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The effect of insulin on plasma-membrane and mitochondrial-membrane potentials in isolated fat-cells

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1. A recently developed technique for the measurement of plasma-membrane and mitochondrial-membrane potentials in intact cells by using the distribution of $^{86}Rb^+$ and [^{3}H]methyltriphenylphosphonium$^+$ has enabled us to characterize a novel insulin effect on fat-cell mitochondria. For control cells the plasma-membrane and mitochondrial-membrane potentials were 75 mV and 152 mV respectively. Insulin (10 munits/ml) caused a 9 mV hyperpolarization of the plasma membrane and a 19 mV depolarization of the mitochondrial membrane. 2. The insulin-dependent mitochondrial depolarization was observed at physiological insulin concentrations (10 μunits/ml) and was apparent when the cells metabolized a wide variety of substrates. 3. Evidence from the uptake of the weak acid 5,5-dimethyloxazolidine-2,4-dione by fat-cells was interpreted as indicating that the mitochondrial pH gradient was increased by insulin. 4. Insulin alters the balance between the electrical and pH-gradient components that form the mitochondrial protonmotive force. A model is proposed.

In recent years a number of workers have proposed that an alteration in the cellular ion balance may play a central role in the mechanism of action of insulin. Zierler (1951) has shown that insulin produces a hyperpolarization of the plasma membrane in skeletal muscle. Clausen (1975) and Czech (1977) have put forward the view that insulin causes an increase in free Ca^{2+} concentrations in the cytoplasm and have suggested that this may be fundamental to the action of the hormone. This proposal is attractive, since many of the intracellular events that are affected by insulin are also affected by Ca^{2+}, and many insulin-mimetic agents would be expected to produce a rise in the cytoplasmic free Ca^{2+} concentration. However, an experimental demonstration of the elevation of the free Ca^{2+} concentrations in the cytoplasm has been difficult to achieve.

In the present study we have extended the method of Hoek *et al.* (1980) and Scott & Nicholls (1979, 1980) by using the distribution of $^{86}Rb^+$ and [^{3}H]TPMP$^+$ to measure the plasma-membrane and mitochondrial-membrane potentials of intact fat-cells. The method offers the possibility of examining the effect of insulin and other hormones on cellular ion balance.

Abbreviations used: $\Delta\psi_p$, plasma-membrane potential; $\Delta\psi_m$, mitochondrial-membrane potential; ΔpH_m, mitochondrial pH gradient; $\Delta\bar{\mu}_{H^+}$, mitochondrial proton electrochemical gradient (protonmotive force); TPMP$^+$, methyltriphenylphosphonium; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

Experimental

Materials

[^{3}H]Methyltriphenylphosphonium bromide (lot no. 1133-022) was bought from New England Nuclear Chemicals, Dreieich, West Germany. ^{86}RbCl, ^3H_2O, [^{3}H]inulin, [U-^{14}C]sucrose, 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione, [1-^{3}H]glucose, potassium [^{14}C]thiocyanate and sodium [U-^{14}C]-acetate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Valinomycin, ouabain, bovine serum albumin (fraction V), insulin and collagenase (type II) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. All other enzymes were from Boehringer Corp. (London), Lewes, East Sussex, U.K. Dinonyl phthalate was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Defatted albumin was prepared by chloroform extraction, and acetyl-CoA was prepared by the method of Simon & Shemin (1953). All other reagents were of analytical grade.

Preparation of fat-cells

Fat-cells from rat epididymal fat-pads were isolated by the method of Rodbell (1964) with minor

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modifications as described by Martin & Denton (1970). The rats used weighed 130–180g. As a routine the fat-cells were screened for the lipogenic response with insulin (10 munits/ml). Fat-cells (50–100 μl of cells/ml of medium) were incubated in a bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing half the stated Ca^{2+} concentration (1.27 mM), 1% (w/v) albumin and 1 mM-glucose and gassed with O_2/CO_2 (19:1). The cells were pre-incubated in a shaking water bath at 37°C for 40 min, then [1-^{3}H]glucose (0.5 μCi/ml) was added with or without insulin. After a further 40 min of incubation, 200 μl samples were taken and put in small polythene tubes containing 100 μl of dinonyl phthalate. The tubes were centrifuged on a Beckman Microfuge for 45 s to separate the cells from the medium (Gliemann *et al.*, 1972). The tubes were sliced through the oil layer, and the fat-cell cakes were placed in scintillation vials. The radioactivities of the samples were counted after addition of 6 ml of scintillation fluid (4 ml of xylene, 2 ml of Triton X-114 and 18 mg of 2,5-diphenyloxazole). A crude measure of the lipogenic effect is given by the ratio of radioactivities obtained with insulin-treated and control cells. Cells with less than a 4-fold response were rejected. This was the case in about 10% of the fat-cell preparations.

Preparation of fat-cell mitochondria

Mitochondria were prepared from fat-cells by the method of Martin & Denton (1970). Briefly, the cells were disrupted by vortex-mixing at 0°C in a medium containing 250 mM-sucrose, 10 mM-Tris/HCl buffer, pH 7.4, 2% (w/v) defatted albumin, 7.5 mM-glutathione (reduced) and 2 mM-EGTA. The fat-plug and cell debris were separated by centrifugation at 1000 **g** for 60 s at 0°C, and the mitochondria were then sedimented from the infranatant by centrifugation at 14000 **g** for 1 min. Finally, the mitochondria were washed once and stored at 0°C until use. The mitochondria were screened for a respiratory control ratio greater than 4 with succinate as substrate by using an oxygen electrode. Preparations of mitochondria with a respiratory control ratio less than 4 were rejected.

Determination of Rb^+, $TPMP^+$ and 5,5-dimethyloxazolidine-2,4-dione accumulation by fat-cells

Before any experiment to determine the acculullation of Rb^+, $TPMP^+$ or dimethyloxazolidinedione was performed, the fat-cells were pre-incubated for 40 min in the standard bicarbonate-buffered medium [containing 1% (w/v) albumin and 1 mM-glucose and gassed with O_2/CO_2 (19:1)]. Rb^+ and $TPMP^+$ uptakes were measured by a dual-label method. The incubation was started by adding 50 μM-^{86}RbCl (1.0 μCi/ml) and 0.3 μM-[^{3}H]$TPMP^+$ (1.0 μCi/ml) to a suspension of fat-cells (50–100 μl of cells/ml of medium) in the standard bicarbonate-buffered medium. After defined times 200 μl samples were taken and the cells were separated from the medium as described above. Samples of the medium and the fat-cell cakes were taken for scintillation counting of radioactivities in the Triton X-114/xylene-based scintillation fluid. The measured radioactivities were corrected for quenching by external standardization and cross-over as described by Grower & Bransome (1970). A parallel experiment was performed to determine the total water space and extracellular space of the fat-cell cake. Fat-cells were incubated for 3 min with ^3H_2O (4 μCi/ml) and in a separate vial with 50 μg of [^{3}H]inulin/ml (4 μCi/ml). The apparent spaces of the $^{86}Rb^+$ and [^{3}H]-$TPMP^+$ within the fat-cells were calculated after allowance for the extracellular space estimated from the [^{3}H]inulin radioactivity of the fat-cell cake. In a typical experiment with a suspension of 60 μl of cells/ml of medium and with 200 μl samples being taken, the following apparent spaces were obtained: ^3H_2O, 0.26 μl; [^{3}H]inulin, 0.06 μl; $^{86}Rb^+$, 3.3 μl; [^{3}H]$TPMP^+$, 29.4 μl. The accumulation ratios for the ions were then calculated by dividing the apparent spaces by the water-permeable inulin-impermeable space. In the typical experiment quoted the accumulation ratios are 16.2 and 147 for $^{86}Rb^+$ and [^{3}H]$TPMP^+$ respectively.

The accumulation of dimethyloxazolidinedione was measured in a single-labelled incubation with 13 μM-dimethyl[^{14}C]oxazolidinedione (0.75 μCi/ml). The apparent space and the accumulation ratio of the weak acid in the fat-cells were determined in an analogous manner to the procedure described for $^{86}Rb^+$ and [^{3}H]$TPMP^+$.

Determination of Rb^+, $TPMP^+$, 5,5-dimethyloxazolidine-2,4-dione and acetate accumulation by isolated mitochondria

The accumulation of Rb^+, $TPMP^+$, dimethyloxazolidinedione and acetate by isolated mitochondria was investigated by a centrifugation method. Mitochondria (approx. 2 mg of protein/ml) were incubated for 5 min in a medium containing 10 mM-Tes/NaOH buffer, pH 7.0, 160 mM-NaCl, 2 mM-succinate, 2 mM-EGTA and 1% (w/v) defatted bovine serum albumin at 30°C. For membrane-potential determinations three different incubations were performed in the medium containing added radioisotopically labelled compounds: (1) 50 μM-^{86}RbCl (0.5 μCi/ml) and ^3H_2O (2 μCi/ml); (2) 0.3 μM-[^{3}H]$TPMP^+$ (1 μCi/ml) and 40 μM-[U-^{14}C]sucrose (0.25 μCi/ml); (3) 40 μM-[U-^{14}C]sucrose (0.25 μCi/ml) and ^3H_2O (2 μCi/ml). Valinomycin (0.5 μM) and KCl were added as stated in Fig. 4. To measure the pH gradient 160 mM-KCl was used rather than NaCl, and again three incubations were made: (1) 13 μM-dimethyl[^{14}C]oxazolidinedione (0.75 μCi/ml)

and ^3H_2O (2 μCi/ml); (2) 20 μM-[^{14}C]acetate (0.75 μCi/ml) and ^3H_2O (2 μCi/ml); (3) 40 μM-[U-^{14}C]sucrose (0.25 μCi/ml) and ^3H_2O (2 μCi/ml).

The incubations were stopped by rapid centrifugation in a Beckman Microfuge to pellet the mitochondria. The supernatant was decanted and samples were taken for scintillation counting of radioactivities. The pellet was disrupted by adding 100 μl of 100 mM-potassium phosphate buffer, pH 7.4, and freezing, thawing and vortex-mixing the pellet three times. Samples of the pellet extract were taken for scintillation counting of radioactivities. To calculate the accumulation of the radioisotopic labels, the matrix space of the mitochondria was estimated as the sucrose-impermeable water space of the pellet from incubation (3). The intramitochondrial apparent space of [^{3}H]TPMP$^+$ was calculated by subtracting the [U-^{14}C]sucrose space of the pellet in the same incubation. A similar calculation was performed for the intramitochondrial apparent spaces of Rb^+, dimethyloxazolidinedione and acetate by subtracting the total water space that was determined in the same incubation and adding this to the matrix space determined in incubation (3). Finally, the accumulation is given by the intramitochondrial apparent space of the radioisotopically labelled material divided by the mitochondrial matrix space. In a typical experiment the total water space and sucrose space of the mitochondrial pellet were 12 μl and 11 μl respectively.

Enzyme assays

Pyruvate carboxylase, glutamate dehydrogenase and citrate synthase were extracted from fat-cells and mitochondria and were assayed as described by Martin & Denton (1970), except that the assays were performed at 20°C instead of 25°C.

Results

Intramitochondrial water space as a proportion of fat-cell water space

In order to quantify the accumulation of TPMP$^+$ into the mitochondria, it is necessary to measure the mitochondrial volume. Morphometric studies of electron micrographs have yielded much information for some cell types such as hepatocytes (Weibel *et al.*, 1969). The application of these techniques to fat-cells is difficult because of their unique structure: a thin layer of cytoplasm covering a large fat globule. The problems of these studies are reviewed by Weibel (1969). Most of the present knowledge of the structure of fat-cells is qualitative and not quantitative (Slavin, 1972). It is therefore necessary to use a different approach.

In the present study, the intramitochondrial volume as a proportion of the cytoplasmic volume was measured by using assays for mitochondrial enzymes. Pyruvate carboxylase, glutamate dehydrogenase and citrate synthase are mitochondrial enzymes (Martin & Denton, 1970). These enzymes were assayed and their activities determined for a given amount of fat-cells and also for the mitochondria isolated from the fat-cell preparation. The cytoplasmic volume of the fat-cells was measured as the inulin-impermeable water space by the dinonyl phthalate separation method described in the Experimental section. The matrix space of the mitochondria was measured as the sucrose-impermeable water space by a centrifugation procedure also described in the Experimental section (Table 1).

The results can be compared with other published data by introducing a number of conversion factors. It was found that 100 μl of packed cells on average contained 1.9 μl of cytoplasm (inulin-impermeable water space). This agrees well with the value reported by Gliemann *et al.* (1972). A 1 ml volume of packed cells is equivalent to 0.7 g of lipid (Dole & Meinertz, 1960) and will therefore approximate fairly closely to 0.7 g dry weight. The activities of the enzymes can be calculated as 0.27, 0.41 and 0.81 unit (μmol/min)/g dry wt. of fat-cells for glutamate dehydrogenase, pyruvate carboxylase and citrate synthase respectively at 20°C. These values are somewhat larger than those reported by Martin & Denton (1970), but the relative activities of the three enzymes are the same. In a more recent paper (Coore *et al.*, 1971) the same group reported a value for glutamate dehydrogenase of 1 unit/g dry wt. of cells at 30°C. The value for glutamate dehydrogenase reported in the present paper is about half this, after allowance for the difference in temperature, and therefore lies within the range of values in these previous reports. If it is assumed that the mitochondrial volume determined *in vitro* is the same as that in the intact cell, it can be calculated that the mitochondria account for 1% of the intracellular water. There is a close agreement between the results for the three enzymes.

The percentage volume of the cytoplasm that can be accounted for by the mitochondria can also be assessed by the method of Scott & Nicholls (1980) on the basis of the yield of isolated mitochondria. Martin & Denton (1970) isolated 1 mg of mitochondrial protein from 1 g dry wt. of epididymal fat-pad. A 1 ml volume of packed fat-cells contains 0.7 g of extractable lipid (Dole & Meinertz, 1960) and has 19 μl of cytoplasm (the present paper). Therefore 1 mg of mitochondria can be isolated from 27 μl of cytoplasm. Fat-cell mitochondria have a matrix space of 0.6 μl/mg of mitochondrial protein (Table 1). Therefore the mitochondria can be calculated to account for 2% of the cytoplasmic volume. This estimate will be affected by the recovery and purity of the mitochondria isolated by Martin & Denton (1970). However, the estimate is

Table 1. *Mitochondrial volume as a proportion of cytoplasmic volume*

The results are expressed as means ± S.E.M., with the numbers of determinations performed in parentheses. The assays of enzyme activities and volumes were performed in parallel, and calculations are based on the same arbitrary quantity of fat-cells or mitochondria. Three determinations were performed on each fat-cell and mitochondrial preparation. The enzymes were extracted and assayed as described in the Experimental section. The cytoplasmic volume of fat-cells was defined as the inulin-impermeable water space of a cell suspension. Two types of incubation of fat-cells were made in the standard bicarbonate-buffered medium containing albumin (10mg/ml) and glucose (1mM). In the former 50μg of [^{3}H]inulin/ml (4μCi/ml) and in the latter ^3H_2O (4μCi/ml) was added. Spaces were determined as described in the Experimental section. For isolated mitochondria the matrix volume was defined as the sucrose-impermeable water space. Mitochondria were incubated in a medium containing 250mM-sucrose, 8mM-succinate, 2% (w/v) bovine serum albumin, 10mM-Tris/HCl buffer, pH7.4, and 2mM-EGTA. In a double-label experiment ^3H_2O (2μCi/ml) and [U-^{14}C]sucrose (0.25μCi/ml) were added. Spaces were measured as described in the Experimental section. Protein was determined as described by Lowry *et al.* (1951) after the mitochondria had been washed five times in albumin-free medium. It was found that the mitochondrial matrix space was 0.6 ± 0.05 μl/mg of protein (mean ± S.E.M. for four different preparations).

	Fat-cells		Mitochondria		Calculated mitochondrial volume (% of cytoplasmic volume)
Enzyme	Enzyme activity (munits)	Cytoplasmic volume (μl)	Enzyme activity (munits)	Matrix volume (μl)	
Glutamate dehydrogenase	1.9 ± 0.02 (12)	0.19 ± 0.005 (12)	730 ± 10 (12)	0.82 ± 0.02 (12)	1.1 ± 0.06
Pyruvate carboxylase	3.8 ± 0.03 (3)	0.21 ± 0.008 (3)	1740 ± 30 (3)	0.89 ± 0.04 (3)	0.92 ± 0.04
Citrate synthase	5.7 ± 0.02 (12)	0.19 ± 0.005 (12)	2390 ± 50 (12)	0.82 ± 0.02 (12)	1.03 ± 0.04

of the same order of size as that determined on the basis of enzyme activities.

The mitochondrial matrix space was determined in a sucrose-based medium. A possible cause of systematic error in the measurement of cell water space that can be accounted for by the mitochondria is that the mitochondria change size during isolation. The calculated accumulation of TPMP$^+$ into the mitochondria in whole-cell experiments is subject to this systematic error. Consequently a precise value for the mitochondrial membrane potential cannot be determined. However, the technique of Scott & Nicholls (1980) does allow changes in the mitochondrial membrane potentials of fat-cells *in situ* to be observed. The logarithmic nature of ion accumulation by a membrane potential causes the apparent mitochondrial membrane potential to change by 18mV for each 2-fold error in the determination of mitochondrial volume. Thus, although the uncertainty in the mitochondrial volume introduces a systematic error into the calculated mitochondrial membrane potential, the systematic error does not affect the calculation of changes in mitochondrial membrane potential. In the accumulation studies using isolated mitochondria in NaCl or KCl media, the intramitochondrial volume was determined in each experiment.

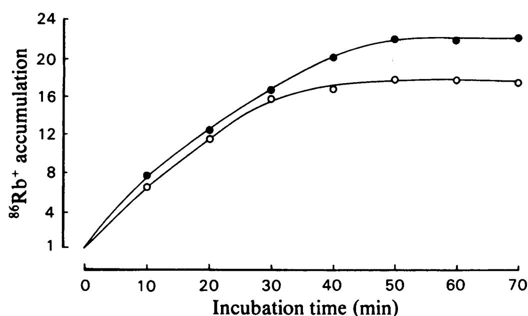

Fig. 1. *Accumulation of Rb$^+$ by intact fat-cells*
Isolated fat-cells were preincubated for 40min in the standard bicarbonate-buffered medium containing glucose (1mM) and albumin (10mg/ml) at a concentration of 50–100μl of packed cells/ml. The incubation was started by the addition of 50μM-^{86}RbCl (1μCi/ml). After defined times 200μl samples were taken and the accumulation of the $^{86}Rb^+$ by the fat-cells was determined as described in the Experimental section. The cytoplasmic volume of the fat-cells was defined as the inulin-impermeable water space of the fat-cells and was assessed as described in the Experimental section. Symbols: ○, control cells; ●, insulin (10 munits/ml)-treated cells. Each point is the mean for four separate experiments performed on different preparations of fat-cells.

Accumulation of Rb$^+$ by fat-cells

The plasma membrane of fat-cells has a low permeability to ions in relation to the total cellular content of these ions. It was found that the time for equilibration of $^{86}Rb^+$ was 50min (Fig. 1). Consequently only steady-state accumulation ratios can be determined for these cells, except under con-

ditions of enhanced permeability (such as the presence of valinomycin). The $^{86}Rb^+$ accumulation will give an estimate of the K^+ diffusion potential across the plasma membrane. Scott & Nicholls (1979, 1980) used the assumption that the K^+ diffusion potential was a good estimate of the plasma-membrane potential of synaptosomes. For fat-cells this assumption may not be so good, since it has been suggested that the K^+ accumulation is not in equilibrium with the plasma-membrane potential (Beigelman & Hollander, 1964*a*). The $^{86}Rb^+$ diffusion potential may overestimate the plasma-membrane potential. The $^{86}Rb^+$ diffusion potential can be calculated by the Nernst equation:

$$^{86}\mathrm{Rb}^+ \text{ diffusion potential} = 61.5\log\left(\frac{[\mathrm{Rb}^+]_c}{[\mathrm{Rb}^+]_e}\right) \quad (1)$$

The subscripts c and e refer to the cytoplasmic and extracellular compartments respectively.

In 36 determinations the equilibrium $^{86}Rb^+$ diffusion potential was 75 ± 1.1 mV in control cells and 84 ± 1.8 mV in cells treated with insulin (10 munits/ml). Thus insulin causes a hyperpolarization of the plasma membrane.

Addition of 150 mM-KCl and 0.5 μM-valinomycin caused the $^{86}Rb^+$ accumulation of pre-loaded cells to fall to 1.3 on average in four experiments (Table 2). This demonstrates that potential-independent binding of $^{86}Rb^+$ is low.

There is a wide variation in the reported values for the resting plasma-membrane potentials of fat-cells determined by micro-electrodes. Beigelman & Hollander (1962, 1963, 1964*b*, 1965) have reported the results of many micro-electrode experiments, and mean plasma-membrane potentials ($\Delta\psi_p$) of 50.8 mV, 57 mV, 37.7 mV and 30.7 mV respectively can be calculated from their papers. The variation appears to depend on the number of very low $\Delta\psi_p$ values (20 mV) that are recorded. Because of the problems of micro-electrode insertion, it is probable that the higher $\Delta\psi_p$ results are more accurate. For cells treated with insulin (1 munit/ml) hyperpolarizations of 30.3 mV, 28 mV, 14.7 mV and 22 mV are reported in the papers by Beigelman & Hollander (1962, 1963, 1964*b*, 1965) respectively. Thus mean $\Delta\psi_p$ values of 44.1 mV and 67.9 mV can be calculated for control and insulin-treated fat-cells respectively. The true $\Delta\psi_p$ may be overestimated by the $^{86}Rb^+$ diffusion potential and probably underestimated by the micro-electrode technique. An attempt was made to measure the plasma-membrane potential by the exclusion of [^{14}C]thiocyanate. However, the scatter of the results made this estimate unreliable.

Accumulation of TPMP⁺ by fat-cells

The permeability of membranes to $TPMP^+$ was increased by Scott & Nicholls (1980) by the addition of 3 μM-tetraphenylboron. However, since in fat-cells the equilibration time for Rb^+ was 50 min and for $TPMP^+$ alone was 40 min (Fig. 2), no advantage can

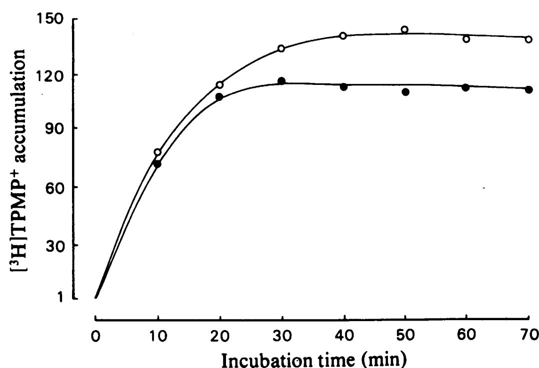

Fig. 2. *Accumulation of TPMP⁺ by intact fat-cells*
The accumulation of $TPMP^+$ was measured by the method described in the legend to Fig. 1, except that 0.3 μM-[^{3}H]TPMP$^+$ (1 μCi/ml) was used rather than 50 μM-^{86}RbCl (1 μCi/ml). Symbols: ○, control cells; ●, insulin (10 munits/ml)-treated cells. Each point is the mean for four separate experiments performed on different preparations of fat-cells.

Table 2. *Accumulation of Rb⁺ and TPMP⁺ by fat-cells*
$^{86}Rb^+$ and [^{3}H]TPMP$^+$ accumulation ratios were measured as described in the Experimental section. Additions when made were at the same time as the incubations with radioisotopically labelled material were started. All incubations were for 40 min. The high-K^+ medium was made iso-osmolar with the control medium by substituting K^+ for Na^+. The results are expressed as means ± S.E.M., with the numbers of separate experiments performed on different preparations of fat-cells given in parentheses.

Experiment	$^{86}Rb^+$ accumulation	[^{3}H]TPMP$^+$ accumulation
Control	16.3 ± 0.7 (36)	146.4 ± 3.7 (36)
Insulin (10 munits/ml)	22.8 ± 1.5 (36)	119.7 ± 3.3 (36)
Tetraphenylboron (3 μM)	16.7 ± 1.4 (4)	149.3 ± 8.2 (4)
Valinomycin (0.5 μM)	15.3 ± 1.1 (4)	25.4 ± 2.1 (4)
Insulin (10 munits/ml) + valinomycin (0.5 μM)	14.4 ± 1.4 (4)	24.2 ± 2.5 (4)
KCl (150 mM) + valinomycin (0.5 μM)	1.3 ± 0.2 (4)	2.5 ± 0.3 (4)

be gained from the use of tetraphenylboron to accelerate the $TPMP^+$ equilibration. Therefore it was not used. The accumulation ratio of $TPMP^+$ was not significantly different in presence and in the absence of tetraphenylboron (Table 2). The variation of the accumulation of $TPMP^+$ with the concentration of the lipophilic ion is presented in Fig. 3. Highest accumulation is given by 0.3 μM-$TPMP^+$. The variation of $TPMP^+$ accumulation with concentration is very similar to that observed by Scott & Nicholls (1980).

Since $TPMP^+$ is lipophilic, the uptake of the ion into the fat was investigated. After the cells had been loaded with $TPMP^+$, they were broken by being drawn through a fine needle five times, and the fat was separated from the aqueous phase by centrifuging through dinonyl phthalate as described in the Experimental section. It was found that the water space, inulin space and $TPMP^+$ space were not significantly different, indicating that little of the $TPMP^+$ is taken up into the fat.

Potential-independent binding of the $TPMP^+$ in the cell preparation was investigated by adding 0.5 μM-valinomycin and 150 mM-KCl to cells pre-loaded with $TPMP^+$. This treatment depolarizes both the mitochondrial membrane and the plasma membrane, as demonstrated by the $^{86}Rb^+$ accumulation ratio of 1.3 under these conditions. $TPMP^+$ was found to have an accumulation ratio of 2.5 (four experiments), thus indicating a low potential-independent binding of $TPMP^+$.

In 36 experiments it was found that the $TPMP^+$ accumulation ratio was 146.4 ± 3.7 for control cells and was decreased to 119.7 ± 3.3 for cells treated with 10 munits of insulin/ml (mean ± S.E.M.) at a $TPMP^+$ concentration of 0.3 μM. The effect of insulin was also observed at 1.0 μM-$TPMP^+$. This accumulation compares with the 184-fold accumulation of $TPMP^+$ reported by Hoek *et al.* (1980) for isolated hepatocytes. The results of the studies of fat-cell Rb^+ and $TPMP^+$ accumulation are summarized in Table 2.

The far greater accumulation of $TPMP^+$ than of Rb^+ by fat-cells that is observed may be due to two reasons. First, the $TPMP^+$ may have a low activity

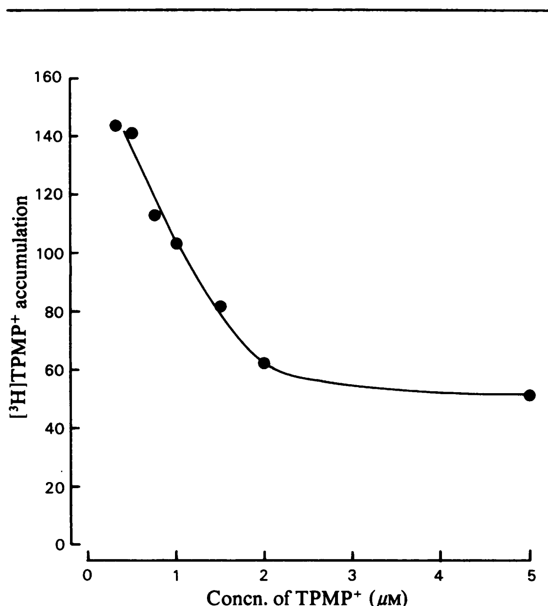

Fig. 3. *Effect of varying the concentration of $TPMP^+$ on the accumulation of $TPMP^+$ by fat-cells*
Packed fat-cells (50–100 μl/ml of incubation medium) were incubated for 40 min with [^{3}H]-$TPMP^+$ (1 μCi/ml). Then 200 μl samples were taken and the accumulation was measured as described in the Experimental section. The concentration of $TPMP^+$ was varied between 0.3 μM and 5 μM, and the results presented represent the means for four separate experiments performed on different preparations of fat-cells.

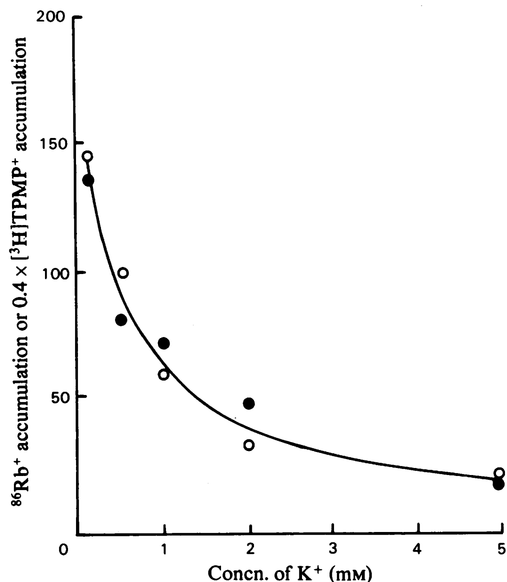

Fig. 4. *Effect of K^+ and valinomycin on the accumulation of Rb^+ and $TPMP^+$ by isolated fat-cell mitochondria*
Mitochondrial (2 mg/ml) were incubated for 5 min at 30°C in the medium described in the Experimental section with added KCl from 0 to 5 mM. The accumulations of $^{86}Rb^+$ and [^{3}H]$TPMP^+$ by the mitochondria were measured by a centrifugation method described in the Experimental section. Symbols: ○, $^{86}Rb^+$ accumulation ratio; ●. $TPMP^+$ accumulation ratio assuming an activity coefficient of 0.4 in the mitochondrial matrix relative to that in the external medium. The results are expressed as means for four experiments performed on different preparations of fat-cell mitochondria.

coefficient in the cell owing to binding to a component of the cytoplasm. Secondly, the $TPMP^+$ might be additionally accumulated by an organelle within the cytosol possessing a high membrane potential, such as a mitochondrion. To resolve these two factors, the effect of valinomycin on fat-cells was investigated.

The high cytoplasmic K^+ concentration will cause the depolarization of organelles in the presence of valinomycin, including the mitochondria (Mitchell & Moyle, 1969). If $TPMP^+$ were to be accumulated within a cellular organelle, the addition of valinomycin ought to cause a decrease in the accumulation of $TPMP^+$ by the fat-cells, and this is in fact what is observed (Table 2). Under these conditions the plasma-membrane potential will be fixed by the K^+ diffusion potential, and the $^{86}Rb^+$ accumulation by the fat-cells is only slightly decreased (Table 2). This demonstrates that there is little accumulation of $^{86}Rb^+$ into cellular organelles. In the presence of valinomycin it would be expected that the accumulation of Rb^+ and $TPMP^+$ would be the same. However, it was found that the accumulation of $TPMP^+$ was always greater than that of $^{86}Rb^+$. Similar results were obtained by Scott & Nicholls (1980) with synaptosomes. They suggested that the difference was due to a lower activity of the $TPMP^+$ in the cytoplasm relative to the external medium. If the Rb^+ activity is assumed to be the same in both compartments, the activity coefficient for the $TPMP^+$ in the cytoplasm relative to the medium can be calculated. In four experiments the $TPMP^+$ was accumulated 1.7 times more than the Rb^+. The relative activity coefficient (a_c) is 0.6. This is the same as that determined by Scott & Nicholls (1980).

The activity coefficient of $TPMP^+$ in isolated mitochondria was assessed by the method of Scott & Nicholls (1980). Isolated fat-cell mitochondria were incubated with a variety of K^+ concentrations in the presence of 0.5μM-valinomycin, and the Rb^+ and $TPMP^+$ accumulation ratios were measured. The results are shown in Fig. 4. There is a good fit between the two isotope-label distributions if it is assumed that the activity of the $TPMP^+$ in the mitochondrion is 0.4 of that in the external medium. This is the same as the value obtained for guinea-pig cerebral-cortical mitochondria by Scott & Nicholls (1980). No difference in the Rb^+ and $TPMP^+$ uptakes was found between control mitochondria and mitochondria isolated from fat-cells that had been pretreated with 10 munits of insulin/ml for 40 min (four experiments).

Calculation of the mitochondrial-membrane potential

Accumulation of $TPMP^+$ by organelles will only be a significant part of the total accumulation by the cell if the organelle has a high membrane potential, because of the logarithmic nature of the Nernst equation. The mitochondria are the obvious candidates for the high accumulation of $TPMP^+$ by fat-cells. Scott & Nicholls (1980) used the assumption that all the cellular $TPMP^+$ accumulation that could not be accounted for by the plasma-membrane potential was accumulated by the mitochondria. Thus the concentration gradient of $TPMP^+$ across the mitochondrial membrane can be calculated. The Nernst equation will then give the mitochondrial membrane potential, $\Delta\psi_m$ (Scott & Nicholls, 1980):

$$\Delta\psi_m = 61.5 \log \left\{ \frac{V_c a_m}{V_m} \cdot \left[\frac{[Rb^+]_e}{[Rb^+]_c} \cdot \frac{[TPMP^+]_s}{[TPMP^+]_e} \cdot \frac{1}{a_e} - \frac{1}{a_c} \right] \right\} \quad (2)$$

where the subscripts m, c, e and s refers to mitochondria, cytoplasm, medium and the whole cell respectively, a is the $TPMP^+$ relative activity coefficient and V is the volume. Substituting the estimated values for $a_m = 0.4$, $a_c = 0.6$, $a_e = 1$ and $V_m/V_c = 0.01$ into eqn. (2) gives mitochondrial-membrane potentials of 152 ± 0.6 mV and 133 ± 1.6 mV for control and insulin-treated fat-cells respectively (means ± S.E.M.).

Since the calculation assumes that the $^{86}Rb^+$ diffusion potential is a good estimate of the plasma-membrane potential, this may lead to an error in the calculated $\Delta\psi_m$. The mitochondrial-membrane potential can therefore be re-calculated on the basis of the micro-electrode measurements made by Beigelman & Hollander (1962, 1963, 1964*b*, 1965). As discussed above, the $^{86}Rb^+$ diffusion potential probably overestimates $\Delta\psi_p$ and will therefore cause an underestimation of $\Delta\psi_m$. The micro-electrode data will underestimate $\Delta\psi_p$ and consequently overestimate $\Delta\psi_m$. By using the mean values of the micro-electrode-determined plasma-membrane potentials, 44.1 mV and 67.9 mV, the mitochondrial-membrane potentials can be calculated as 180 mV and 148 mV for control and insulin-treated fat-cells respectively. These results are summarized in Table 3. The insulin-dependent depolarizations of the mitochondrial-membrane potentials are 19 mV and 32 mV when calculated from the results of $^{86}Rb^+$ accumulation and micro-electrode experiments respectively.

The size of the calculated mitochondrial-membrane potential is dependent on the estimation used for the proportion of the cytoplasmic volume that can be accounted for by the mitochondria by

using the two estimates for the plasma-membrane potential (which are based on $^{86}Rb^+$ distribution and the use of micro-electrodes); the $\Delta\psi_m$ can be calculated as being between 180mV and 152mV, if one assumes that the percentage mitochondrial volume is 1% (Table 3). A systematic error in the determination of the mitochondrial volume will result in an 18mV change in the calculated $\Delta\psi_m$ for each 2-fold error. Thus if the mitochondrial volume were 2%, the $\Delta\psi_m$ would be calculated as being between 162mV and 134mV. However, the decrease in the calculated $\Delta\psi_m$ when insulin is added is independent of the assessment of mitochondrial volume.

There are two alternative ways of interpreting the decline in $TPMP^+$ accumulation by isolated cells in the presence of insulin. First, the mitochondria may change size when fat-cells are treated with insulin. To account for the data, the mitochondria would have to shrink to half their original size when the cells are treated with insulin in order to maintain the same $\Delta\psi_m$ as control cells. Such large decreases in mitochondrial volume appear to be unlikely. Secondly, the change in apparent accumulation may be due to an insulin-dependent change of the activity coefficient of the $TPMP^+$. There are two possibilities: an increase of the activity coefficient of the $TPMP^+$ in the cells or a decrease of that in the medium would cause a decrease in the apparent accumulation of $TPMP^+$. Such changes caused by insulin may occur because of the profound effect of insulin on fat-cell metabolism. For example, the concentration of free fatty acids in the insulin-treated cells would be expected to be much lower than that in the control cells. These large changes in metabolism may affect the $TPMP^+$ activity coefficient. To decrease the activity coefficient of $TPMP^+$ in the medium, the $TPMP^+$ would be expected to bind to a component of the medium. The simple salts and glucose contained in the medium are not likely to bind to the $TPMP^+$, but it is possible that the albumin is responsible. It was found that the binding of $TPMP^+$ to albumin (10mg/ml) in bicarbonate-buffered medium was not detectable by equilibrium dialysis. If insulin increases the activity coefficient of the $TPMP^+$ in the fat-cell cytoplasm, the $TPMP^+$ must bind to some component of the control cells. It has been demonstrated that potential-independent binding of the $TPMP^+$ is low in control cells, by the addition of 0.5 μM-valinomycin and 150mM-KCl. A diminution in this binding would not account for the insulin-dependent decrease in apparent $TPMP^+$ accumulation. To test whether insulin causes the proposed changes in activity coefficient, fat-cells were incubated with valinomycin (0.5 μM), 50 μM-^{86}RbCl (1.0 μCi/ml) and 0.3 μM-[^{3}H]$TPMP^+$ (1.0 μCi/ml) in the presence and in the absence of insulin (Table 2). As discussed above, the ratio of apparent $TPMP^+$ accumulation to Rb^+ accumulation can be used under the conditions of the incubation as a measure of the relative activity coefficient of the $TPMP^+$ in the cytoplasm relative to the medium. To account for the insulin effect on the apparent $TPMP^+$ accumulation by fat-cells, the relative activity coefficient of the $TPMP^+$ in the cytoplasm should decline in the presence of insulin. The method does not differentiate between an insulin-dependent increase in the activity coefficient in the cytoplasm or a decrease in the medium. It was found that there was no significant difference between the relative activity coefficient of $TPMP^+$ in the cytoplasm in the presence or in the absence of insulin.

Determination of the mitochondrial pH gradient

Under the conditions of a depolarization of the mitochondrial membrane potential, the mitochondrial proton electrochemical potential gradient ($\Delta\bar{\mu}_{H^+}$) may be diminished or alternatively may be maintained by an increase in the pH gradient. To investigate these two possibilities, the accumulation

Table 3. *Calculated mitochondrial-membrane potentials*

The mitochondrial-membrane potential was calculated as described in the Results section by using two estimates for the plasma-membrane potential. Method I estimates $\Delta\psi_p$ as the K^+ diffusion potential measured by the $^{86}Rb^+$ distribution. Method II estimates $\Delta\psi_p$ from the results of micro-electrode experiments by Beigelmann & Hollander (1962, 1963, 1964*b*, 1965). The results calculated by Method I are expressed as means ± S.E.M., with the numbers of fat-cell preparations used in parentheses. The calculation assumes that the mitochondria account for 1% of the cytoplasmic volume. If there is an error in this volume, the calculated $\Delta\psi_m$ will change by 18mV for each 2-fold error.

	Calculated mitochondrial-membrane potentials (mV)		
	Control cells	Insulin (10 munits/ml)-treated cells	Insulin-dependent depolarization
Method I	152 ± 0.6 (36)	133 ± 1.6 (36)	19 ± 1.7
Method II	180	148	32

Table 4. *Accumulation of 5,5-dimethyloxazolidine-2,4-dione by fat-cells*
Fat-cells were incubated in the standard bicarbonate-buffered medium containing glucose (1 mM) and bovine serum albumin (10 mg/ml) with 13 μM-dimethyl[^{14}C]oxazolidinedione (0.75 μCi/ml). The inulin space and water space were determined in separate incubations. In the former 50 μg of [^{3}H]inulin (4 μCi/ml) and in the latter ^3H_2O (4 μCi/ml) was added. Broken fat-cells were made by passing a suspension of fat-cells through a hypodermic syringe five times. All spaces were determined as described in the Experimental section, and the accumulation of dimethyloxazolidinedione was calculated after allowance for the dimethyloxazolidinedione bound to the fat cake of broken cells as described in the Results section. The results are expressed as means ± S.E.M. for four different fat-cell preparations. Abbreviation: DMO, 5,5-dimethyloxazolidine-2,4-dione.

	Intact cells		Broken cells	
Experiment	Water space − inulin space (μl)	DMO space − inulin space (μl)	DMO space − water space (μl)	Calculated accumulation
Control	0.18 ± 0.01	0.247 ± 0.01	0.04 ± 0.005	1.15 ± 0.10
Insulin	0.18 ± 0.01	0.321 ± 0.02	0.04 ± 0.005	1.56 ± 0.11

of dimethyloxazolidinedione by fat-cells was measured.

About 20% of the dimethyloxazolidinedione in intact control cells was found to be associated with the fat cake from broken cells. The amount of dimethyloxazolidinedione bound to the fat was independent of insulin treatment. It is assumed that this dimethyloxazolidinedione is either bound to or dissolved in the cell fat globule. A cytoplasmic accumulation ratio of dimethyloxazolidinedione can be calculated by correcting the total accumulation of dimethyloxazolidinedione by that bound to the fat cake from broken cells. The data are presented in Table 4. In four separate experiments the dimethyloxazolidinedione accumulation ratios were found to be 1.15 ± 0.10 and 1.56 ± 0.11 for control and insulin (10 munits/ml)-treated cells respectively (means ± S.E.M.). A similar increase in dimethyloxazolidinedione accumulation caused by insulin (250 munits/ml) was reported by Moore (1979) for frog skeletal muscle.

Moore (1979) interpreted this result as indicating that insulin caused an alkalinization of the frog muscle. This was confirmed by Gupta & Moore (1980) by ^{31}P n.m.r. However, the distribution of dimethyloxazolidinedione will be affected by the pH gradients of both the plasma and the mitochondrial membranes (Waddell & Butler, 1959; Addanki *et al.*, 1968). Therefore the dimethyloxazolidinedione accumulation will be a function of the pH of the cytoplasmic and mitochondrial compartments of the intact cell. The derivation of an equation (eqn. 3) describing the mitochondrial pH is given in the Appendix:

$$pH_m = pK_a + \log\left[\left(\frac{V_s}{V_m}\right)\cdot\left(\frac{[DMO]_s}{[DMO]_e}\right)\cdot\left(1 + 10^{(pH_e - pK_a)}\right) - \frac{V_c}{V_m}\left(1 + 10^{(pH_c - pK_a)}\right) - 1\right] \quad (3)$$

where DMO represents 5,5-dimethyloxazolidine-2,4-dione. This equation assumes that there is no significant accumulation of the dimethyloxazolidinedione into any cellular compartment other than the mitochondria. This assumption obviously limits the validity of the equation applied to cells. There are two unknowns in eqn. (3), pH_m and pH_c, so that a value for the pH_m cannot be calculated. Deutsch *et al.* (1979) have calculated from weak acid accumulation in mouse neuroblastoma cells that the maximum mitochondrial pH gradient, ΔpH_m, is 1.2 units. In their experiments pH_c was assessed by using micro-electrodes and was shown to be within 0.1 pH unit of the external medium. Hoek *et al.* (1980) investigated the distribution of dimethyloxazolidinedione in isolated hepatocytes by a disruption technique. The accumulation ratio of dimethyloxazolidinedione was 0.80, and it was calculated that the cytoplasmic pH was 7.00 and that the mitochondrial pH gradient was 0.97. In the present study a bicarbonate buffer was used, so that the pH_c is probably near to the pH of the medium, 7.4. The variation in calculated ΔpH_m with pH_c for control and insulin-treated cells can be determined from eqn. (3). Over the range of pH_c from 7.1 to 7.6, with pH of the medium set at 7.4, the calculated ΔpH_m of insulin-treated cells is always higher (Fig. 5).

Errors in the calculated mitochondrial volume have a small effect on the calculated mitochondrial-membrane potential because of the logarithmic nature of the ion distribution. However, the mitochondrial pH gradient is low, so that an error in the mitochondrial volume will have a large effect on the calculated ΔpH_m. Therefore the size of the ΔpH_m presented in Fig. 5 is subject to a possible systematic error. The calculation assumes that the mitochondrial volume is not changed by insulin. To account

for the data, the mitochondria would have to swell with insulin, whereas to account for the decreased apparent $TPMP^+$ accumulation the mitochondria would have to shrink. Evidence for an alkalinization of the cytoplasm by 0.1 pH unit caused by insulin should be borne in mind when inspecting Fig. 5 (Gupta & Moore, 1980). A comparable alkalinization of the fat-cell cytoplasm would decrease, but not abolish, the calculated insulin-dependent increase in pH gradient.

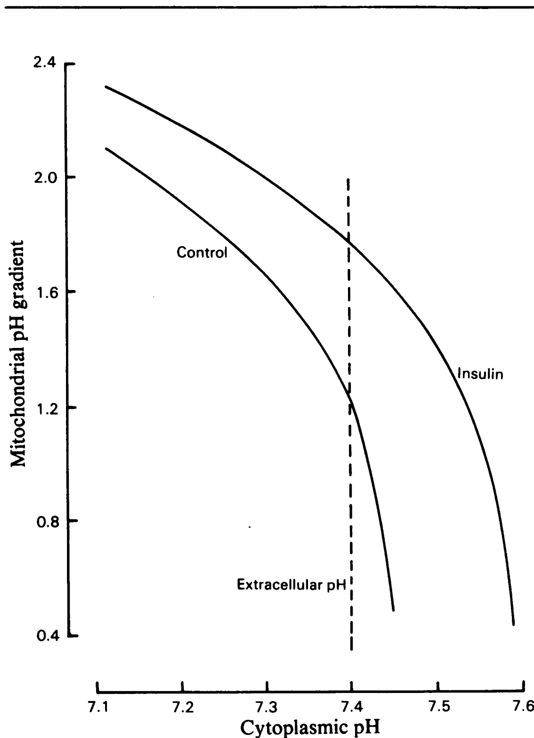

Fig. 5. *Variation of the calculated mitochondrial pH gradient with possible values for the pH of the cytoplasm*
Dimethyloxazolidinedione accumulation by isolated fat-cells was determined as described in the Experimental section after allowance for the dimethyloxazolidinedione associated with the fat cake from broken cells. The mitochondrial pH gradient, ΔpH_m, was calculated by using eqn. (3). The pK_a of dimethyloxazolidinedione at 37°C was taken as 6.13 (Addanki *et al.*, 1968). The results are calculated from mean data from four separate preparations of fat-cells (presented in Table 5).

It is possible that the activity of the dimethyloxazolidinedione is diminished in the mitochondrion relative to the external medium. To investigate this, the accumulation of dimethyloxazolidinedione by isolated fat-cell mitochondria was compared with that of acetate (Table 5). Acetate is a small charged molecule, and would be expected to have a very similar activity in the medium and mitochondrial matrix. The ΔpH_m deduced from the Henderson–Hasselbach equation was 0.29 for dimethyloxazolidinedione and 0.33 for acetate. Thus it appears that the dimethyloxazolidinedione and acetate have a similar change in activity coefficient between the two compartments.

Effect of variation of insulin concentrations

The insulin treatment used as a routine (10 munits/ml) was a higher concentration than that observed physiologically. It was found that the mitochondrial depolarization, plasma-membrane hyperpolarization and lipogenic effects were apparent with 10 μunits of insulin/ml, which is within the physiological range (Fig. 6).

Effect of variation of substrate on the insulin response

It was found that the hyperpolarization of the plasma membrane and the mitochondrial-membrane depolarization caused by insulin were observed in the presence of glucose, glucose together with acetate, fructose or no substrate (Table 6).

Discussion

The hyperpolarization of the plasma membrane has been characterized in rat adipose tissue by micro-electrode techniques (Beigelman & Hollander, 1962, 1963, 1964*b*, 1965) and by the fluorescence

Table 5. *pH gradients of isolated fat-cell mitochondria*
Mitochondria were incubated for 5 min as described in the Experimental section. The medium contained 10 mM-Tes/NaOH buffer, pH 7.0, 160 mM-KCl, 2 mM-succinate, 2 mM-EGTA and 1% (w/v) defatted bovine serum albumin at 30°C. The pH gradient was calculated from the accumulation by the Henderson–Hasselbach equation. The results are expressed as means ± S.E.M., with the numbers of different fat-cell mitochondrial preparations used in parentheses.

Weak acid	pK_a (30°C)	Accumulation	pH gradient
Acetate	4.76*	2.13 ± 0.15 (2)	0.33
Dimethyloxazolidinedione	6.28†	1.8 ± 0.2 (2)	0.29

* Taken from Kortüm *et al.* (1961).
† Taken from Addanki *et al.* (1968).

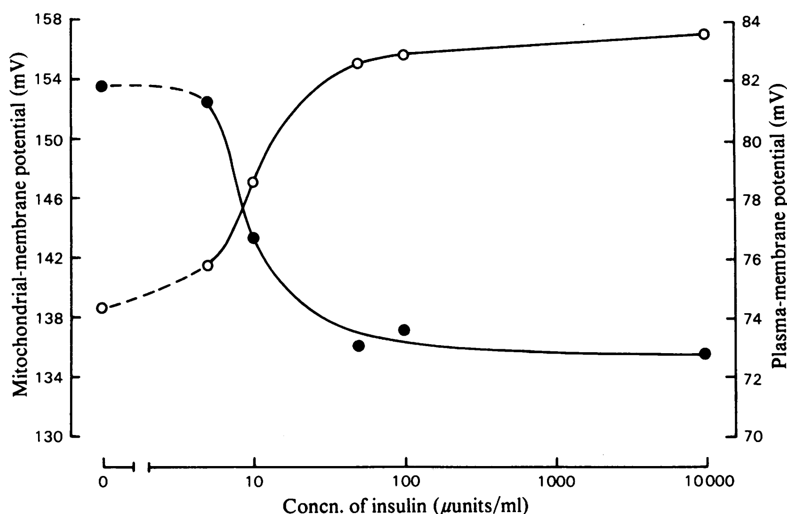

Fig. 6. *Effect of variation of insulin concentration on membrane potentials of fat-cells*
Membrane potentials of fat-cells were determined by the method of Scott & Nicholls (1980) by incubating the cells with $^{86}Rb^{+}$ and $[^{3}H]TPMP^{+}$ for 40min as described in the Experimental section and calculated as described in the Results section at a variety of insulin concentrations. Symbols: ○, plasma-membrane potential; ●, mitochondrial-membrane potential. The results are those of a typical experiment. Maximal responses were observed consistently at 50–100 μunits of insulin/ml. The partial response at 10 μunits/ml was less consistent with different preparations of cells.

Table 6. *Effect of substrate on membrane potentials*
Fat-cells were incubated with $^{86}Rb^{+}$ and $[^{3}H]TPMP^{+}$ for 40min as described in the Experimental section in media containing the substrates as stated. In the case of no substrate, the cells were not preincubated. Insulin at a concentration of 10 munits/ml was used. The results are expressed as means ± S.E.M., with the numbers of different fat-cell preparations used in parentheses. The plasma-membrane potential was assessed from the $^{86}Rb^{+}$ distribution, and the mitochondrial-membrane potential was calculated as described in the Results section.

		Accumulation ratio		Calculated membrane potentials (mV)	
Substrate	Treatment	$^{86}Rb^{+}$	$[^{3}H]TPMP^{+}$	Plasma membrane	Mitochondrial membrane
1 mM-Glucose	—	16.3 ± 0.7 (36)	146.4 ± 3.7 (36)	74.5 ± 1.1	151.7 ± 0.6
1 mM-Glucose	Insulin	22.8 ± 1.5 (36)	119.7 ± 3.3 (36)	83.5 ± 1.8	132.6 ± 1.6
1 mM-Glucose + 0.5 mM-acetate	—	16.1 ± 0.9 (4)	111.1 ± 5.4 (4)	74.2 ± 1.5	142.7 ± 1.1
1 mM-Glucose + 0.5 mM-acetate	Insulin	20.0 ± 1.1 (4)	96.3 ± 2.1 (4)	80.0 ± 1.5	129.2 ± 1.4
1 mM-Fructose	—	16.3 ± 0.8 (4)	154.4 ± 6.3 (4)	74.5 ± 1.3	153.4 ± 0.5
1 mM-Fructose	Insulin	20.6 ± 1.0 (4)	123.4 ± 3.6 (4)	80.8 ± 1.2	137.6 ± 0.4
No substrate	—	16.8 ± 1.2 (4)	149.2 ± 6.6 (4)	75.4 ± 1.9	151.3 ± 0.9
No substrate	Insulin	24.6 ± 1.4 (4)	118.7 ± 4.1 (4)	85.5 ± 1.5	129.2 ± 0.9

changes of carbocyanine dye (Petrozzo & Zierler, 1976). The 9mV increase in the $^{86}Rb^{+}$ diffusion potential reported in the present paper is rather lower than the hyperpolarization found by Beigelman & Hollander (1963) of 28mV. This suggests that insulin may decrease the Na^{+} permeability of the plasma membrane, as proposed by Zierler (1972). Rat skeletal muscle has also been found to be hyperpolarized by insulin (Zierler, 1951, 1959, 1960).

The interpretation of the insulin-dependent change in the apparent accumulation of $TPMP^{+}$ by fat-cells is discussed in the Results section. If it is assumed that large decreases in mitochondrial volume do not occur, there are two possible causes of the change in accumulation. First, the activity coefficient of

$TPMP^+$ may change. However, the amount of insulin-dependent decrease in apparent $TPMP^+$ accumulation is greater than the amount of potential-independent binding of $TPMP^+$ to the cell. Thus a decrease in this binding cannot account for all of the insulin-dependent decrease in apparent $TPMP^+$ accumulation. Furthermore, the accumulation of $TPMP^+$ is not significantly altered in the presence of valinomycin (where there is no mitochondrial component of the accumulation) with or without insulin (10 munits/ml). A difference would be expected if insulin caused a change in the activity coefficient of the $TPMP^+$ in the cells or in the medium. Secondly, the decrease in apparent $TPMP^+$ accumulation may be due to a decrease in the mitochondrial-membrane potential.

The insulin-dependent depolarization of the mitochondrial membrane is a phenomenon that has not been previously reported. The response appears to be labile in that the Rb^+ and $TPMP^+$ accumulation by mitochondria isolated from control cells and from insulin-treated cells are the same. This is in contrast with the insulin-dependent activation of pyruvate dehydrogenase in fat-cell mitochondria, which is persistent (Denton *et al.*, 1971). It is also in contrast with the reported effect of glucagon pretreatment on the protonmotive force generated by mitochondria subsequently isolated from rat liver (Halestrap, 1978).

There are two basic interpretations for the insulin-dependent decrease in $\Delta\psi_m$: first, that there is an alteration in the total mitochondrial protonmotive force; secondly, that there are ion movements across the mitochondrial inner membrane.

Insulin causes a 23% increase in the O_2 consumption of whole epididymal fat-pads (Jungas & Ball, 1963). The insulin-activated mitochondria might be thought analogous to State-3 mitochondria *in vitro* because of the increase in O_2 consumption (Chance & Williams, 1956). The transition from State 4 to State 3 is characterized by a decrease in protonmotive force (Mitchell & Moyle, 1969). Nicholls (1974) demonstrated a $\Delta\bar{\mu}_{H^+}$ of 228 mV and 170 mV for rat liver mitochondria in States 4 and 3 respectively. The $\Delta\psi_m$ acounted for 18 mV of the decrease in $\Delta\bar{\mu}_{H^+}$ under the particular conditions of his experiments. In the present paper, $\Delta\psi_m$ was found to fall by 19 mV, so that a transition from State 4 to State 3 might be able to account for the effect.

However, it is unlikely that the lower $\Delta\psi_m$ due to insulin is because of increased oxidative phosphorylation, for five reasons. First, the insulin effect on O_2 uptake is observed in the presence of glucose, but not in its absence (Jungas & Ball, 1963). The insulin response of $\Delta\psi_m$ was apparent in the presence and in the absence of glucose. Secondly, whereas insulin increases O_2 uptake of fat-pads by 23%, adrenaline and insulin together (in the presence of glucose) increase the O_2 uptake by 300% (Jungas & Ball, 1963). Therefore the insulin stimulation of respiration is by no means a maximal response by the electron-transport chain. Thirdly, the calculations made by Flatt (1970) demonstrate that the conversion of glucose into fat in adipose tissue is limited by the tissue's ability to use the high-energy bonds produced in excess during this process. It might therefore be expected that, in the presence of insulin, fat-cell mitochondria will be closer to State-4 conditions that to State 3. Fourthly, when glucose is the substrate the [lactate]/[pyruvate] ratio is raised by insulin (Del Boca & Flatt, 1969). Glucose together with acetate or fructose, when used as substrates, result in the [lactate]/[pyruvate] ratio being lowered (Del Boca & Flatt, 1969; Coore *et al.*, 1971). The insulin effect on apparent $TPMP^+$ accumulation was observed in the presence of all the substrates. It is unlikely therefore that the calculated decrease in $\Delta\psi_m$ is due to any change in the [NADH]/[NAD^+] ratio. Fifthly, evidence from the uptake of dimethyloxazolidinedione by fat-cells has been obtained that can be interpreted as indicating that insulin causes an increase in the mitochondrial pH gradient (Fig. 5). The reliability of this evidence is discussed in the Results section.

If the insulin-dependent decrease in $\Delta\psi_m$ is equivalent to the rise in ΔpH_m, as suggested by the uptake of dimethyloxazolidinedione by fat-cells, any change in $\Delta\bar{\mu}_{H^+}$ will be small. Such a change in the relative size of the electrical and pH components of the $\Delta\bar{\mu}_{H^+}$ would be expected from the uptake of cation or loss of anion by a uniport mechanism. Electroneutral proton symport or antiport transport could also account for the observed effect of insulin.

Ca^{2+} has been widely proposed as the possible second messenger for insulin action (for review see Czech, 1977). Evidence supporting this hypothesis has come from metabolic pathways that are both insulin- and Ca^{2+}-sensitive (Kissebah *et al.*, 1974; Denton *et al.*, 1975). Clausen (1975) has pointed out that a wide variety of agents that raise cytoplasmic Ca^{2+} concentrations have an insulin-mimetic effect. The difficulty in resolving free and bound Ca^{2+} in the cell has made evidence for a raised free Ca^{2+} concentration in the cytoplasm difficult to interpret. However, Clausen *et al.* (1974, 1975) demonstrated that insulin increases the resting tension of rat soleus muscles in hyperosmolar media. This suggests that insulin causes a rise in the cytoplasmic free Ca^{2+} concentration.

The uptake of Ca^{2+} into the mitochondrion is by electrogenic uniport (Selwyn *et al.*, 1970; Rottenberg & Scarpa, 1974; Reynafarje & Lehninger, 1977). Thus net Ca^{2+} uptake by insulin-treated fat-cell mitochondria would lower $\Delta\psi_m$. Respiration would then increase the ΔpH_m, so that the $\Delta\bar{\mu}_{H^+}$ is

maintained. This would be consistent with the insulin effect on mitochondrial protonmotive force. An efflux pathway from the mitochondrion for Ca^{2+} has been found to be independent of the influx pathway (for review see Nicholls & Crompton, 1980). A steady state of Ca^{2+} cycling across the mitochondrial membrane can be achieved, and it has been proposed that rat liver mitochondria can act as efficient Ca^{2+} buffers for the cytoplasm (Puskin *et al.*, 1976; Nicholls, 1978). Fat-cell mitochondria buffer Ca^{2+} in the medium at about pCa^{2+} 6.2 at pH 7.0 (R. J. Davis, unpublished work). The hypothesis that insulin causes a rise in the cytoplasmic free Ca^{2+} concentration needs to be considered in the light of this Ca^{2+} buffering.

It is most probable that in the resting cell the free Ca^{2+} concentration in the cytoplasm is regulated by the plasma membrane and possibly the endoplasmic reticulum at a concentration that is below the buffering point of the mitochondria (Carafoli & Crompton, 1978; Denton *et al.*, 1980; Schweitzer & Blaustein, 1980). Such mitochondria will be Ca^{2+}-depleted, and the efflux pathway for Ca^{2+} from the mitochondria will not be saturated. Nicholls & Crompton (1980) argue that under these conditions the matrix free Ca^{2+} concentration will be a function of the cytoplasmic concentration. If insulin causes a rise in the cytoplasmic free Ca^{2+} concentration, the mitochondria will take up Ca^{2+} by electrogenic uniport, causing a decrease in $\Delta\psi_m$. As the Ca^{2+} is taken up, the concentration of Ca^{2+} in the matrix will rise and eventually saturate the efflux pathway. The mitochondria will then buffer the cytoplasmic free Ca^{2+} concentration. The buffering power of the mitochondria therefore forms an upper limit to rises in cellular free Ca^{2+} concentration (except for very transient changes). In this model the decrease in $\Delta\psi_m$ observed with insulin is due to net Ca^{2+} uptake as the cytoplasmic free Ca^{2+} concentration rises to the mitochondrial buffering point. The model requires that re-equilibration of the ΔpH_m, for example by uptake of weak acid, must occur slowly or to a smaller extent than Ca^{2+} uptake. A variety of other models can be described for the insulin effect on $\Delta\psi_m$ if the cytoplasmic free Ca^{2+} concentration was regulated in resting fat-cells by the mitochondria.

In all of these models the source of the Ca^{2+} to raise the cytoplasmic Ca^{2+} concentration is not the mitochondria. Insulin has been reported to affect calcium metabolism in a wide variety of subcellular fractions of adipocytes. Insulin increases Ca^{2+} uptake by fat cell endoplasmic reticulum (McDonald *et al.*, 1978), so that it seems unlikely that the endoplasmic reticulum is the source of Ca^{2+}. However, insulin decreases Ca^{2+} binding to fat-cell 'ghost' membranes (Kissebah *et al.*, 1975) and increases the Ca^{2+} permeability of intact tissue (Clausen, 1969). A requirement for extracellular Ca^{2+} has been demonstrated for the stimulation of protein synthesis by insulin (Jacobs & Krahl, 1973), but not for the insulin effects on glucose metabolism (Letarte & Renold, 1969). Thus it appears that the plasma membrane is the most likely source for the proposed increase in cytoplasmic Ca^{2+} concentration. This is consistent with a report that insulin inhibits a Ca^{2+}-stimulated ATPase in the fat-cell plasma membrane (Pershadsingh & McDonald, 1979).

The general model of insulin action that we propose can be summarized as follows. Insulin binds to the plasma membrane and causes a rise in the cytoplasmic free Ca^{2+} concentration by increasing Ca^{2+} uptake from the medium and by displacement of Ca^{2+} from binding sites on the plasma membrane. The increased cytoplasmic Ca^{2+} concentrations leads to increased uptake of Ca^{2+} into the mitochondrion by uniport. This results in a decreased $\Delta\psi_m$, and, in order to maintain $\Delta\mu_{H+}$, respiration increases ΔpH_m. Ca^{2+} uptake by mitochondria to give a higher matrix concentration of the free ion may activate pyruvate dehydrogenase phosphate phosphatase (Severson *et al.*, 1974). This is consistent with the insulin-dependent activation of pyruvate dehydrogenase (Denton *et al.*, 1971).

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APPENDIX

5,5-Dimethyloxazolidine-2,4-dione accumulation

$$\text{Un-ionized (U)} \rightleftharpoons \text{H}^+ + \text{Ionized}^- \text{ (I)}$$

The un-ionized dimethyloxazolidinedione is assumed to equilibrate with all compartments and to have the same activity in each compartment. It is also assumed that no organelles other than the mitochondria significantly accumulate the dimethyloxazolidinedione.

$$[\text{U}]_e = [\text{U}]_c = [\text{U}]_m \quad (4)$$

where the subscripts m, c and e refer to the mitochondria, cytoplasm and external media respectively. The Henderson–Hasselbach equation can be applied to the system:

$$[I]_e = [U]_e(10^{(pH_e - pK_a)}) \quad (5)$$

The total dimethyloxazolidinedione in any compartment = [C] = [U] + [I]. Therefore:

$$[C]_e = [I]_e + [U]_e = [U]_e \cdot (1 + 10^{(pH_e - pK_a)}) \quad (6)$$

Similar equations can be derived for $[C]_m$ and $[C]_c$. The total concentration of dimethyloxazolidinedione averaged over all the compartments in the cell is $[C]_s$:

$$[C]_s = \frac{V_c[C]_c + V_m[C]_m}{V_s} \quad (7)$$

where V is the volume and the subscripts refer to the same compartments as before. By using eqns. (4) and (5) this can be expanded:

$$[C]_s = \frac{[U]}{V_s}[V_c \cdot (1 + 10^{(pH_c - pK_a)} + V_m \cdot (1 + 10^{(pH_m - pK_a)})] \quad (8)$$

But from eqn. (6):

$$[U] = \frac{[C]_e}{1 + 10^{(pH_e - pK_a)}} \quad (9)$$

Substitute eqn. (9) into eqn. (8) and re-arrange to give pH_m:

$$pH_m = pK_a + \log\left[\left(\frac{V_s}{V_m}\right) \cdot \left(\frac{[C]_s}{[C]_e}\right) \cdot (1 + 10^{(pH_e - pK_a)}) - \frac{V_c}{V_m} \cdot (1 + 10^{(pH_c - pK_a)}) - 1\right] \quad (10)$$