The effect of β -adrenergic agonists on the membrane potential of fat-cell mitochondria *in situ*

Roger J. DAVIS and B. Richard MARTIN

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(Received 19 April 1982/Accepted 18 May 1982)

1. The accumulation of [^3H]methyltriphenylphosphonium by isolated fat-cells was used to estimate the membrane potential of mitochondria *in situ*. 2. Adrenaline caused a large decrease in the accumulation of [^3H]methyltriphenylphosphonium. Mitochondria in fat-cells incubated in the presence of adrenaline had a very low calculated membrane potential. This effect was also given by isoprenaline (a β -adrenergic agonist) and was blocked by propranolol (a β -adrenergic antagonist). 3. The effect of isoprenaline could be partially antagonized by the use of media with high albumin concentrations. Addition of sodium oleate to saturate the fatty acid-binding sites on the albumin reversed this antagonism. 4. It is proposed that the decrease in the calculated mitochondrial membrane potential is due to the uncoupling effect of the non-esterified fatty acids released by the stimulation of lipolysis observed in the presence of β -adrenergic agonists.

In a previous paper (Davis *et al.*, 1981) we described the use of [^3H]methyltriphenylphosphonium to estimate the mitochondrial membrane potential ($\Delta\psi_m$) of rat white epididymal fat-cells by an extension of the method of Deutsch *et al.* (1979), Hoek *et al.* (1980) and Scott & Nicholls (1980). It was demonstrated that the $\Delta\psi_m$ of the mitochondria of resting fat-cells was about 148 mV. The problems associated with calibrating the uptake of [^3H]TPMP $^+$ are similar to those found with the measurement of $\Delta\psi_m$ of mitochondria *in situ* with safranin (Akerman, 1979; Akerman & Jarvisalo, 1980; Akerman & Nicholls, 1981). However, the [^3H]TPMP $^+$ method, although not providing a precise value for $\Delta\psi_m$ of mitochondria *in situ*, can provide a quantitative indication of changes in the $\Delta\psi_m$.

In this paper we have investigated the effect of adrenaline and β -adrenergic agonists on the membrane potentials of isolated white fat-cells.

Experimental

Materials

[^3H]Methyltriphenylphosphonium bromide (lot nos. 1133-022 and 1314-115) was bought from New

England Nuclear Chemicals, Dreieich, Germany. $^{86}\text{RbCl}$, $^3\text{H}_2\text{O}$ and [^3H]inulin were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (fraction V), methyltriphenylphosphonium bromide, crude firefly extract, adrenaline, phenylephrine [(–)-1-(*m*-hydroxyphenyl)-2-methylaminoethanol hydrochloride] and isoprenaline [(+)-1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride] were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. (±)-Propranolol hydrochloride [1-isopropylamino-3-(1-naphthyl)propan-2-ol hydrochloride; Inderal] was from ICI Pharmaceuticals Division, Macclesfield, Cheshire, U.K. Collagenase, the enzymes and coenzymes for glycerol assays and all other biochemicals were from Boehringer, Mannheim, Germany. Dinonyl phthalate was bought from BDH Chemicals, Poole, Dorset, U.K. 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 modifications as described by Martin & Denton (1970). The rats that were used weighed between 130 and 180 g. The medium used for the digestion of the fat-pads and for the incubation of the isolated cells was a bicarbonate-buffered medium (Krebs & Henseleit, 1932) but with half the stated Ca^{2+} concentration (120 mM-NaCl/4.8 mM-KCl/1.28 mM- CaCl_2 /1.12 mM- KH_2PO_4 /1.2 mM- MgSO_4 /25.3 mM-

Abbreviations used: $\Delta\psi_p$, plasma-membrane potential; $\Delta\psi_m$, mitochondrial membrane potential; $\Delta\bar{\mu}_{\text{H}^+}$, mitochondrial proton electrochemical gradient; ΔpH_m , mitochondrial pH gradient; TPMP $^+$, methyltriphenylphosphonium.

NaHCO₃), and containing 1% (w/v) bovine serum albumin and 1 mM-glucose; the medium was gassed with O₂/CO₂ (19:1). Before each experiment the isolated fat-cells were pre-incubated for 30 min at 37°C in a shaking water bath. In all experiments the cell density was 50–70 μ l of packed cells per ml of medium.

Determination of ⁸⁶Rb⁺ and [³H]TPMP⁺ accumulation of isolated fat-cells

The accumulation of ⁸⁶Rb⁺ and [³H]TPMP⁺ was measured by a dual-label method described by Davis *et al.* (1981). Briefly, the cells were incubated in the standard bicarbonate-buffered medium in a shaking water bath at 37°C with 50 μ M-⁸⁶Rb⁺ (0.2 μ Ci/ml) and 0.3 μ M-[³H]TPMP⁺ (0.5 μ Ci/ml). At defined times 200 μ l samples were taken and the cells were separated from the medium by centrifugation with 100 μ l of diindanyl phthalate (Gliemann *et al.*, 1972). The accumulation was calculated from the radioactivity in the cells and in the medium after allowing for the extracellular space of the fat-cell cake. To determine the total water space and the extracellular space of the fat-cell cake two incubations were done in parallel, one with ³H₂O to estimate the total water space of the fat-cell cake and the other with [³H]-inulin to estimate the extracellular space of the fat-cell cake.

In these experiments the accumulation of [³H]-TPMP⁺ by isolated fat-cells was higher than that which we have published previously (Davis *et al.*, 1981). It was also observed that the cells used in these experiments had a greater response to insulin as estimated from the stimulated rate of fat synthesis from glucose. The reason for this is not known, but it would be consistent with an increase in the cell viability. It is possible that it may be due to a change in the way in which the fat-cells were prepared. In these experiments crude collagenase from Boehringer Mannheim (lot no. 1230234) was used, whereas previously the source of the collagenase was from Sigma (lot no. 28C-6900).

Glycerol and ATP assays

Fat-cell suspensions were deproteinized by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The samples were then centrifuged and the supernatants were removed to be extracted three times with 5 vol. of diethyl ether. Glycerol was assayed by the method of Garland & Randle (1962). ATP was assayed by injecting 20 μ l of the sample into a solution of crude firefly lantern extract (10 mg/ml) in a DuPont 760 luminescence biometer calibrated with standard ATP solutions that had been treated in the same way.

Calculation of mitochondrial membrane potentials in situ

Mitochondrial membrane potentials were calculated from the accumulation of [³H]TPMP⁺ by fat-cells as described by Davis *et al.* (1981). To calculate the $\Delta\psi_m$ an estimate for the plasma-membrane potential has to be made. In the Figures and Tables it is assumed for the purposes of the calculation of the $\Delta\psi_m$ that the rubidium diffusion potential is in equilibrium with the $\Delta\psi_p$. The rubidium diffusion potential probably over-estimates the $\Delta\psi_p$ and will cause a lower $\Delta\psi_m$ to be calculated. This systematic error in the calculated $\Delta\psi_m$, however, does not alter the calculation of changes in $\Delta\psi_m$. This assumption and other assumptions made during the calculation of the $\Delta\psi_m$ are discussed in detail by Davis *et al.* (1981).

Results

Effect of adrenaline on fat-cell membrane potentials

Adrenaline markedly increases the O₂ uptake of fat-cells and most of this increase is not sensitive to oligomycin (Hepp *et al.*, 1968a). This indicates that the increased O₂ uptake is not linked to oxidative phosphorylation but to ion movements across the mitochondrial inner membrane unrelated to the reversible ATPase. Adrenaline markedly activates lipolysis in fat-cells and it has been suggested that the non-esterified fatty acids that are released uncouple the mitochondria (Bihler & Jeanrenaud, 1970; Angel *et al.*, 1971). Thus adrenaline would be expected to cause a depolarization of the mitochondrial inner membrane. It was found that the [³H]TPMP⁺ accumulation ratio decreased from 262.4 $\pm$ 2.4 in control cells to 18.9 $\pm$ 1.5 in cells treated with 5 μ M-adrenaline for 40 min (means $\pm$ S.E.M. of three separate preparations of fat-cells). It was also observed that the ⁸⁶Rb⁺ accumulation was decreased and this may be a result of the decreased ATP levels observed in adrenaline-treated fat-cells (Hepp *et al.*, 1968b; Halperin & Denton, 1969; Touabi & Jeanrenaud, 1970; Bihler & Jeanrenaud, 1970). Addition of an uncoupler (0.1 mM-dinitrophenol) to fat-cells has a similar effect to 5 μ M-adrenaline. The decrease in the apparent accumulation of [³H]TPMP⁺ indicates that the mitochondria become depolarized. The [³H]TPMP⁺ method of determining the membrane potential of mitochondria *in situ* therefore provides a direct demonstration of the uncoupling effect of adrenaline-induced lipolysis in isolated fat-cells. The results of the effects of 5 μ M-adrenaline and 0.1 mM-dinitrophenol are summarized in Table 1. To further characterize the response of fat-cells to adrenaline, experiments with specific agonists and antagonists are necessary.

Table 1. *Effect of adrenaline and dinitrophenol on the accumulation of [³H]TPMP⁺ by fat-cells*

Fat-cells were pre-incubated for 30min at 37°C in a shaking water bath and then 50 μ M-⁸⁶Rb⁺ (0.2 μ Ci/ml) and 0.3 μ M-[³H]TPMP⁺ (0.5 μ Ci/ml) were added. ⁸⁶Rb⁺ and [³H]TPMP⁺ accumulation ratios after 40min of incubation were measured as described in the Experimental section. The medium containing 0.1 mM-dinitrophenol was dialysed overnight with 20vol. of medium without albumin to allow for the binding of the dinitrophenol to the albumin. The results are expressed as means of three separate preparations of fat-cells $\pm$ S.D.

	⁸⁶ Rb ⁺ accumulation ratio	[³ H]TPMP ⁺ accumulation ratio
Control	17.4 $\pm$ 1.4	262.4 $\pm$ 24.0
Adrenaline (5 μ M)	6.0 $\pm$ 0.8	18.9 $\pm$ 1.5
Dinitrophenol (0.1 mM)	4.9 $\pm$ 0.7	23.2 $\pm$ 2.3

β -Adrenergic effects on fat-cell membrane potentials

Isoprenaline is a specific agonist that can be used to study the effects of adrenaline mediated by the β -receptors on fat-cell plasma membranes (for review, see Fain, 1973). The effect of isoprenaline on fat-cell membrane potentials is summarized in Fig. 1. Low concentrations of isoprenaline (0.01 μ M) had a large effect on the apparent [³H]TPMP⁺ accumulation by fat-cells, without significantly altering the apparent ⁸⁶Rb⁺ accumulation. Higher concentrations of isoprenaline (10 μ M) decreased the apparent [³H]TPMP⁺ accumulation even further and also decreased the accumulation of ⁸⁶Rb⁺. At the higher concentrations of isoprenaline the decrease in the apparent [³H]TPMP⁺ accumulation was associated with a fall in the level of ATP. After 40min of incubation in the presence of 0, 0.01, 0.1, 1.0 and 10.0 μ M-isoprenaline the ATP levels were 94, 79, 47, 33 and 18% respectively (mean of three separate experiments). The lower level of ATP may account for the decrease in the ⁸⁶Rb⁺ uptake (Hepp *et al.*, 1968b; Halperin & Denton, 1969; Touabi & Jeanrenaud, 1970; Bihler & Jeanrenaud, 1970) and may result in a depolarization of the plasma membrane (Petrozzo & Zierler, 1976). The extensive changes in the ⁸⁶Rb⁺ uptake and ATP levels that are observed at high concentrations of the agonist may have secondary effects on the fat-cells. Therefore in the following experiments 0.1 μ M-isoprenaline was routinely used as this has a marked effect on the apparent [³H]TPMP⁺ accumulation (Fig. 1) without causing a significant change in the ⁸⁶Rb⁺ accumulation (Figs. 1 and 2). Under these conditions the ATP level was decreased to 47% after 40min of incubation.

The time course of the [³H]TPMP⁺ and ⁸⁶Rb⁺ uptake by cells treated with isoprenaline is presented in Fig. 2. After 5min the accumulation of [³H]-

Fig. 1. *Effect of agonist concentration on the accumulation of ⁸⁶Rb⁺ and [³H]TPMP⁺*

The accumulation ratios of (a) ⁸⁶Rb⁺ and (b) [³H]TPMP⁺ after 40min of incubation were measured as described in the Experimental section. The mitochondrial membrane potential (c) was calculated as described by Davis *et al.* (1981) with the assumption that the rubidium diffusion potential is in equilibrium with the plasma-membrane potential. The results are those of one representative experiment and are expressed as means of three separate incubations. The bars indicate the standard deviation. Similar results were obtained with three other preparations of fat-cells. Symbols: $\circ$, phenylephrine; $\bullet$, isoprenaline.

TPMP⁺ by fat-cells in the presence of 0.1 μ M isoprenaline was only slightly less than that of control cells. With longer periods of incubation the accumulation of [³H]TPMP⁺ by isoprenaline-treated fat cells was progressively diminished. The lag in the effect of isoprenaline was also observed when the agonist was added to fat-cells prelabelled with [³H]TPMP⁺ (Fig. 3). Angel *et al.* (1971) have measured the level of intracellular non-esterified fatty acids during nor-

Fig. 2. Effect of isoprenaline and phenylephrine on the accumulation of $^{86}\text{Rb}^+$ and $[\text{H}] \text{TPMP}^+$

The accumulation ratios of (a) $^{86}\text{Rb}^+$ and (b) $[\text{H}] \text{TPMP}^+$ were measured as described in the Experimental-section. The results are those of one representative experiment and are expressed as means of three separate incubations. The bars indicate the standard deviation. Similar results were obtained with two other preparations of fat-cells. Symbols: ●, control; ■, 1 μM -phenylephrine; ▲, 0.1 μM -isoprenaline.

adrenaline-stimulated lipolysis in fat-cells. They have reported that after an initial rise in the level of non-esterified fatty acids there is a lag before any further increase occurs during which the medium albumin becomes saturated with fatty acids. The period after the addition of isoprenaline before the marked decrease in $[\text{H}] \text{TPMP}^+$ accumulation was observed may be related to the time required to saturate the medium albumin with fatty acid. To investigate this lag, the time course of the effect of isoprenaline on the $[\text{H}] \text{TPMP}^+$ accumulation of prelabelled fat-cells was studied in media that had a much higher capacity to bind fatty acids (5% albumin) and a much lower capacity to bind fatty acids (5% albumin and 4.5 mM-sodium oleate) compared with the control medium, which contained 1% albumin (Fig. 3). The fatty acid/albumin molar ratio in the medium containing oleate was 6 and this corresponds to a saturation of sites I and II of the albumin molecule (Goodman, 1958). This would be expected to inhibit fatty acid release from the fat-cells so that they accumulate intracellularly (Scow *et al.*, 1965). The

Fig. 3. Effect of isoprenaline on the accumulation ratio of $[\text{H}] \text{TPMP}^+$ in media with different capacities to bind fatty acids

The glycerol production and the accumulation ratio of $[\text{H}] \text{TPMP}^+$ were measured as described in the Experimental section. The results are those of one representative experiment and are expressed as means of three separate incubations. Similar results for each experiment were obtained with two other preparations of fat-cells. (a) Standard medium (1% albumin); (b) 5% albumin; (c) 5% albumin with 4.5 mM-sodium oleate. The medium containing oleate was prepared from Krebs-Henseleit bicarbonate buffer (without the CaCl_2) with 10% albumin and was incubated with sodium oleate. Care was taken that the pH remained at 7.4. Immediately before use the medium was diluted with fresh buffer to give a final CaCl_2 concentration of 1.28 mM. Symbols: ● and ○, $[\text{H}] \text{TPMP}^+$ accumulation ratio; ▲ and △, glycerol production. Filled symbols indicate the control and the open symbols the effect of 0.1 μM -isoprenaline, which was added at 30 min.

lag that was observed in 1% albumin before the large decrease in $[^3\text{H}]\text{TPMP}^+$ accumulation was increased in 5% albumin and was abolished in media that contained 4.5 mM-oleate bound to the 5% albumin. The ATP levels in the cells used for this experiment are presented in Table 2. In the absence of isoprenaline there was a slow decline in the level of ATP in all three media. Isoprenaline caused a large decrease in the ATP content of fat-cells in 1% albumin and this effect was prevented by media with 5% albumin. However, a small decrease was found in 5% albumin with 4.5 mM-oleate. There is therefore a good correlation between the decrease in the $[^3\text{H}]\text{TPMP}^+$ accumulation and the ATP levels in isoprenaline-treated fat-cells. The observation that oleate partially blocks the protective effects of high albumin concentrations is consistent with the suggestion that the level of intracellular fatty acids mediates the effect of isoprenaline on the level of ATP and $[^3\text{H}]\text{TPMP}^+$.

The O_2 uptake stimulated by adrenaline does not have this lag phase (Hepp *et al.*, 1968a). The reason for this is probably because uncontrolled respiration is allowed by relatively small changes in the $\Delta\bar{\mu}_{\text{H}^+}$ (Nicholls, 1974; Nicholls & Bernson, 1977), which may occur a short time after the stimulation of lipolysis, perhaps due to the initial increase in the level of non-esterified fatty acids (Angel *et al.*, 1971). Further decreases in the $\Delta\bar{\mu}_{\text{H}^+}$ that are observed after a lag will not then cause an additional increase in O_2 uptake.

In the experiments using 5% albumin it was observed that the apparent $[^3\text{H}]\text{TPMP}^+$ accumulation ratios measured were much lower than those using

the standard medium (1% albumin). $[^3\text{H}]\text{TPMP}^+$ ($0.3\text{ }\mu\text{M}$) binds poorly to 1% albumin (Davis *et al.*, 1981). However, albumin is known to bind small hydrophobic ligands so that it is possible that the decreased apparent accumulation is due to $[^3\text{H}]\text{TPMP}^+$ binding to media containing high concentrations of albumin. The binding of $0.3\text{ }\mu\text{M}$ - $[^3\text{H}]\text{TPMP}^+$ to Krebs–Henseleit bicarbonate-buffered medium with different concentrations of albumin was investigated by equilibrium dialysis (Fig. 4). It was observed that the $[^3\text{H}]\text{TPMP}^+$ bound very strongly to 5% albumin. This result means that the binding of $[^3\text{H}]\text{TPMP}^+$ to media containing high concentrations of albumin should be taken into account if a quantitative interpretation of the accumulation of $[^3\text{H}]\text{TPMP}^+$ by cells is made. The strong binding of $[^3\text{H}]\text{TPMP}^+$ to 5% albumin could lead to an artefact in the determination of the accumulation of $[^3\text{H}]\text{TPMP}^+$ by cells if the level of fatty acids in the medium changes during the course of the incubation. Fatty acids bind to albumin (Goodman, 1958) and could therefore displace the $[^3\text{H}]\text{TPMP}^+$. The effect of sodium oleate on the $[^3\text{H}]\text{TPMP}^+$ binding was investigated (Fig. 4). Oleate at high albumin concentrations caused a small increase in the binding of $[^3\text{H}]\text{TPMP}^+$. The small increase in binding is not

Table 2. Effect of isoprenaline on the ATP levels of fat-cells in media with different capacities to bind fatty acids

Samples of fat-cell suspensions from the experiment described in Fig. 3 were taken to assay ATP. The ATP levels at time zero for the fat-cells in 1% albumin, 5% albumin and 5% albumin with 4.5 mM-oleate were 190, 240 and 221 nmol/g dry wt. respectively. The values in the Table express the level of the ATP remaining after 75 min of incubation as a percentage of that at time zero. Isoprenaline was added at 30 min and the incubation was terminated after a further 45 min. Similar results for each experiment were obtained with two other preparations of fat-cells.

	ATP remaining after 75 minutes of incubation (%)	
	Control	Isoprenaline ($0.1\text{ }\mu\text{M}$)
Albumin (1%)	91	54
Albumin (5%)	91	92
Albumin (5%)+ 4.5 mM-oleate	92	78

Fig. 4. Binding of $[^3\text{H}]\text{TPMP}^+$ to albumin
Equilibrium dialysis of media containing different concentrations of albumin was carried out with $0.3\text{ }\mu\text{M}$ - $[^3\text{H}]\text{TPMP}^+$ ($0.2\text{ }\mu\text{Ci/ml}$). Samples ($50\text{ }\mu\text{l}$) were taken for scintillation counting after 24 h. The results are means of three separate incubations for each point. Media containing sodium oleate were prepared as described in the legend to Fig. 3. The fatty acid/albumin molar ratio in each case was 6. Symbols: O, control; ●, with sodium oleate.

Table 3. *Effect of propranolol on the accumulation of [³H]TPMP⁺ by fat-cells*

Fat-cells were pre-incubated for 30 min at 37°C in a shaking water bath before 50 μ M-⁸⁶RbCl (0.2 μ Ci/ml) and 0.3 μ M-[³H]TPMP⁺ (0.5 μ Ci/ml) were added. ⁸⁶Rb⁺ and [³H]TPMP⁺ accumulation ratios were measured after 40 min of incubation as described in the Experimental section. The results are those from a representative experiment and are expressed as means of three separate incubations $\pm$ s.d. Similar results were obtained with three other preparations of fat-cells. The mitochondrial membrane potential was calculated as described by Davis *et al.* (1981) with the assumption that the rubidium diffusion potential is in equilibrium with the plasma-membrane potential.

	⁸⁶ Rb ⁺ accumulation ratio	[³ H]TPMP ⁺ accumulation ratio	Calculated mitochondrial potential
Control	16.5 $\pm$ 0.4	256.4 $\pm$ 6.8	168.8
Propranolol (1 μ M)	16.1 $\pm$ 0.5	233.2 $\pm$ 9.1	166.7
Isoprenaline (0.1 μ M)	15.9 $\pm$ 0.3	92.3 $\pm$ 6.4	136.5
Isoprenaline (0.1 μ M) + 1 μ M-propranolol	16.2 $\pm$ 0.4	186.3 $\pm$ 13.2	159.6

sufficient to account for the marked effect of isoprenaline on the apparent [³H]TPMP⁺ accumulation by fat-cells because of the release of fatty acids.

The β -antagonist propranolol (for review, see Fain, 1973) was found to prevent the effect of isoprenaline on the apparent accumulation of [³H]-TPMP⁺ and ⁸⁶Rb⁺ (Table 3).

To study the possibility that there may be α -receptor-mediated adrenergic effects on fat-cell membrane potentials, an agonist with low β -potency was used. Phenylephrine has found wide use as a selective α -agonist and was used in initial experiments. Phenylephrine had no effect on the accumulation of ⁸⁶Rb⁺ by fat-cells but did reduce the apparent accumulation of [³H]TPMP⁺. This effect could be demonstrated at concentrations of phenylephrine as low as 0.1 μ M (Fig. 1). The time course of the accumulation of ⁸⁶Rb⁺ and [³H]TPMP⁺ in the presence of 1 μ M-phenylephrine is presented in Fig. 2. It is possible that this effect of phenylephrine is due to its weak β -agonist activity (Lawrence & Larner, 1977). The effect of α -adrenergic agonists on fat-cells has been further characterized in the following paper (Davis & Martin, 1982).

Discussion

Measurement of the plasma-membrane potential

Cheng *et al.* (1980) have reported the use of [³H]TPMP⁺ to measure the plasma-membrane potential of isolated fat-cells. In these experiments the accumulation of [³H]TPMP⁺ by organelles was not taken into account. A plasma-membrane potential of 58.3 mV was calculated from the uptake of [³H]TPMP⁺. Cheng *et al.* (1980) used 10 μ M-[³H]-TPMP⁺ in the incubation medium, whereas in experiments to determine the membrane potential of mitochondria *in situ* (Davis *et al.*, 1981) 0.3 μ M-[³H]TPMP⁺ was used. If there is a plasma-membrane potential of 50 mV and a high mitochondrial membrane potential (150 mV) the concentration of

[³H]TPMP⁺ in the mitochondrial matrix under the conditions of Cheng *et al.* (1980) would be about 18 mM. It is likely that this large uptake of [³H]-TPMP⁺ will cause substantial depolarization of the mitochondria and that the accumulation of [³H]TPMP⁺ under these conditions may give an estimate of the plasma-membrane potential. This would provide an explanation for the observation of an insulin-dependent increase in the apparent [³H]TPMP⁺ accumulation by Cheng *et al.* (1981), which they interpret as being due to a plasma-membrane hyperpolarization. Under the conditions used by Davis *et al.* (1981) the matrix concentration of [³H]TPMP⁺ would be 0.5 mM. The value of 58.3 mV derived by Cheng *et al.* (1980) for the plasma-membrane potential therefore appears to be an over-estimate because of uptake of [³H]TPMP⁺ by internal organelles. However, Cheng *et al.* (1980) did not calculate the accumulation ratio of [³H]-TPMP⁺ by fat-cells but measured the uptake. The $\Delta\psi_p$ was determined in their experiments by comparing the total uptake of [³H]TPMP⁺ by control cells with that by depolarized cells incubated in the presence of valinomycin and a high potassium concentration. They report that about 700 pmol of [³H]TPMP⁺ are taken up by 10⁶ fat cells. If the cytoplasmic volume of a fat-cell is assumed to be 1.4 pl (Siegel & Olefsky, 1980) the apparent accumulation ratio of [³H]TPMP⁺ by the fat-cells can be calculated to be about 50-fold. In the presence of 10 μ M-[³H]TPMP⁺ the accumulation of the cation by fat-cells was found to be 89.4 $\pm$ 20.4-fold in 1% albumin-containing media (mean $\pm$ s.d. of four experiments: R. J. Davis, unpublished work). The difference in the accumulation ratio that can be calculated from the data of Cheng *et al.* (1980) and that reported here may be related to the binding of [³H]TPMP⁺ to the 3% albumin medium used in their experiments. In 3% albumin medium 55% of the total [³H]TPMP⁺ is bound (Fig. 4). The accumulation of [³H]TPMP⁺ therefore appears to be greater than that which can be accounted for by

the value of 58.3 mV for the plasma-membrane potential calculated by Cheng *et al.* (1980).

If [^3H]TPMP $^+$ is used to estimate the plasma-membrane potential the accumulation by organelles has to be taken into account. Addition of valinomycin depolarizes the mitochondria so that under these conditions the accumulation of [^3H]TPMP $^+$ will reflect the plasma-membrane potential (Scott & Nicholls, 1980). However, the plasma-membrane potential will be set by the potassium diffusion potential, which is probably different to the resting plasma-membrane potential. Another technique that can be used to depolarize the mitochondria is to add an uncoupler. Dinitrophenol treatment of fat-cells resulted in an accumulation ratio of 23.2-fold (Table 1). If a relative activity coefficient of 0.6 is assumed for the [^3H]TPMP $^+$ in the cytoplasm (Davis *et al.*, 1981) a plasma-membrane potential of about 70 mV can be calculated. [In this calculation the accumulation of [^3H]TPMP $^+$ is quite low compared with control cells, so that the small amount of potential independent binding of [^3H]TPMP $^+$ to fat cells ought to be taken into account (Davis *et al.*, 1981). After correcting for this binding a $\Delta\psi_p$ of about 68 mV can be calculated.] The reliability of this estimate of the $\Delta\psi_p$ can be questioned because of the possible effect of an uncoupler on the plasma-membrane potential. The addition of an uncoupler will perturb the plasma-membrane potential if there is a $\Delta\mu_H$ across the membrane and also because of the drop in ATP levels. Furthermore the collapse of the $\Delta\psi_m$ may affect the $\Delta\psi_p$, for example, because of Ca^{2+} efflux from the mitochondria (Nicholls & Crompton, 1980). This is therefore not a satisfactory estimate of the plasma-membrane potential.

If the [^3H]TPMP $^+$ accumulation is a poor estimate of the plasma-membrane potential of fat-cells there are three other measurements that can be used. First, there are experiments in which micro-electrodes are embedded into fat-cells. The mean value for the resting membrane potential obtained by Beigelmann & Hollander (1962, 1963, 1964, 1965) was 44.1 mV. However, this value includes a large number of very low measurements (<20 mV) where correct insertion of the micro-electrode had probably not occurred and the more usual value obtained was in the region of 50 mV. Secondly, the accumulation of $^{86}\text{Rb}^+$ can be measured and used as an estimate of the potassium diffusion potential across the plasma membrane. We have reported that the rubidium diffusion potential is about 75 mV (Davis *et al.*, 1981). However, the potassium diffusion potential is probably greater than the resting plasma-membrane potential. Thirdly, the equilibrium distribution of $^{36}\text{Cl}^-$ has been suggested to be a measure of the fat-cell plasma-membrane potential because fat-cells are very permeable to Cl^- (Perry & Hales, 1969). The chloride diffusion potential across

the plasma membrane of fat-cells has been estimated to be 28.7 mV (Perry & Hales, 1969). In order for the chloride diffusion potential to be a good estimate of the plasma-membrane potential the principal chloride movement across the plasma membrane must be electrogenic. However, 30% of the chloride efflux from isolated fat-cells can be inhibited by suspending the cells in chloride-free media (Perry & Hales, 1970), which suggests that there may be Cl^-/Cl^- exchange. In some cell types, such as erythrocytes, there is evidence for an inwardly directed chloride pump (for review, see Knauf, 1979). If this was present in fat-cells it would cause the chloride diffusion potential to be smaller than the resting plasma-membrane potential. Until a reliable estimate of the $\Delta\psi_p$ is available the calculation of the $\Delta\psi_m$ is subject to a systematic error according to which estimate of the $\Delta\psi_p$ is used. Although this systematic error will affect the absolute size of the calculated $\Delta\psi_m$ it has no effect on the calculation of changes in the $\Delta\psi_m$ (unless there is also a change in the $\Delta\psi_p$).

β -Adrenergic effects on the apparent [^3H]TPMP $^+$ accumulation

The effect of β -adrenergic agonists to stimulate lipolysis and to increase O_2 uptake in an oligomycin-insensitive manner (Hepp *et al.*, 1968a) indicates that the great reduction in the [^3H]TPMP $^+$ accumulation by fat-cells when incubated with adrenaline or isoprenaline is due to a depolarization of the mitochondria. The non-esterified fatty acids released during lipolysis have been suggested to uncouple fat-cell mitochondria (Bihler & Jeanrenaud, 1970; Angel *et al.*, 1971) and the data here support this hypothesis.

In brown fat-cells β -adrenergic stimulation has been shown to be associated with an increase in O_2 uptake and an increase in the proton conductance of the mitochondrial inner membrane resulting in an uncoupling of oxidative phosphorylation (for review, see Nicholls, 1979). Recently it has been demonstrated that the increased O_2 uptake by brown fat mitochondria during β -adrenergic stimulation is due to the effect of fatty acids to increase the proton permeability of the mitochondrial inner membrane (Locke & Nicholls, 1981; Bukowiecki *et al.*, 1981). This specific effect of fatty acids accounts for the greater stimulation of O_2 uptake observed with brown fat-cells than with white fat-cells in the presence of lipolytic hormones (Joel, 1966) and for the greater sensitivity to uncoupling by non-esterified fatty acids that has been observed for mitochondria isolated from brown fat than from other tissues (Heaton & Nicholls, 1976). Brown adipose tissue is the major site of catecholamine-induced thermogenesis in mammals and the stimulation of mitochondrial O_2 uptake by fatty acids is physiologically

relevant to this function. In contrast with this effect the non-specific uncoupling by non-esterified fatty acids that has been suggested in white fat-cells is of uncertain physiological importance (Bihler & Jeanrenaud, 1970; Angel *et al.*, 1971).

R. J. D. holds an S.E.R.C. Studentship.

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