et al.* (1974) have reported appreciable deposits of calcium associated with the endoplasmic reticulum in fat-cells. Cytoplasmic Ca^{2+} could account for only a minute fraction of the total ^{45}Ca incorporation, assuming the concentration of cytoplasmic Ca^{2+} is less than $1\mu M$, as accepted for muscle and nerve tissue (Baker, 1970, 1972).

Surprisingly, Ruthenium Red, $NiCl_2$ and $MnCl_2$ all increased total ^{45}Ca incorporation into fat-cells, with the greatest increase in the supernatant fraction. It is possible that these compounds inhibit the membrane system involved in pumping Ca^{2+} out of fat-cells. Marked increases in ^{45}Ca incorporation into the supernatant fraction of fat-cells were also observed with ionophore A23187, high P_i concentrations and Li^+ replacement. The increase with ionophore A23187 is presumably because the compound enhances the inward flux of Ca^{2+} across the cell membrane down the concentration gradient, as has been observed with bovine epididymal sperm and rat erythrocytes (Reed & Lardy, 1972). The increase with high P_i concentrations has been observed with fat-cells (Martin *et al.*, 1975). In kidney cells the increase in the size of the slowly exchanging compartment by increasing extracellular phosphate has been used as indirect evidence that this represents a pool of calcium in the mitochondria (Borle, 1972, 1973); the results of the present study suggest that this pool is largely extramitochondrial. Finally, the increase in ^{45}Ca incorporation with Li^+ replacement has been observed with squid axon, and probably reflects enhanced Ca^{2+} influx and a decreased Na^+-dependent Ca^{2+} efflux (Baker, 1970, 1972). It should be noted that in all these instances a large increase in total ^{45}Ca incorporation into fat-cells was accompanied by no change or only modest increases in the mitochondrial ^{45}Ca incorporation. In other words, the size of the pool of calcium within mitochondria that exchanges with $^{45}Ca^{2+}$ in the medium is not related in a simple way to the amount of ^{45}Ca associated with extramitochondrial cell components.

The ^{45}Ca incorporation into fat-pad mitochondria showed marked differences to that into fat-cell mitochondria. This was reflected in a decreased total calcium content, a decrease in the percentage of total calcium that was exchangeable, and a decrease in mitochondrial ^{45}Ca as a proportion of the total ^{45}Ca incorporated into the fat-pad. These alterations in cellular calcium metabolism may be related to some effect of collagenase on the cell membrane, or may be a general phenomenon associated with isolated cell preparations due to an increase in the total surface area of the cell exposed to the medium. The possibility is raised that the calcium content of mitochondria in other isolated cell preparations (e.g. liver cells, kidney tubules) may be also much greater than *in vivo*.

Role of calcium in the regulation of pyruvate dehydrogenase activity

The present studies were initiated in an attempt to try and obtain evidence that insulin increases the activity of adipose-tissue pyruvate dehydrogenase by increasing the mitochondrial concentration of Ca^{2+}. However, increasing the ^{45}Ca incorporation into fat-cell mitochondria by 1.5–2-fold with high P_i concentrations, ionophore A23187 or Li^+ replacement or greatly decreasing the ^{45}Ca incorporation into fat-cell mitochondria by incubating with EGTA was without effect on the pyruvate dehydrogenase activity. Thus it appeared that exchangeable calcium in mitochondria of fat-cells may have little relevance in terms of regulating pyruvate dehydrogenase phosphate phosphatase and the ratio of inactive/active forms of pyruvate dehydrogenase.

On the other hand, it is tempting to ascribe the increased activity of pyruvate dehydrogenase in fat-cells and the smaller activation by insulin in comparison with fat-pads to the much greater amounts of calcium in the fat-cell mitochondria. The total

amount of calcium in the mitochondrial fraction from fat-pads (42.3 ng-atoms of calcium/unit of glutamate dehydrogenase) does approach the content of fat-cell mitochondria, in which pyruvate dehydrogenase phosphatase activity has been observed to be inactive after depletion of calcium with ionophore A23187 in the presence of EGTA (24 ng-atoms of calcium/unit of glutamate dehydrogenase; Severson *et al.*, 1974). The persistence of the effect of insulin on pyruvate dehydrogenase activity during preparation of mitochondrial fractions from fat-pads (Table 8) and subsequent incubation with respiratory substrates (Denton *et al.*, 1975) is consistent with alteration in a non-metabolizable effector of the pyruvate dehydrogenase system (such as Ca^{2+}) that is not metabolized by or lost from mitochondria under these conditions. However, other evidence obtained in the present studies indicates that the concentration of Ca^{2+} in fat-pad mitochondria is probably not sufficiently low to play a role in the regulation of pyruvate dehydrogenase activity. No changes in the size of the exchangeable pool of calcium in the mitochondrial fraction of fat-pads were found associated with the changes in pyruvate dehydrogenase activity observed with insulin or with Ruthenium Red in the presence of insulin; and moreover incubation with EGTA did not appreciably alter the activity of pyruvate dehydrogenase, although some 45% of the ^{45}Ca content of the mitochondrial fraction had been lost.

If the rapidly exchangeable pool of calcium in the mitochondrial fraction of fat-pads were all unbound and intramitochondrial, then the concentration of Ca^{2+} within mitochondria would exceed 1 mM. This is some three orders of magnitude greater than the concentration range that affects the activity of preparations of the phosphatase from fat-cells, heart muscle and kidney and the activity of the kinase from heart muscle (Denton *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974; Cooper *et al.*, 1974). It seems very probable that a proportion of the rapidly exchangeable pool of calcium is bound but rather unlikely that this proportion accounts for 99.9% of the pool. If Ca^{2+} is distributed across the mitochondrial inner membrane according to the membrane potential, then it can be calculated from the Nernst equation that the intramitochondrial concentration will be about 10 times the cytoplasmic concentration for either every 30 mV potential difference across the mitochondrial membrane, assuming that Ca^{2+} ions enter with a net positive charge of two (Rottenberg & Scarpa, 1974), or every 60 mV potential difference if Ca^{2+} ions enter by a process in which the net charge is effectively one (Rossi & Lehninger, 1963; Reynafarje & Lehninger, 1974). As estimates of this potential have been as high as 200 mV (Mitchell & Moyle, 1969; Nicholls, 1974), a concentration of Ca^{2+} within mitochondria orders of magnitude greater than 10 μM would be expected in the absence of any known mechanisms for pumping Ca^{2+} out of mitochondria.

On balance, then, we feel the conclusion must be drawn that under most conditions the intramitochondrial concentration of Ca^{2+} is likely to be too high to be important in the physiological regulation of pyruvate dehydrogenase in most tissues; one exception might be liver, because pyruvate dehydrogenase phosphatase in extracts of rat liver mitochondria has been found to be sensitive to concentrations of Ca^{2+} in the 0.1–1 mM range (Denton *et al.*, 1972). In particular, it seems unlikely that insulin exerts its effect on pyruvate dehydrogenase activity in rat adipose tissue by increasing the mitochondrial concentration of Ca^{2+}. The possibility remains, however, that some of the intracellular effects of adrenaline, including its effect on pyruvate dehydrogenase activity in adipose tissue, may involve a decrease in mitochondrial calcium content. Borle (1974) has reported that under certain conditions cyclic AMP can markedly diminish calcium accumulation by isolated mitochondria. The observed decrease in ^{45}Ca incorporation into the mitochondrial fraction of fat-pads incubated with adrenaline may be related to the increase in tissue cyclic AMP, but a number of alternative explanations could be given, including partial uncoupling of mitochondria leading to a lowering in membrane potential and in turn a smaller accumulation of Ca^{2+} within the mitochondria.

D. L. S. held a Research Fellowship of the Medical Research Council of Canada during these studies, which were also supported in part by grants from the Medical Research Council (U.K.), the British Diabetic Association and the British Insulin Manufacturers. We are also very grateful to Dr. J. Morris, Department of Anatomy, University of Bristol Medical School, for the electron-microscopic examination of mitochondrial fractions.

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