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Intracellular mitochondrial membrane potential as an indicator of hepatocyte energy metabolism: further evidence for thermodynamic control of metabolism

Michael N. Berry, Roland B. Gregory, Anthony R. Grivell, Debra C. Henly, Catherine D. Nobes, John W. Phillips and Patricia G. Wallace

Department of Medical Biochemistry, School of Medicine, Flinders University of South Australia, Bedford Park (Australia)

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The lipophilic triphenylmethylphosphonium cation ($TPMP^+$) has been employed to measure $\Delta\Psi_m$, the electrical potential across the inner membrane of the mitochondria of intact hepatocytes. The present studies have examined the validity of this technique in hepatocytes exposed to graded concentrations of inhibitors of mitochondrial energy transduction. Under these conditions, $TPMP^+$ uptake allows a reliable measure of $\Delta\Psi_m$ in intracellular mitochondria, provided that the ratio $[TPMP^+]_i/[TPMP^+]_e$ is greater than 50:1 and that at the end of the incubation more than 80% of the hepatocytes exclude Trypan blue. Hepatocytes, staining with Trypan blue, incubated in the presence of Ca^{2+}, do not concentrate $TPMP^+$. The relationships between $\Delta\Psi_m$ and two other indicators of cellular energy state, ΔG_{P_c} and E_h, or between $\Delta\Psi_m$ and J_0, were examined in hepatocytes from fasted rats by titration with graded concentrations of inhibitors of mitochondrial energy transduction. Linear relationships were generally observed between $\Delta\Psi_m$ and ΔG_{P_c}, E_h or J_0 over the $\Delta\Psi_m$ range of 120–160 mV, except in the presence of carboxyatractyloside or oligomycin, where $\Delta\Psi_m$ remained constant. Both the magnitude and the direction of the slope of the observed relationships depended upon the nature of the inhibitor. Hepatocytes from fasted rats synthesized glucose from lactate or fructose, and urea from ammonia, at rates which were generally linear functions of the magnitude of $\Delta\Psi_m$, except in the presence of oligomycin or carboxyatractyloside. Linear relationships were also observed between $\Delta\Psi_m$ and the rate of formation of lactate in cells incubated with fructose and in hepatocytes from fed rats. The linear property of these force–flow relationships is taken as evidence for the operation of thermodynamic regulatory mechanisms within hepatocytes.

Abbreviations: ΔG_P, ATP-phosphorylation potential. Subscript 'c' denotes the phosphorylation potential of the cytoplasmic compartment; E_h, mitochondrial redox potential; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical potential gradient across the inner mitochondrial membrane; $\Delta\Psi$, membrane electrical potential. Subscripts 'm' or 'p' denote the membrane potential across the inner mitochondrial or the plasma membrane, respectively; ΔpH_m, proton chemical potential gradient across the inner mitochondrial membrane; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TDGA, tetradecylglycidic acid; $TPMP^+$, triphenylmethylphosphonium cation. Subscripts 'i', 'e', 'c' and 'm' refer to intracellular, extracellular, cytoplasmic and mitochondrial pools, respectively.

Correspondence: M.N. Berry, Department of Medical Biochemistry, School of Medicine, Flinders University of South Australia, Bedford Park, SA 5042, Australia.

Introduction

The determination of $\Delta\Psi_m$ in isolated mitochondria by measurement of the uptake of a lipophilic cation such at $TPMP^+$ is a now well-established technique [1–3]. More recently, the same approach has been successfully employed to measure $\Delta\Psi_m$ within whole cells [4–8]. The measurement of $\Delta\Psi_m$ with $TPMP^+$ or other lipophilic cations in intact cells is complicated by the presence of an electrical potential difference across the plasma membrane ($\Delta\Psi_P$) which will influence cation distribution [1,2], and by the multiplicity of organellar compartments, which might be expected to lead to non-homogeneous distribution of $TPMP^+$. However, it appears that, in practice, only mitochondria have sufficient membrane potential and volume to give measurable cellular $TPMP^+$ uptake above that resulting from the influence of $\Delta\Psi_P$ [4]. Thus, by making appropriate corrections, a number of workers have succeeded in obtaining values for $\Delta\Psi_m$ in whole cells, including hepatocytes [4,5], adipocytes [6,7] and lymphocytes [8].

Although several groups have observed a fall in $\Delta\Psi_m$ on exposure of cells to a respiratory inhibitor or an uncoupling agent [4,5,7], as yet there has been no systematic study of the relationship of $\Delta\Psi_m$ to other measures of 'energy state' or metabolic activity. We report here a number of relationships of this kind, which confirm that $\Delta\Psi_m$, as derived from cellular $TPMP^+$ uptake, is a useful indicator of the hepatocyte's capacity for energy-dependent processes. The linear nature of many of the observed relationships provides additional evidence for the operation of thermodynamic regulatory mechanisms within the liver cell [9,10].

Materials and Methods

Materials

Collagenase and enzymes for metabolite determination were from Boehringer Mannheim (F.R.G.), as was bovine serum albumin (fraction V) which was defatted by the method of Chen [11]. TDGA was a gift from Dr. G.F. Tutwiler (McNeil Pharmaceuticals, PA, U.S.A.). Palmitate, digitonin, rotenone, valinomycin, oligomycin, carboxyatractyloside and antimycin were obtained from Sigma (U.S.A.), DL-2-bromopalmitate came from Fluka (Switzerland) and FCCP from Aldrich (U.S.A.). Fatty acids were neutralized, and dissolved in 0.15 M NaCl containing 9% bovine serum albumin, and other water-insoluble compounds were dissolved in dimethylsulphoxide. NCS tissue solubilizer, ACS II liquid scintillation fluid, [^{14}C]$TPMP^+$ iodide, [^{14}C]hydroxymethylinulin, [^{3}H]inulin, $^{36}Cl^-$ and ^3H_2O were purchased from Amersham International (U.K.). Percoll was obtained from Pharmacia (Sweden) and silicone oil from DOW Corning (U.S.A.). Other chemicals were of the highest quality commercially available.

Methods for cell preparation, metabolite analysis, microscopy and morphometry

Isolated liver cells from male Hooded Wistar rats (250–280 g body wt.), starved for 24 h to deplete liver glycogen, were prepared by a modification [12] of the method of Berry and Friend [13]. The cells (90–120 mg wet wt.) were incubated at 37 °C in 2 ml balanced bicarbonate-saline medium [14,15] containing albumin, 2.25% (w/v), with a gas phase of 95% O_2/5% CO_2. O_2 consumption in the presence of CO_2 was measured manometrically [16]. At the completion of the incubation period, a sample was taken for $\Delta\Psi_m$ estimation (see below) and the remainder deproteinized with an equal volume of 1 M perchloric acid and neutralized. DNA was measured according to Ref. (17). Metabolites were measured by standard enzymatic techniques as in Ref. (18), by means of a Cobas FARA automated analyser (Roche Diagnostics, Basle), the data being transferred to a PDP 11/73 computer (D.E.C., U.S.A.) for subsequent processing. All fitted, straight-line relationships were calculated by least-squares linear regression analysis of untransformed data.

Hepatocytes were counted using a Neubauer haemocytometer and the proportion of damaged cells which stained with 0.2% Trypan blue was estimated. For determination of mitochondrial volume, cells were fixed by standard procedures for electron microscopy using glutaraldehyde and osmium tetroxide, dehydrated and embedded in Araldite [19]. Thin sections were stained with uranyl acetate and lead citrate [13] and examined with a Philips 201 electron microscope. Mitochon-

drial volume was determined by the morphometric method of Weibel [20].

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) in intact hepatocytes

The mitochondrial membrane potential ($\Delta\Psi_m$) of isolated hepatocytes was measured by including 2.5 μM [^{14}C]TPMP$^+$ in the incubation medium. At the end of the incubation period, (generally 40 min), 0.5 ml of the incubation mixture was rapidly centrifuged (10000 $\times g$, 30 s) and the TPMP$^+$ content of the supernatant determined by scintillation counting to estimate $[TPMP^+]_e$. Alternatively, the cell pellet was dissolved in 0.5 ml NCS tissue solubilizer and a portion (0.2 ml) counted to provide $[TPMP^+]_i$. The sum of these measurements invariably resulted in a quantitative recovery of the added [^{14}C]TPMP$^+$.

$\Delta\Psi_m$ is derived on the assumption that the outer mitochondrial membrane is freely permeable to TPMP$^+$ and that there is a Nernstian distribution of the cation in free solution across the inner mitochondrial membrane according to:

$$\Delta\Psi_m = 61.5\ ^{10}\log\frac{[TPMP^+]_m}{[TPMP^+]_c} \quad (1)$$

Initial studies showed that, under controlled conditions, steady-state levels for $[TPMP^+]_i$ were obtained within 15 min of incubation, more than 95% of the $[TPMP^+]_i$ being found by digitonin fractionation studies [21] to represent $TPMP^+_m$. On subsequent addition of a respiratory inhibitor, $[TPMP^+]_i$ fell to a new steady state within 15 min. This fall in $[TPMP^+]_i$ within the hepatocytes, following the addition of graded concentrations of inhibitors, was shown by the fractionation studies to be a consequence of a decrease in $[TPMP^+]_m$. We consider that these observations justify the derivation of $[TPMP^+]_m$ from $[TPMP^+]_i$. Accordingly, $[TPMP^+]_i$ has been calculated on the basis of a cell-water volume of 2.1 mg/g dry weight and total $[TPMP^+]_m$ has been further estimated by assuming a mitochondrial matrix space of 12% of cell volume [4]. Hence,

$$[TPMP^+]_m \cong \frac{[TPMP^+]_i}{0.12}$$

At high inhibitor concentrations, our fractionation studies showed a significant rise in $[TPMP^+]_c$ so that $[TPMP^+]_m$ could no longer be derived accurately from $[TPMP^+]_i$. However, the studies indicated that provided the ratio $[TPMP^+]_i/[TPMP^+]_e$ is greater than 50:1, $[TPMP^+]_i/0.12$ can be substituted for $[TPMP^+]_m$ in Eqn. 1, with the introduction of a maximal error of ± 6 mV. All data presented in this paper comply with this requirement.

Brown and Brand [22] have argued that, over a wide range of mitochondrial membrane potential, 67% of the TPMP$^+$ associated with the mitochondria is bound in a nonspecific manner. The digitonin fractionation studies described above revealed that less than 5% of the TPMP$^+$ was associated with the non-mitochondrial fraction in intact cells and on this basis we have adopted the same correction factor as used by Brown and Brand [22] for isolated mitochondria. Thus, for the purposes of Eqn. 1, $[TPMP^+]_m$ in free solution was taken as 33% of total $[TPMP^+]_m$. A correction has also been necessary for the measured binding of 10% of the extracellular TPMP$^+$ to bovine serum albumin which was present at a concentration of 2.25% in all cell incubations. The contribution of the plasma membrane potential ($\Delta\Psi_p$) was calculated from the $^{36}Cl^-$ distribution between the intracellular and extracellular spaces [23] after centrifugation of the cells through silicone oil. The extracellular space was measured with [^{14}C]hydroxymethylinulin and the intracellular space with ^3H_2O [8,24]. In all experiments in which $\Delta\Psi_P$ was measured, a value of 32 ± 2 mV was obtained, indicating a concentration gradient of TPMP$^+$ across the plasma membrane of 3.31, so that $[TPMP^+]_c \cong 3.31 \times 0.9 \times [TPMP^+]_e$.

With these assumptions and corrections Eqn. 1 may be restated as follows:

$$\Delta\Psi_m = 61.5\ ^{10}\log\frac{[TPMP^+]_i \times 0.33}{[TPMP^+]_e \times 3.31 \times 0.9 \times 0.12} \quad (2)$$

Measurement of mitochondrial redox potential

Values for mitochondrial redox potential (E_h), defined as the half-cell reaction potential of free [NAD$^+$]/free [NADH] according to the equation (see Ref. 25):

$$E_h = E^{\circ\prime} + \frac{RT}{nF}\ln\frac{[\text{acetoacetate}]}{[\text{3-hydroxybutyrate}]}$$

were obtained by measurement of the concentrations of acetoacetate and 3-hydroxybutyrate, on the assumption of an $E^{0'}$ at 37°C of −0.297 V [26].

Separation of damaged from intact cells

In order to separate cells stained with Trypan blue from unstained cells, advantage was taken of the fact that the stained cells have a considerably lower density due to loss of cytoplasmic contents [27]. Percoll was mixed with 1.54 M NaCl solution (9 : 1, v/v) and the resultant mixture diluted with bicarbonate-free incubation medium (42 : 58, v/v) to yield a Percoll-saline solution of appropriate density. Samples (1.4 ml) from cell incubations were mixed with this Percoll-saline in a total volume of 10 ml and centrifuged for 1 min at $1550 \times g$. The pelleted cells were gently resuspended in a total volume of 1 ml incubation medium (bicarbonate-free). Contamination of the pellet with the original incubation medium was assessed using [^{3}H]inulin. Cells accumulating at the top of the supernatant were collected and washed in an equal volume of bicarbonate-free incubation medium, recovered by centrifugation for 1 min at $1550 \times g$, and gently resuspended in 0.5 ml of incubation medium. Samples from both fractions were taken for determination of DNA, $TPMP^+$ content and Trypan blue uptake. Over 90% of the cells forming the pellet excluded Trypan blue, whereas all cells collected from the top of the supernatant stained.

Results

Measurement of $\Delta\Psi_m$ in hepatocytes

A number of groups who have successfully determined $\Delta\Psi_m$ from measurements in whole cells rather than isolated mitochondria have discussed in detail the various factors that must be taken into consideration [4–8]. In deriving $\Delta\Psi_m$ from cellular $TPMP^+$ uptake, it is necessary to know the dry weight of the cell mass and the proportion of cell water represented by the mitochondrial matrix, as well as the distribution and degree of binding of $TPMP^+$ within the hepatocyte. Other possible factors that need to be taken into account include cellular heterogeneity, cell damage and barriers to $TPMP^+$ uptake, as well as the effects of high $[TPMP^+]_m$ on mitochondrial metabolism.

In our studies, light microscopy revealed that cells exposed to high concentrations of inhibitor, e.g., 2–4 μM antimycin, acquired a characteristic 'blebbing' similar to that described by Ross et al. [28], but otherwise were not grossly changed in size. Those cells that took up Trypan blue after exposure to inhibitor, had rounded, grossly swollen mitochondria, whereas cells that excluded Trypan blue had mitochondria of normal shape but increased volume. Whereas a population of isolated mitochondria can undergo substantial and variable swelling [29], in hepatocytes mitochondrial swelling appears minimal unless gross cell damage exists. For example, electron-microscopic morphometric studies showed that the mitochondria of cells incubated with 25 μM FCCP but not staining with trypan blue were increased in volume by 25%. If this increase, which was the greatest observed, were not taken into account, the value of $\Delta\Psi_m$ would be overestimated by only 5 mV. Moreover, for lower concentrations of inhibitors, substantially less swelling was observed. Accordingly, correction of $\Delta\Psi_m$ for increase in mitochondrial volume was considered to be unnecessary in the context of the current studies.

Conventional methods for the preparation of isolated hepatocytes yield suspensions comprising greater than 95% parenchymal cells [13]. Hence, in studying $TPMP^+$ uptake, heterogeneity of cell type does not present a problem. However, it is not possible to prepare suspensions in which all cells exclude Trypan blue, and an increase in the number staining invariably occurs during incubation. In a normal preparation, about 5–8% of the cells are stained initially and this increases to 10–15% over an incubation period of 40 min. When cells are exposed to potent inhibitors of respiration or energy transduction, a much greater degree of staining can occur, so that a suspension of cells treated with high concentrations of FCCP or cyanide may have more than 50% of the population taking up Trypan blue. We therefore investigated whether cells susceptible to staining can concentrate $TPMP^+$. Cells were incubated for 40 min with various metabolic inhibitors and then rapidly mixed with Percoll-saline and centrifuged. Pelleted cells (of which more than 90% excluded Trypan blue) consistently retained higher levels of $TPMP^+$ per mg DNA than were found in the

TABLE I

UPTAKE OF $TPMP^+$ BY INTACT HEPATOCYTES

Hepatocytes were incubated for 40 min with palmitate (2 mM) and [^{14}C]$TPMP^+$ (2.5 μM) in the presence of various inhibitors of energy transduction. Intact cells, which exclude Trypan blue, were recovered using the Percoll technique described in the Materials and Methods section. The results are taken from a representative experiment.

Inhibitor	$TPMP^+$/DNA (nmol/mg)	
	whole suspension	intact cells
None	29	31
Antimycin (4 μM)	16	21
FCCP (30 μM)	6.5	8
Oligomycin (1 μM)	23.5	28
Valinomycin (200 nM)	10	13

original whole cell suspension (Table I). In contrast, cells from the supernatant layer (all of which took up Trypan blue) were unable to concentrate $TPMP^+$. The failure of the mitochondria of cells stained with Trypan blue to maintain an electrical potential difference across the inner mitochondrial membrane can be explained on the basis that such cells are readily permeable to Ca^{2+} [27,30]. Exposure of mitochondria to the concentrations of Ca^{2+} found in the incubation medium is known to lead to their destruction and loss of respiratory activity [31].

In our subsequent studies we avoided inaccuracies in determination of $\Delta\Psi_m$, ΔG_P and E_h due to high levels of cellular damage, either by accurately counting the percentage of staining cells after the incubation, or by using inhibitor concentrations which maintained the percentage of intact cells at levels at least 80% of those seen in control incubations. Failure to take into account a loss of 20% of the intact cell mass during incubation with an inhibitor would lead to an underestimation of $\Delta\Psi_m$ of only 6 mV.

Prior to studying the relationship between J_0 and $\Delta\Psi_m$, we investigated possible deleterious effects of $TPMP^+$ on liver cell metabolism. These were seen only at added concentrations of $TPMP^+$ of 10 μM or greater, corresponding to $[TPMP^+]_m$ of 25 mM. The effects observed included a fall in J_0 and [ATP]/[ADP], a rise in the ratios [3-hydroxybutyrate]/[acetoacetate] and [lactate]/[pyruvate] and a decrease in the rate of gluconeogenesis. At added concentrations of 2.5 μM $TPMP^+$ no inhibitory effects on metabolism were observed. Hence, this concentration of $TPMP^+$ was chosen for determining $\Delta\Psi_m$ in the experiments reported here. A similar concentration has been used in studies with isolated mitochondria [22].

Relationships between $\Delta\Psi_m$ and J_0

We examined the relationship between J_0 and $\Delta\Psi_m$ by incubating liver cells from fasted rats with added palmitate, which is the main hepatic fuel of respiration [32]. Under these circumstances, J_0 could be suppressed either with inhibitors of fatty acid oxidation or with inhibitors of electron transport. However, for similar degrees of inhibition of J_0, rotenone and antimycin were more effective in

TABLE II

COMPARISON OF THE EFFECTS OF RESPIRATORY INHIBITORS AND INHIBITORS OF FATTY ACID OXIDATION ON $\Delta\Psi_m$ AND RESPIRATORY RATE

Hepatocytes were incubated as described in Materials and Methods, with 2 mM palimitate and the inhibitors as listed below. In the case of TDGA, cells were pre-incubated with the inhibitor for 5 min at 37 °C. Results are expressed as mean ± S.E. and are derived from at least 12 experiments except in the case of bromopalmitate (2 experiments).

Inhibitor	J_0		$\Delta\Psi_m$	
	μmol per g wet wt. cells per min	% inhibition	mV	% decrease
None	3.39 ± 0.03	–	164.9 ± 0.7	–
TDGA (0.25 μM)	2.36 ± 0.05	30	155.1 ± 1.0	6
Bromopalmitate (100 μM)	2.24	34	153.7	7
Rotenone (1 μM)	2.46 ± 0.08	27	142.0 ± 2.4	14
Antimycin (2 μM)	2.24 ± 0.08	34	134.5 ± 1.6	18

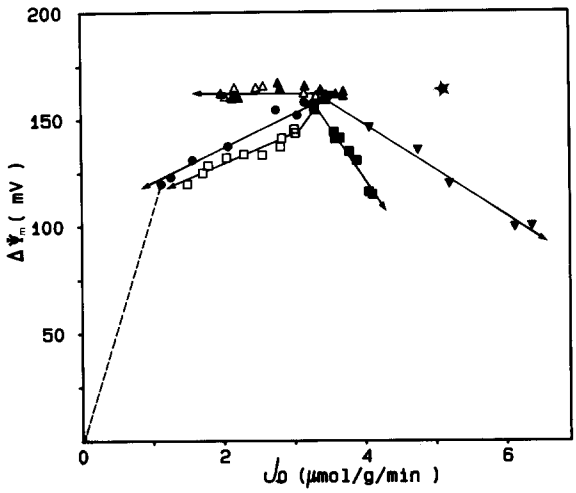

Fig. 1. The effect of inhibitors of energy transduction on $\Delta\Psi_m$ and J_0. Hepatocytes from fasted rats were incubated with 2 mM palmitate for 40 min at 37 °C as described in Materials and Methods. Graded concentrations of inhibitors and ionophores were added as follows: ●, rotenone (0–8 µM); □, antimycin (0–4 µM); ■, FCCP (0–18 µM); ▼, valinomycin (0–25 nM); ▲, oligomycin (0–0.72 µM) and △, carboxyatractyloside (0–175 µM). Arrowheads indicate the direction of increasing concentration of inhibitor or ionophore. The data are taken from one or two representative experiments for each inhibitor, and hence not all inhibitor titrations share a common control point. The single point (*) represents mean values from 10 experiments for $\Delta\Psi_m$ and J_0 for cells incubated with 2 mM palmitate, 10 mM lactate and 1 mM pyruvate.

lowering $\Delta\Psi_m$ than were bromopalmitate [33] and TDGA [34] (Table II). This lack of a constant relationship between J_0 and $\Delta\Psi_m$ was especially evident when we studied the effects of graded concentrations of various inhibitors on mitochondrial energy transduction. For hepatocytes exposed to rotenone, the plot for J_0 vs. $\Delta\Psi_m$, measured over the range for which reliable values of $\Delta\Psi_m$ could be obtained (i.e., between 120 and 165 mV), was best represented by a straight line, each alteration in J_0, in response to variation in rotenone concentration being accompanied by a proportional change in $\Delta\Psi_m$ (Fig. 1). With antimycin, this linearity was apparent only over the latter part of the titration. Graded concentrations of the uncoupling agent FCCP, or the ionophore valinomycin, stimulated J_0 while depressing $\Delta\Psi_m$, an inverse linear relationship between J_0 and $\Delta\Psi_m$ being maintained over a considerable range of inhibitor concentration. In contrast to the action of FCCP or valinomycin, addition of a gluconeogenic substrate such as lactate brought about a substantial stimulation of J_0 but caused no detectable decline in $\Delta\Psi_m$. The ATP synthase inhibitor, oligomycin, or the inhibitor of adenine nucleotide translocation, carboxyatractyloside, depressed J_0 but had almost no effect on $\Delta\Psi_m$.

Relationship between $\Delta\Psi_m$ and ΔG_{P_c}

A systematic examination of the effects of metabolic inhibitors, employing digitonin fractionation, indicated that, except in the case of carboxyatractyloside, extramitochondrial [ATP]/[ADP] ratios closely followed mitochondrial ratios (data not presented). As the extramitochondrial compartment contains approx. 75% of the total cellular adenine nucleotides [35,36], we concluded that for all inhibitors studied, including carboxyatractyloside where intramitochondrial levels of adenine nucleotides were unchanged, the measurement of cellular ATP and ADP provides a reliable indication of the concentration ratios of these nucleotides in the extramitochondrial compartment. Cytoplasmic [Pi] did not vary substantially in cells capable of excluding Trypan blue over the ranges of inhibitors used, levels between 6 and 9 mM being maintained. Values for [Pi] of 8 mM [36] and for $\Delta G_P^{\circ\prime}$ of 300 mV [37] have been employed in calculating ΔG_{P_c}.

The relationships between ΔG_{P_c} and $\Delta\Psi_m$ in the presence of graded concentrations of inhibitors of the respiratory chain, FCCP or valinomycin (where more than 80% of cells excluded Trypan blue) are shown in Fig. 2. Except in the presence of antimycin, where the plot of ΔG_{P_c} vs. $\Delta\Psi_m$ was sigmoidal, ΔG_{P_c} tended to be a linear function of $\Delta\Psi_m$, the value of the slope for $\Delta\Psi_m$ vs. ΔG_{P_c} varying from about 0.7 to 2.6, dependent upon the agent added. No correlation between $\Delta\Psi_m$ and ΔG_{P_c} was seen in the presence of oligomycin and carboxyatractyloside (Fig. 2). Whereas $\Delta\Psi_m$ was maintained almost constant over a wide range of concentrations of oligomycin or carboxyatractyloside, ΔG_{P_c} fell as the inhibitor concentration was increased. Except in preparations of severely damaged cells, where levels of ATP were immeasurably low, the value of ΔG_{P_c} generally did not fall below 400 mV, i.e., equivalent to an [ATP]/[ADP] ratio of 0.3.

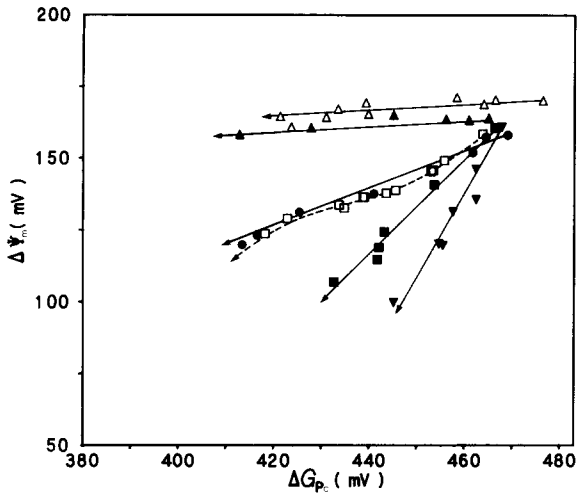

Fig. 2. Correlation between $\Delta\Psi_m$ and ΔG_{P_c} with various inhibitors of energy transduction. Hepatocytes from fasted rats were incubated as described for Fig. 1 together with graded concentrations of inhibitors as follows: ●, rotenone (0–8 μM); □- - - - - -□, antimycin (0–3 μM); ■, FCCP (0–25 μM); ▼, valinomycin (0–20 nM); ▲, oligomycin (0–0.6 μM) and △, carboxyatractyloside (0–100 μM). Each plot shows data from one or two representative experiments and hence not all the plots share a common control point. The arrowheads indicate the direction of increasing inhibitor concentration.

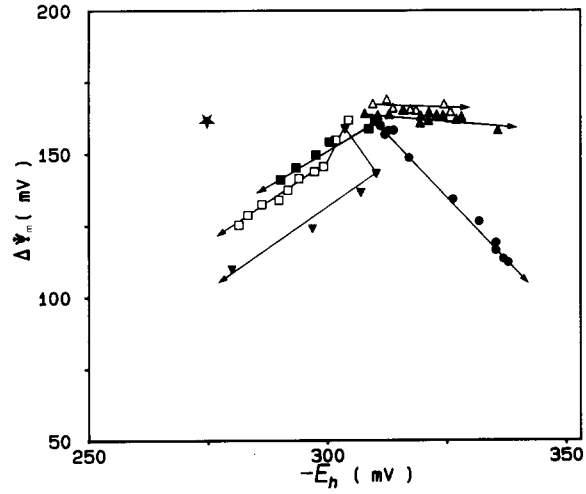

Fig. 3. The effect of inhibitors of energy transduction on $\Delta\Psi_m$ and mitochondrial redox potential. Hepatocytes from fasted rats were incubated as described for Fig. 1. The concentration ranges for the various inhibitors were: ●, rotenone (0–8 μM); □, antimycin (0–4 μM); ■, FCCP (0–12.5 μM); ▼, valinomycin (0–20 nM); ▲, oligomycin (0–0.65 μM) and △, carboxyatractyloside (0–100 μM). Each plot shows data from one or two representative experiments and hence not all plots share a common control point. The arrowheads indicate the direction of increasing inhibitor concentration. The isolated point (*) represents the mean value from 15 experiments for incubations carried out in the absence of added palmitate.

Relationship between $\Delta\Psi_m$ and E_h

In numerous experiments with a selection of inhibitors of energy transduction, linear relationships between $\Delta\Psi_m$ and E_h were found over a range of inhibitor concentrations (Fig. 3). For some agents, e.g., rotenone and FCCP, linearity was maintained over the whole titration. On the other hand, for antimycin and valinomycin this was not the case. Fig. 3 demonstrates some of these quantitative relationships. In the presence of antimycin, FCCP or higher concentrations of valinomycin, E_h and $\Delta\Psi_m$ both declined with increasing concentrations of inhibitor, whereas at very low valinomycin concentrations $\Delta\Psi_m$ fell but a small rise in E_h was noted. However, the fall in $\Delta\Psi_m$ associated with exposure of cells to rotenone was accompanied by a rise in E_h. Changes in E_h were not invariably accompanied by alterations in $\Delta\Psi_m$. For example, treatment of cells with oligomycin or carboxyatracyloside increased E_h but had little effect on $\Delta\Psi_m$. Moreover, the substantial stimulation of respiration induced by fatty

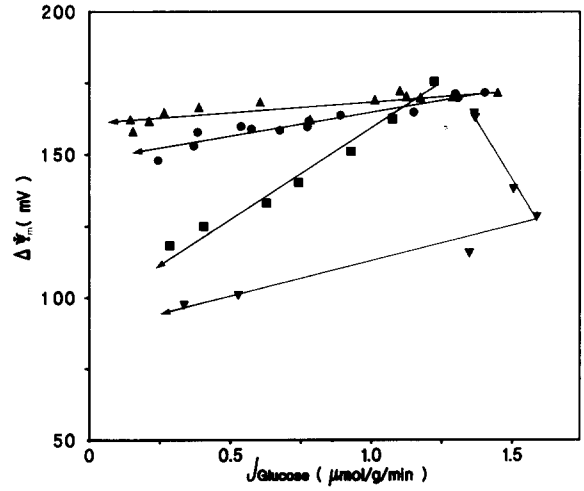

Fig. 4. The effect of various inhibitors of energy transduction on $\Delta\Psi_m$ and the rate of glucose formation from lactate. Hepatocytes from fasted rats were incubated at 37° C for 40 min in the presence of 2 mM palmitate, 10 mM lactate and 1 mM pyruvate, with graded concentrations of the following inhibitors: ●, rotenone (0–2 μM); ■, FCCP (0–30 μM); ▼, valinomycin (0–40 nM) and ▲, oligomycin (0–0.7 μM). Arrowheads indicate the direction of increasing inhibitor concentration. Each plot is data from a single but different representative experiment, and hence the individual titrations do not share a common control point.

acid also increased E_h yet had only a trivial effect on $\Delta\Psi_m$.

Relationship between $\Delta\Psi_m$ and metabolic flux

The ability of hepatocytes from fasted rats to synthesize glucose or urea is a well-recognized indicator of cellular integrity. We measured rates of gluconeogenesis from lactate in the presence of palmitate and graded concentrations of various inhibitors and found in a number of instances that there was a linear correlation between $J_{glucose}$ and the magnitude of $\Delta\Psi_m$ (Fig. 4). A linear relationship between $\Delta\Psi_m$ and $J_{glucose}$ was observed in the presence of rotenone and FCCP. However, this relationship was lost in cells exposed to oligomycin, or carboxyatractyloside (not shown) both of which decreased $J_{glucose}$ but did not affect $\Delta\Psi_m$. Valinomycin was of particular interest in that low concentrations caused a sharp fall in $\Delta\Psi_m$ yet had a small stimulatory effect on $J_{glucose}$ (Fig. 4). At higher concentrations of valinomycin, $\Delta\Psi_m$ and $J_{glucose}$ fell concomitantly.

Linear relationships were also demonstrated between $\Delta\Psi_m$ and rates of other synthetic processes, e.g., gluconeogenesis from fructose in the presence of graded concentrations of rotenone and antimycin but not of oligomycin (Fig. 5). When ureogenesis from ammonia was examined, we found a linear relationship between $\Delta\Psi_m$ and J_{urea} over the whole of the range of the titration with rotenone. In the case of antimycin, FCCP and valinomycin, there was an initial sharp decline in $\Delta\Psi_m$ at low concentrations of inhibitor, followed by a linear relationship between $\Delta\Psi_m$ and J_{urea} (Fig. 6).

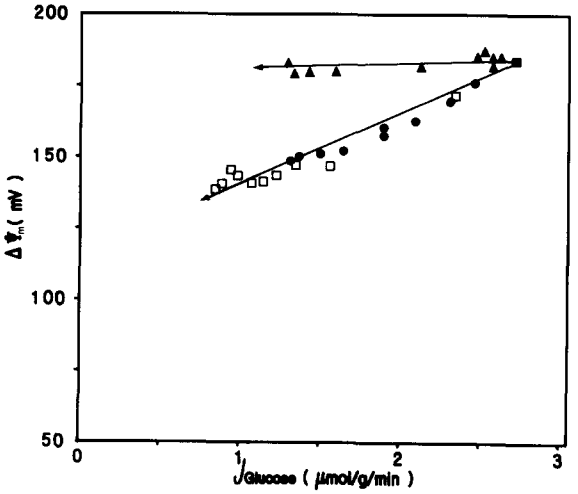

Fig. 5. The effect of inhibitors of energy transduction on $\Delta\Psi_m$ and the rate of glucose formation from fructose. Hepatocytes from fasted rats were incubated for 30 min at 37 °C in the presence of 10 mM fructose and graded concentrations of the following inhibitors: ●, rotenone (0–4 μM); □, antimycin (0–4 μM) and ▲, oligomycin (0–1.1 μM). The data are derived from a single representative experiment. Arrowheads indicate increasing concentrations of inhibitors.

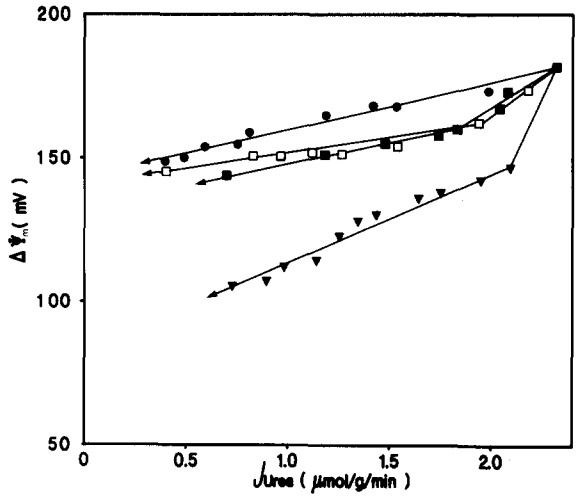

Fig. 6. The effect of inhibitors of energy transduction on $\Delta\Psi_m$ and the rate of urea production from ammonia. Hepatocytes from fasted rats were incubated for 35 min at 37 °C in the presence of 2 mM palmitate, 10 mM lactate, 1 mM pyruvate, 12 mM ammonium chloride and 2 mM ornithine, as well as graded concentrations of the following inhibitors: ●, rotenone (0–4 μM); □, antimycin (0–1 μM); ■, FCCP (0–20 μM) and ▼, valinomycin (0–100 nM). Arrowheads indicate the direction of increasing concentrations of inhibitors. The data are derived from one or two representative experiments.

Although for each individual inhibitor of energy transduction there was a consistent relationship between the rate at which the cells synthesized glucose (or urea) and the magnitude of $\Delta\Psi_m$, this relationship was highly dependent on the nature of the substrate and inhibitor used. Thus for rotenone, a small fall in $\Delta\Psi_m$ was associated with a substantial decrease in $J_{glucose}$, whereas this degree of depression of gluconeogenesis was accompanied by a much greater fall in $\Delta\Psi_m$ in the presence of FCCP or valinomycin (Fig. 4).

The relationship between $\Delta\Psi_m$ and glycolytic flux was examined in hepatocytes from fed rats. In

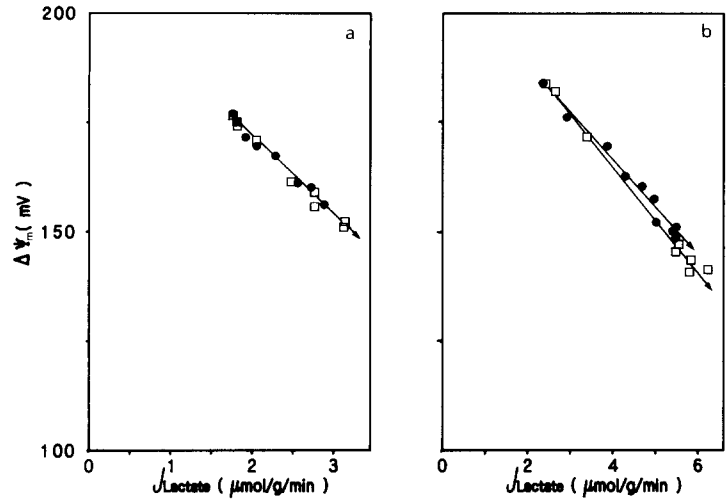

Fig. 7. (a) The effect of the respiratory inhibitors antimycin and rotenone on $\Delta\Psi_m$ and the rate of lactate production from endogenous glycogen. Hepatocytes from fed rats were incubated in the presence of 1 mM 3-hydroxybutyrate and 0–4 µM antimycin (□) or 0–8 µM rotenone (●). The data are derived from a single representative experiment. Arrowheads indicate increasing concentrations of inhibitors. (b) The effect of antimycin and rotenone on $\Delta\Psi_m$ and the rate of lactate production from fructose. Hepatocytes from fasted rats were incubated as described for Fig. 5. Rotenone (●) and antimycin (□) were present at 0–4 µM and 0–2 µM, respectively. The plot shows a single representative experiment with inhibitor concentration increasing in the direction of the arrowheads.

the fed state and in the absence of inhibitors, $\Delta\Psi_m$ was approx. 10 mV higher than that in cells from fasted animals. Linear relationships were observed between $\Delta\Psi_m$ and $J_{lactate}$ when hepatocytes from fed rats were exposed to graded concentrations of rotenone or antimycin (Fig. 7a). In the presence of fructose, hepatocytes from fasted rats exhibited a value for $\Delta\Psi_m$ of the same order of magnitude as that seen in cells from fed animals. $J_{lactate}$ from fructose was also found to be linearly related to $\Delta\Psi_m$ for cells exposed to rotenone or antimycin (Fig. 7b).

Discussion

Limitations of $\Delta\Psi_m$ measurement with TPMP$^+$

Several groups have used the lipophilic cation $TPMP^+$ to measure $\Delta\Psi_m$ across the inner membrane of the intracellular mitochondria of intact hepatocytes [4–5]. The work described here confirms the validity of these studies, but identifies certain limitations on the use of $TPMP^+$. In hepatocytes exposed to high concentrations of inhibitor where $[TPMP^+]_i/[TPMP^+]_e$ is less than 50 : 1, $[TPMP^+]_c$ increases substantially, with the consequence that $[TPMP^+]_i$ can no longer be considered solely to reflect $[TPMP^+]_m$. Moreover, suspensions containing a high percentage of cells susceptible to staining with Trypan blue will provide erroneous values for $\Delta\Psi_m$, since such cells incubated in the presence of Ca^{2+} do not take up $TPMP^+$. Nevertheless, if extreme experimental stresses are avoided, the use of $TPMP^+$ uptake would appear to provide a valuable approach to the measurement of $\Delta\Psi_m$ in intracellular mitochondria. Certainly, the difficulties associated with the large and variable degrees of swelling observed with isolated mitochondria [29] are avoided with the intact cell preparation.

In studies with intact cells it has been assumed that the binding of $TPMP^+$ to the intracellular mitochondria of hepatocytes is similar in nature to its binding to isolated mitochondria. Unfortunately, the measurement of mitochondrial binding of lipophilic cations is fraught with uncertainty and consequently there is no consensus as to the degree of binding [4,22,38,39]. However, in most studies it is the relative value of $\Delta\Psi_m$ under differing experimental circumstances, rather than its absolute value, that is of interest. Hence, if as assumed by others [22], the degree of binding remains constant over the range of values for $\Delta\Psi_m$ under study, an incorrect estimate of binding is unlikely to lead to erroneous conclusions, pro-

vided these are based on the relative differences in $\Delta\Psi_m$ obtained under various experimental conditions, rather than on absolute values.

Relationships between $\Delta\Psi_m$ and J_0

In addition to validating the measurement of $\Delta\Psi_m$ in intact cells, a major aim of this study has been to examine the relationships between $\Delta\Psi_m$ and other indicators of cellular energy state. According to chemiosmotic convention, the magnitude of $\Delta\tilde{\mu}_{H^+}$ (and hence, at least in animal mitochondria [2], of $\Delta\Psi_m$) is determined by the balance between its generation by means of electron transport and its dissipation due to backflow of protons through the inner mitochondrial membrane. This backflow can be the result of nonspecific leakage or passage of protons through the ATP synthase during oxidative phosphorylation. The shape of the curve for J_0 vs. $\Delta\Psi_m$ can be interpreted on this basis.

In the presence of inhibitors of electron transport (e.g., rotenone), the plot of J_0 vs. $\Delta\Psi_m$ can be regarded as consisting of two parts, a region where $\Delta\Psi_m$ can be readily measured, i.e., $\Delta\Psi_m$ lying approximately between 120 and 165 mV, and a region where $\Delta\Psi_m$ is less than 120 mV and where accurate measurement of $\Delta\Psi_m$ is technically difficult due to cell damage. However, if it is assumed that in cells from fasted rats no $\Delta\Psi_m$ is generated when J_0 is zero, it can be inferred that the portion of the putative curve from 0 to 120 mV (Fig. 1, broken lines) is much steeper than the measured slope from 120 to 165 mV. Over the latter range, linear relationships between $\Delta\Psi_m$ and J_0 and between $\Delta\Psi_m$ and ΔG_{P_c} are demonstrable in the presence of some, but not all, inhibitors.

The biphasic nature of the plot of J_0 vs. $\Delta\Psi_m$ appears to reflect the fact that ATP synthesis occurs only at values for $\Delta\Psi_m$ above approx. 120 mV. At values of $\Delta\Psi_m$ below this, intracellular [ATP] and the [ATP]/[ADP] ratio do not alter, and there is no evidence of ATP synthesis. Hence, it appears that in the intact hepatocyte a substantial portion of the oxygen uptake, although mitochondrial in origin, is not directly linked to phosphorylation. The slope of J_0 vs. $\Delta\Psi_m$ flattens sharply above 120 mV, where an increment in the ratio [ATP]/[ADP] and a rise in [ATP] levels signal the onset of ATP synthesis. This pattern is consistent with the suggestion that the ATP synthase may be 'gated', proton flow through the enzyme not occurring if $\Delta\Psi_m$ is below about 110 mV [40]. Whether or not this is the case, it seems likely that the flatter slope for J_0 vs. $\Delta\Psi_m$ occurring at values for $\Delta\Psi_m$ above 120 mV reflects proton flow back to the matrix through the ATP synthase [41]. If this backflow is prevented with oligomycin or carboxyatractyloside, $\Delta\Psi_m$ is maintained at maximal levels despite the marked reduction in J_0 brought about by these inhibitors.

Linear relationships between J_0 and $\Delta\Psi_m$ have occasionally been observed with isolated mitochondrial preparations and have been explained on the basis that the inner mitochondrial membrane exhibits ohmic properties [42]. The fall in $\Delta\Psi_m$ and associated rise in J_0 on exposure of hepatocytes to uncoupling agents or ionophores has also been observed with isolated mitochondria and is readily explicable in terms of chemiosmotic theory [41]. The inverse linear relationship between $\Delta\Psi_m$ and J_0 which occurs over a considerable range of uncoupler or ionophore concentrations is not observed, however, when J_0 is stimulated by the increased ATP demand induced by addition of a gluconeogenic substrate. Corresponding differences between the effects of uncoupling agents and ATP demand on $\Delta\Psi_m$ have been reported for isolated mitochondrial preparations, but a complete explanation for these findings has not yet been advanced [43,44].

Measurement of mitochondrial 'energy state': relationships between $\Delta\Psi_m$, ΔG_{P_c} and E_h

In many studies on whole cells, ΔG_{P_c} has been used as an indirect measure of mitochondrial 'energy state', on the assumption that ΔG_{P_c} reflects ΔG_{P_m}. In the fasted state, this will generally be the case, although the presence of fructose in high concentration can cause a marked reduction in cytoplasmic adenine nucleotides [45]. In the fed state, glycolysis can partially maintain hepatic cytoplasmic levels of ATP, even when mitochondrial respiration is markedly inhibited (unpublished observations). Under these circumstances, ΔG_{P_c} gives less insight into mitochondrial energy status, and $\Delta\Psi_m$ is perhaps the more useful parameter. However, under most experimental conditions, $\Delta\Psi_m$ tends to parallel ΔG_{P_c} so that measurement of

either will provide a guide to the cell's capacity for energy-dependent function, such as gluconeogenesis. An important exception relates to hepatocytes treated with oligomycin or carboxyatractyloside, which both lower ΔG_{P_c} without reducing $\Delta\Psi_m$.

Another example of a disparity between ΔG_{P_c} and $\Delta\Psi_m$ is seen in hepatocytes treated with valinomycin. Low concentrations of this inhibitor bring about a substantial fall in $\Delta\Psi_m$ with little effect on ΔG_{P_c} or gluconeogenic capability. A possible explanation is that the fall in $\Delta\Psi_m$ is compensated by a rise in ΔpH_m [2] so that $\Delta\tilde{\mu}_{H^+}$ remains constant. However, the fall in $\Delta\Psi_m$ induced by valinomycin is associated with a two-fold increment in J_0. Such an increase would not be expected if the decrease in $\Delta\Psi_m$ was largely compensated by a rise in ΔpH_m. Moreover, ΔpH_m is though to make little contribution to $\Delta\tilde{\mu}_{H^+}$ in animal mitochondria [2,46] and it seems unlikely that a rise in ΔpH_m sufficient to compensate for the fall in $\Delta\Psi_m$ occurs on exposure of hepatocytes to valinomycin. Strzelecki et al. [47] detected no change in ΔpH_m in hepatocytes in the presence of 5 nM valinomycin, a level which induces a fall in $\Delta\Psi_m$ of about 30 mV in our cells but no fall in ΔG_{P_c}. The mechanisms whereby ΔG_{P_c}, E_h and $J_{glucose}$ are maintained, or even increased, in the presence of low concentrations of valinomycin that cause a substantial fall in $\Delta\Psi_m$ and corresponding rise in J_0, would seem an important area for further study. To eliminate the possibility that transport of substrates across the plasma membrane may contribute to the observed force–flow relationships, analogous experiments have been undertaken with glycogen-containing hepatocytes isolated from fed animals in which the fluxes of glycogenolysis and glycolysis have been measured. For the various inhibitors used, including FCCP and valinomycin, the correlations between $\Delta\Psi$ and glycolytic rate were similar to those between $\Delta\Psi$ and gluconeogenesis in Fig. 4 (unpublished data).

In suspensions where less than 20% of the cells are susceptible to staining by Trypan blue, values of ΔG_{P_c} below 400 mV are not observed. The maintenance of ΔG_{P_c} at this level (corresponding to an [ATP]/[ADP] ratio of about 0.3), even in glycogen-depleted hepatocytes from fasted rats, is an unexpected property of intact cells that is not emulated with isolated mitochondria. A possible explanation is that maintenance of plasma membrane integrity is not feasible at cellular values of ΔG_{P_c} below 400 mV, and that total loss of ATP is an inevitable consequence of plasma membrane rupture and mitochondrial destruction. The poising of the [ATP]/[ADP] ratio at a minimal level of 0.3 may reflect the activity of adenylate kinase [43]. Another important difference between the behaviour of the intracellular mitochondria of intact hepatocytes and isolated mitochondria relates to the degree of proportionality between $\Delta\Psi_m$ and ΔG_{P_c}. It has been argued on theoretical grounds that the ratio $\Delta\Psi_m/\Delta G_{P_c}$ should be at least 3 : 1, or possibly 4 : 1, if allowance is made for the electrogenic nature of the adenine nucleotide translocator [48]. Remarkably, in the intact hepatocyte, ratios less than this are consistently observed. Although in keeping with Mitchell's original hypothesis [49], these observations would no longer be considered entirely compatible with chemiosmotic theory, but could be taken as suggestive of variable degrees of 'intramembranous coupling' [50].

Although the correlation between $\Delta\Psi_m$ and ΔG_{P_c} is readily explained on the basis that both $\Delta\Psi_m$ and ΔG_{P_c} are generated as a consequence of $\Delta\tilde{\mu}_{H^+}$, the reason for the frequently observed linear relationships between $\Delta\Psi_m$ and E_h is less evident. Such relationships are a prominent feature of cells treated with rotenone and FCCP, but are not observed in the presence of oligomycin or carboxyatractyloside. There is one well-recognized mechanism whereby $\Delta\tilde{\mu}_{H^+}$ can affect E_h, namely the phenomenon generally referred to as 'reversed electron transfer' [51]. The most familiar example of this is the energy-dependent reduction of NAD^+ by reduced flavin [52]. The energy source may be $\Delta\tilde{\mu}_{H^+}$, or alternatively ATP, in which case the process is blocked by oligomycin. The poising of the redox potentials of the mitochondrial NAD-linked reactions away from true thermodynamic equilibrium as a consequence of energy-dependent reversed electron transfer would appear to be analogous to the poising of the redox state of mitochondrial NADP-linked reactions brought about through the operation of the energy-dependent transhydrogenase [53]. In both instances, energy flow, driven by $\Delta\tilde{\mu}_{H^+}$ or ATP, causes a substantial shift in redox state away from values that

would pertain in the de-energized state. It is suggested that the linear relationships between E_h and $\Delta\Psi_m$ observed in the present study reflect a mutual dependence on $\Delta\tilde{\mu}_{H^+}$.

Thus, the linear relationships observed between $\Delta\Psi_m$ and ΔG_{P_c} or $\Delta\Psi_m$ and E_h can be explained on the basis of the common link of these forces with $\Delta\tilde{\mu}_{H^+}$. Less readily explicable are the linear correlations seen between $\Delta\Psi_m$ and various metabolic flows. Elsewhere we have reported that similar correlations pertain for ΔG_P and E_h [9,10]. Linear relationships of this kind are characteristic of systems operating close to equilibrium, or can be the consequence of a balanced interplay between far-from-equilibrium forces [54–56]. Since living cells are open systems, only the latter possibility is considered relevant to the results reported here. The nature of the forces responsible for these linear relationships remains to be elucidated. Elsewhere we have argued that the ability of a complex metabolic pathway to exhibit linear changes in flux as a function of the magnitude of an applied force must reflect a high degree of organisation within the pathway [9,10]. Further, we have suggested that the enzymes of the pathway are sensitive to the applied force and, as a consequence, ΔG for the reactions catalysed may be substantially altered, thereby poising the reactions apparently close to thermodynamic equilibrium for as long as the force is applied.

We conclude that even when determination of ΔpH_m is omitted, the measurement of $\Delta\Psi_m$, like that of ΔG_P and E_h, can provide useful insight into cellular energy state. That the magnitude of an electric field across the inner mitochondrial membrane (measured by its effect on the partitioning of $TPMP^+$) can be shown to correlate with the rate of a cytoplasmic metabolic process, would seem to offer unequivocal evidence for the existence within the cell of long-range forces, the nature of which remains to be elucidated.

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