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Energetics of Ehrlich ascites mitochondria: membrane potential of isolated mitochondria and mitochondria within digitonin-permeabilized cells

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Ehrlich ascites tumour cells were treated with digitonin so that they became permeable for low-molecular-weight compounds but, at certain concentrations of digitonin, retained most of their cytoplasmic proteins. Respiration of mitochondria with exogenous substrates and their membrane potential could thus be measured in situ by means of oxygen electrode and tetraphenylphosphonium-sensitive electrode, respectively. The results were compared with data from similar measurements on mitochondria isolated from such digitonin-permeabilized cells. Isolated mitochondria and mitochondria in situ oxidized succinate at similar rates and developed membrane potential of comparable magnitude. Both preparations also exhibited an identical nonlinear relationship between resting state respiration (titrated with a respiratory inhibitor) and the membrane potential. In the cells permeabilized with low concentrations of digitonin (i.e., retaining most of cytoplasmic proteins) and suspended in medium containing NaCl and other major anions and cations at concentrations close to those in mammalian plasma, anaerobiosis did not produce a decrease in the mitochondrial membrane potential, which was collapsed only after a subsequent addition of oligomycin. In this medium, glucose had little effect on either respiration or the membrane potential.

Introduction

Ascites tumour mitochondria have long attracted attention because they present a convenient model for malignant tissue mitochondria. The drawback of this kind of tissue is, however, that the cells are resistant to disruption and homogenization. Drastic methods like grinding with sand or glass beads, sonication and digestion with proteolytic enzymes, yield mitochondria with variable degrees of damage. Only a few years ago was a relatively gentle procedure of mitochondria isolation from the Ehrlich ascites and some hepatoma tumours published [1]. This method, using digitonin and gentle homogenization with a glass-Teflon homogenizer, provided mitochondrial preparations whose yield and respiratory control ratios were significantly better than those of preparations obtained thus far by other procedures. Moreover, treatment of the cells with digitonin enabled one to obtain 'mitochondria in situ', i.e., mitochondria still located within the network of the cytoskeleton but fully accessible to the external medium due to a complete permeabilization, or destruction, of the plasma membrane.

Although a large body of observations have accumulated on the respiration, oxidative phosphorylation, ATPase, Ca^{2+} transport and other energy-linked functions of Ehrlich ascites mitochondria, much less is known about the membrane potential of these mitochondria and its variations related to changes of the metabolic state. Qualitative measurements of mitochondrial membrane potential in intact and dextran sulphate-permeabilized Ehrlich ascites cells using safranine have been performed by Åkerman [2], who noticed changes upon energization and deenergization. Recently, a quantitative and continuous monitoring of the membrane potential with TPP-sensitive electrode and respi-

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; TPP^+, tetraphenylphosphonium cation; $\Delta\psi$, membrane electric potential.

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ration with oxygen electrode on digitonin-permeabilized Erhlich ascites cells was performed by Olavarría et al. [3].

In the present study, we applied the digitonin procedure [1] for both isolation of Ehrlich ascites mitochondria and preparation of 'mitochondria in situ'. Moreover, by applying lower digitonin concentrations, close to those used by Olavarría et al. [3], we were able to make the plasma membrane essentially permeable to low-molecular-weight compounds, e.g., respiratory substrates, while still maintaining most of the soluble proteins inside the cell boundaries. With this cell preparation we studied the behaviour of the mitochondrial membrane potential under various conditions and with different energy sources (respiration versus glycolysis). We also compared the flux/force characteristics of isolated and in situ mitochondrial preparations.

Materials and Methods

Biological material

Ehrlich ascites cells were cultivated in the peritoneal cavity of White Swiss female mice fed standard laboratory diet. 7 days after inoculation the animals were killed by cervical dislocation and dissected. The ascites cells and mitochondria therefrom were obtained essentially as described by Moreadith and Fiskum [1]. The main differences with respect to the original procedure were (i) addition of glucose to isolation and suspension media and (ii) decreasing digitonin concentration. In brief, the ascites fluid was collected and the peritoneal cavities were washed with cold solution containing 150 mM NaCl, 5 mM KCl, 10 mM glucose and 10 mM Tris-HCl (pH 7.4). The ascites fluid and the washings were combined and filtered through cheese-cloth. The cells were sedimented during 5 min at $180 \times g$ and washed twice with the same medium. They were subsequently suspended in a medium containing 210 mM mannitol, 70 mM sucrose, 10 mM glucose, 5 mM K-Hepes (pH 7.2) and 0.5% defatted bovine serum albumin. This suspension usually contained 50–70 mg cell protein/ml or 10^8–10^{10} cells/ml. All steps of this procedure were carried out at 0–4 °C.

To permeabilize the cells, 10% solution of digitonin (Sigma Chemical Company, St. Louis, MO, U.S.A.) in dimethylsulphoxide was added to the desired final concentration. To fully permeabilize the cells but to preserve mitochondrial functions, the cells were incubated at 0 °C with 0.03–0.05% (0.24–0.40 mM) digitonin. After about 1 min, when most of the cells became permeable to Trypan blue, the suspension was diluted with a double volume of the cold mannitol-sucrose medium and the cells were sedimented at $3000 \times g$ for 3 min. Such cells lost about 40% of protein but retained essentially all membraneous structures. For isolation of mitochondria the permeabilized cells were finally resuspended in the same medium and disrupted by 10 up-and-down passes of a Teflon pestle rotating at approx. 1500 rpm in a glass homogenizer. The subsequent fractional centrifugation was performed as described by Moreadith and Fiskum [1].

Measurements

Oxygen uptake was measured with a Clark-type electrode. The membrane potential ($\Delta\psi$) was determined using a TPP-sensitive electrode [4] connected to a pH-meter (Radiometer, Copenhagen, Denmark; model PHM 72) and a potentiometric recorder. This electrode, together with a calomel reference electrode and the oxygen electrode, was inserted into a stirred thermostated chamber, so that the rate of oxygen uptake and the membrane potential (calculated from the changes of extramitochondrial TPP^+ concentration) could be monitored simultaneously. Before addition of the cells or mitochondria the electrode was calibrated by stepwise additions of TPP^+Cl^-. The potentiometer readings were proportional to the logarithm of TPP^+ concentration within the range 1–15 μM. After each run, 2,4-dinitrophenol (0.1 mM final concentration) was added in order to completely collapse the membrane potential, and TPP^+ concentration was corrected for that amount which still remained inside the cells. The values of $\Delta\psi$ were then further corrected for TPP^+ binding to mitochondria using the formula of Rottenberg [5].

To calculate mitochondrial membrane potential within the cell the contribution of mitochondrial protein to the total cell protein was taken as 6%. This value, which is half of that assumed by Cockrell [6], was deduced from comparing cytochrome oxidase (EC 1.9.3.1) activity (measured by oxygen uptake of the solubilized material in the presence of cytochrome *c* and ascorbate [7]) in whole cells and mitochondrial preparations. A similar value of about 6% was obtained by a rough morphometric estimation of the contribution of mitochondria to the total cell volume. The matrix space of Ehrlich ascites mitochondria was assumed as 0.75 μl/mg protein [8].

ATP and ADP were measured spectrophotometrically in neutralized perchloric acid extracts by enzymatic procedures [9,10]. Lactate dehydrogenase (EC 1.1.1.27) was determined as described by Vassault [11]. Protein was measured by the biuret method using bovine serum albumin as a standard.

Electron microscopy

Ehrlich ascites cells and mitochondria isolated therefrom were fixed by mixing their suspensions with equal volumes of 4% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) and incubating for 1 h at 0 °C. The material was then sedimented by gentle centrifugation, dehydrated, embedded in Epon and thin-sectioned. Ultrathin slices were stained with uranyl acetate and lead

citrate and examined in JEM 100B electron microscope (Jeol, Tokyo, Japan).

Results

Respiration of Ehrlich ascites cells suspended in isotonic NaCl medium containing glucose was inhibited by rotenone by more than 90% (Table I). This is in contrast to rat hepatocytes oxidizing palmitate whose respiration is inhibited by rotenone by less than 30% [12]. In agreement with observations of Müller et al. [10], the respiration was also strongly inhibited by oligomycin, suggesting that, in the intact cell, mitochondria may operate close to active state (State 3) conditions. This is again in contrast to isolated parenchymal cells where oligomycin inhibits the respiration by about 50% only [14]. As shown by the effects of ouabain and colchicine, Na^+/K^+-ATPase and the microtubular system contribute together to less than half of the ATP demand, whereas the contribution of protein synthesis is negligible.

Omission of glucose from the NaCl-rich incubation medium either had no effect or decreased the respiration only slightly (up to 20%, not shown). In the high-potassium medium (for composition see legend to Fig. 1; for reason for using such a medium, see next paragraph) we occasionally observed a brief stimulation of oxygen uptake immediately after addition of glucose (5–10 mM final concentration), followed by a considerable decrease of respiration about 1 min later. In the NaCl medium the total content of cell ATP and ADP amounted to 13 ± 1 and 5.6 ± 1.3 nmol/mg protein, respectively (mean values ± S.D. for three experiments), and did not essentially change either immediately or 5 min after addition of glucose.

TABLE I

Effect of various inhibitors on the respiration of Ehrlich ascites cells

The cells (12–15 mg protein) were suspended in the incubation medium containing 140 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1.5 mM P_i (K salt), 25 mM $NaHCO_3$ and 10 mM glucose and bubbled with 95% O_2 + 5% CO_2. Oxygen uptake was measured at 30 °C in a total volume of 2.7 ml. The results are means for three experiments ± S.D. or are from one experiment. The mean oxygen uptake was 8.3 ngatom O_2/min per mg total cell protein.

Inhibitor	Acting on	Inhibition of oxygen uptake (%)
Rotenone, 2 μM	Electron transport	93 ± 4
Oligomycin, 20 μg/ml	F_0,F_1-ATPase	84 ± 7
Ouabain [a], 2.5 mM	Na^+/K^+-ATPase	20 ± 2
Colchicine [a], 100 μM	microtubular system	16
Cycloheximide [a], 200 μg/ml	protein synthesis	2

[a] The cells were preincubated with the inhibitor for 20 min at 25 °C.

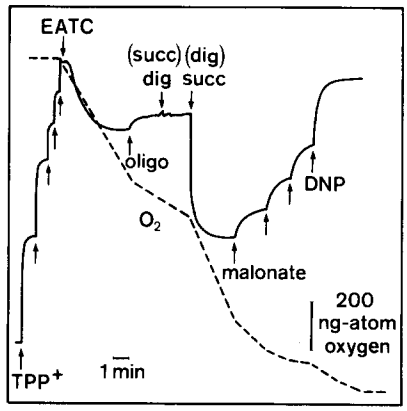


Fig. 1. Oxygen and TPP^+ uptake by Ehrlich ascites cells before and after permeabilization with digitonin. Respiration and membrane potential ($\Delta\psi$) were measured at 30 °C in 2.7 ml of the medium containing 80 mM KCl, 3 mM $MgCl_2$, 5 mM P_i, 1 mM EDTA and 10 mM K-Hepes. The dashed line represents the trace of oxygen electrode and the solid line that of the TPP-sensitive electrode. The TPP electrode was first calibrated with successive additions of TPP^+ (0.6 μM each), followed by the addition of Ehrlich ascites cell suspension (12 mg protein, EATC). Oligomycin (oligo) was added to 4 μg/ml final concentration, followed by digitonin (16.5 μM, dig) and succinate (10 mM, succ) plus rotenone (2 μM). Respiration was then partially inhibited by a stepwise addition of malonate (1.1 mM at each arrow) and finally uncoupled by 2,4-dinitrophenol (0.1 mM, DNP). The same picture was obtained when the order of additions of digitonin and succinate was reversed (succ and dig in parentheses).

In contrast to experiments with intact cells which were carried out in NaCl medium, those with permeabilized cells were routinely performed in KCl medium in order to prevent a deleterious effect of high Na^+ concentration on mitochondria. Such medium produced, however, a depolarization of the plasma membrane (before it was permeabilized by digitonin) and affected cell metabolism by draining energy for futile operation of the Na^+/K^+ pump. As result, respiration of the whole cells was increased by about 15% and the membrane potential of mitochondria inside the cells was decreased (see below).

As expected, carefully isolated cells did not oxidize externally added mitochondrial respiratory substrates, e.g., succinate, unless their plasma membrane was made permeable by treatment with digitonin (Fig. 1). Digitonin at the concentration used in this experiment, 16.5 μM, produced only a partial release, of about 25%, of cytoplasmic lactate dehydrogenase. Fig. 1 also shows that TPP^+ was accumulated by Ehrlich ascites cells. Oligomycin produced a decrease of the rate of oxygen uptake concomitantly with a small release of the accumulated lipophilic cation. Permeabilization of the cells with digitonin had no further effect on both oxygen uptake and TPP^+ accumulation. However, addition of succinate to permeabilized cells resulted in a rapid and potent uptake of TPP^+ in parallel with an increase of oxygen uptake. Titration with malonate decreased the respiration rate and resulted in a partial release of

accumulated TPP^+. An almost complete release was produced by the addition of a protonophore.

No measurable release of TPP^+ after addition of digitonin (Fig. 1) indicates that essentially all TPP^+ taken up by the cells was trapped inside mitochondria. This enables to calculate the membrane potential of mitochondria inside the intact Ehrlich ascites cell. Asuming that mitochondria contribute to 6% of the cell protein (see Material and Methods) and that the volume of the matrix compartment amounts to 0.75 μl/mg protein [8], the mitochondrial membrane potential in the experiment shown in Fig. 1 could be estimated as 123 mV before and 115 mV after addition of oligomycin. However, in the high-Na^+ medium under otherwise similar conditions the in situ mitochondrial membrane potential was estimated as about 150 mV (in the absence of oligomycin, not shown). After permeabilization of the cells with digitonin and energization of mitochondria with succinate (in high K^+ medium, Fig. 1), their membrane potential increased to 170 mV.

In spite of this high membrane potential, the resting state respiration (with succinate as substrate and oligomycin to ascertain State 4) of such mitochondria was rather high and was increased by uncoupler only by a factor of 2–3. This was presumably the result of a deleterious effect of digitonin on the inner mitochondrial membrane at 30 °C. Therefore, the relation between the resting state respiration and the membrane potential was further studied in mitochondria in situ obtained by treatment of the cells with digitonin at 0 °C (see Material and Methods). In spite of a higher digitonin concentration, 0.24–0.40 mM, but presumably because of a lower temperature and a short time of incubation with digitonin (1 min), these mitochondria appeared to be well coupled, with the respiratory control index of more than 7. A comparison of the mitochondria in situ, i.e., inside fully permeabilized cells, and those after isolation is shown in Table II. It can be seen that the respiratory control and the membrane potential in both preparations are virtually the same.

The relation between the membrane potential and the rate of respiration under resting state conditions (in the presence of oligomycin) in permeabilized cells was studied similarly as in Fig. 1, namely, mitochondria were energized with succinate (+rotenone) and their respiration was inhibited by increasing concentrations of malonate. Results from five such experiments, 'normalized' by assuming noninhibited respiration and the corresponding membrane potential as 100%, are presented in Fig. 2. It can be seen that the points for both isolated mitochondria and mitochondria in situ form the outline of a single curve exhibiting a nonlinear dependence between the membrane potential and the resting state respiration.

Behaviour of the mitochondrial membrane potential under anaerobic conditions in cells permeabilized with

TABLE II

Comparison of energetic parameters of isolated Ehrlich ascites mitochondria and mitochondria in situ

Respiration and membrane potential ($\Delta\psi$) were measured at 25 °C in the KCl medium (see legend to Fig. 1) which contained 10 mM succinate, 2 μM rotenone and 1.3–1.6 mg mitochondrial protein/2.7 ml. State 3 was produced by addition of ADP to 0.74 mM final concentration. The cells were permeabilized with 0.24–0.40 mM digitonin at 0 °C for about 1 min and are referred to as 'mitochondria in situ'. When calculating oxygen uptake and membrane potential of these mitochondria their amount was taken as 6% of total cell protein (see Material and Methods). The data are means ± S.D. for three ('mitochondria in situ') or four (isolated mitochondria) preparations.

	Oxygen uptake (ngatom/min per mg mitochondrial protein)	Respiratory control	$\Delta\psi$ (mV)
Mitochondria in situ			
State 4	23 ± 5	7.5 ± 0.4	167 ± 8
State 3	172 ± 13		146 ± 6
Isolated mitochondria			
State 4	16 ± 5	8.1 ± 2.3	176 ± 8
State 3	130 ± 18		153 ± 6

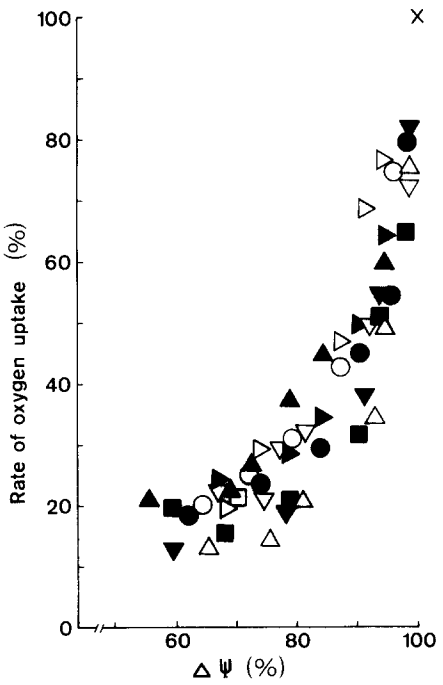


Fig. 2. Relation between the resting state (State 4) respiration and the membrane potential of isolated Ehrlich ascites mitochondria and mitochondria in situ. Cells fully permeabilized by treatment with 0.24–0.40 mM digitonin at 0 °C for about 1 min are referred to as 'mitochondria in situ' (see also Table II). Oligomycin, 4 μg/ml, was present in all incubations. Respiration was modulated by adding malonate up to 7.2 mM concentration. Values for oxygen uptake and membrane potential ($\Delta\psi$) are expressed as percent of the respective values in uninhibited mitochondria (in the absence of malonate). Open symbols, 'mitochondria in situ'; closed symbols, isolated mitochondria from corresponding preparations of permeabilized cells.

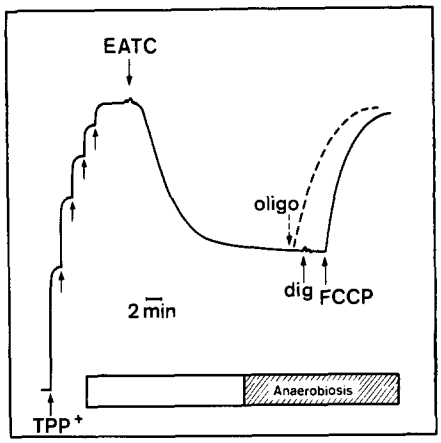
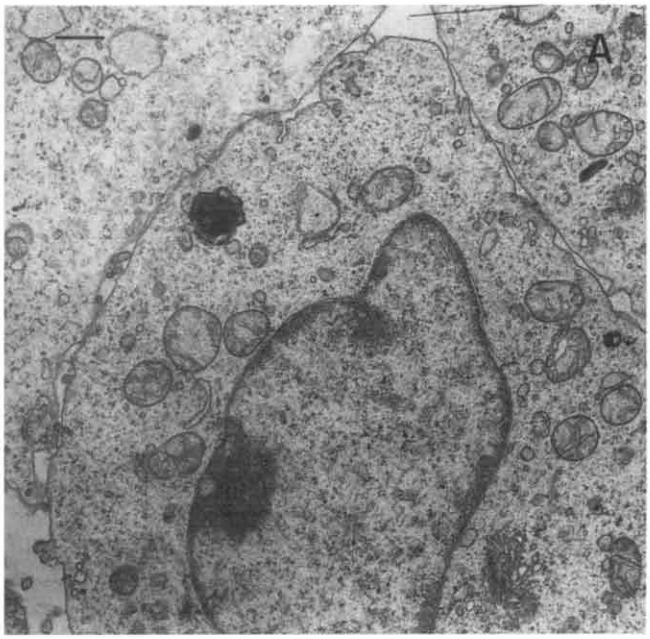
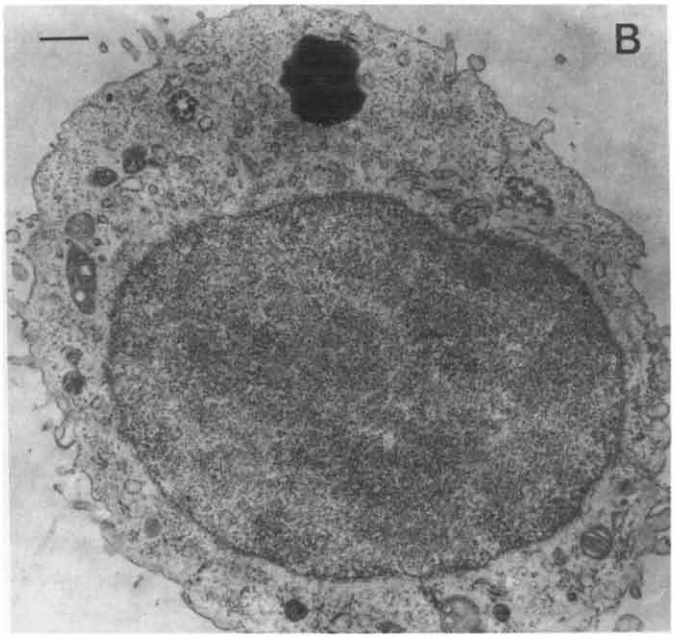


Fig. 3. Effect of anaerobiosis on the membrane potential of Ehrlich ascites mitochondria in permeabilized cells. TPP^+ electrode trace. Conditions as in Table I (NaCl medium). FCCP was added to 1 μM concentration. Dashed line, the trace after addition of oligomycin. The onset of anaerobiosis was signalled by the oxygen electrode (not shown).

low concentrations of digitonin is shown in Fig. 3. Similarly as under aerobiosis (Fig. 1), digitonin produced no decrease in $\Delta\psi$, indicating that the mitochondrial membrane potential within the cell could be maintained by a process other than respiration. Simultaneous measurements of ATP and ADP showed that the content of these nucleotides in the cells was not affected by the onset of anaerobiosis and remained unchanged for up to 5 min. In contrast to aerobiosis, addition of oligomycin resulted in a complete collapse of $\Delta\psi$ (Fig. 3, dashed line). Interestingly, this insensitivity to anaerobiosis was observed only in NaCl medium; the cells suspended in K^+-rich medium were unable to maintain their membrane potential under anaerobic conditions (not shown).

Electron microscopy revealed that upon a brief treatment with 240 μM (0.03%) digitonin the plasma membrane of Erhlich ascites cells remained morphologically intact, although mitochondria within the cell resumed a





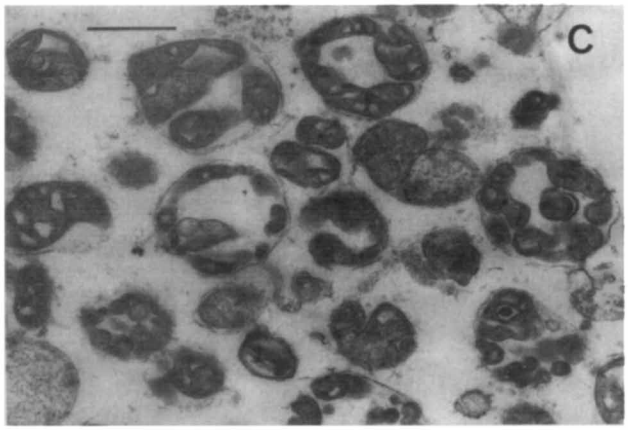


Fig. 4. Electron micrographs of Ehrlich ascites cells and isolated mitochondria. (A) Freshly isolated (control) cell; (B) cell permeabilized by brief (1–2 min) incubation with 0.03% digitonin; (C) mitochondria isolated from digitonin-permeabilized Ehrlich ascites cells. The bar represents 1 μm.

condensed configuration (Fig. 4A and B). A similar picture was found by Fiskum et al. [15] in isolated rat hepatocytes treated with digitonin. Condensation of mitochondria within Ehrlich ascites cells was also observed by Gordon and Bernstein [16] after treatment with valinomycin. Apparently, such transformation results from a change of the ionic composition of the cytoplasm. Mitochondria isolated from digitonin-permeabilized cells retained their intact outer membrane (Fig. 4C).

Discussion

Digitonin has long been used as a means in isolation of cellular organelles [17]. More recent quantitative studies [18,19] on the effect of digitonin on isolated hepatocytes have shown that under controlled conditions the plasma membrane becomes fully permeabilized and even completely disappears but the cytoskeleton and cellular organelles remain virtually intact. The present investigation confirms the observation of Moreadith and Fiskum [1] that mitochondria isolated from digitonin-permeabilized ascites cells and mitochondria in situ in such cells are morphologically intact and energetically tightly coupled as manifested by their high respiratory control and membrane potential.

Determination of the mitochondrial membrane potential within intact cells by means of lipophilic cations is complicated by the fact that the potential difference at the plasma membrane, negative inside, also contributes to the uptake of the probe from the medium. To obtain precise values for the membrane potential at the mitochondrial membrane one should therefore estimate the plasma membrane potential by an independent procedure, e.g., measuring distribution of a penetrant anion [20,21]. Permeabilization of the plasma membrane with digitonin provides another means to measure the membrane potential of mitochondria in situ. However, in case of Ehrlich ascites cells the membrane potential at the plasma membrane appeared to be negligible, at least in media used in the present investigation, since addition of digitonin produced little or no change in the amount of trapped TPP^+ (see, for example, Figs. 1 and 3). Therefore, readings from the TPP^+ electrode can be used directly to calculate, within a certain degree of approximation, the membrane potential of mitochondria within intact (not permeabilized) Ehrlich ascites cells.

The effect of oligomycin requires some attention. Although it decreases the respiration, the membrane potential of mitochondria within the cells is slightly decreased as well (Fig. 1). This paradoxical effect may be interpreted as being due to a deficiency of endogenous respiratory substrate(s). This is compatible with the observation that addition of succinate and digitonin produces an increase of both the respiratory rate and the mitochondrial membrane potential (Fig. 1). One can speculate that in Ehrlich ascites cells, like in another highly glycolytic tumour, S-30D rat hepatoma [22], glycolysis is initiated by phosphorylation of glucose at the outer mitochondrial membrane by bound hexokinase (EC 2.7.1.1) preferentially utilizing ATP generated in mitochondria.

An important point of the present study is the comparison of isolated mitochondria and mitochondria in situ. It shows that both preparations exhibit a nonlinear dependence on the rate of resting state respiration (Fig. 2). This finding strongly suggests that the nonlinear flux/force relation, so characteristic for isolated mitochondria [23–25], is also an intrinsic feature of mitochondria inside the cell and does not merely result from a coupling heterogeneity of isolated orgenelles, as speculated previously [26].

An interesting feature of Ehrlich ascites cell mitochondria in situ is also the fact that their membrane potential is not affected by anaerobiosis (Fig. 3) and could be collapsed only by addition of oligomycin to anaerobic cells. This is compatible with the observation of Åkerman [2] that mitochondria in intact Ehrlich ascites cells were energized by glucose, the process being insensitive to KCN but sensitive to dicyclohexylcarbodiimide, and is in contrast to mitochondria in isolated hepatocytes, whose potential decreased shortly after the onset of anaerobiosis (not shown). It can be, therefore, concluded that glycolytically generated ATP can maintain the mitochondrial membrane potential in the intact Ehrlich ascites cell at the same level as does the respiratory chain. This is corroborated by the observation that the high level of cellular ATP was maintained for at least 5 min of anaerobiosis. It confirms the well-known major role of glycolysis in energetics of these cells [27]. It has to be added that the concentration of digitonin used in the experiment shown in Fig. 3, namely 16.5 μM, produced a release of only about 25% of lactate dehydrogenase and therefore the cells could maintain, at least partly, their glycolytic activity.

Using our NaCl medium, which mimics ionic composition of mammalian extracellular fluid, we were unable to reproduce the effect of addition of glucose observed as early as in 1959 by Chance and Hess [28] and recently confirmed by Olavarría et al. [3], namely that glucose elicits a transient jump of the respiration rate followed by a decrease of respiration (Crabtree effect). In addition, Olavarría et al. [3] observed a long-term decrease of the mitochondrial membrane potential after glucose addition. In our hands, glucose produced a small but long-lasting increase of oxygen uptake and had essentially no effect on the membrane potential. In contrast, Åkerman [2] observed an increase of the membrane potential of Ehrlich ascites mitochondria in situ after addition of glucose. It seems that the response of Ehrlich ascites cells to glucose may

depend on the composition of the incubation medium. The medium used by Chance and Hess [28] and Olavarría et al. [3] was highly unphysiological by its high phosphate concentration and the lack of bicarbonate, Ca^{2+} and Mg^{2+}, whereas that of Åkerman [2] was supplemented with Mg^{2+} and was lacking phosphate.

In conclusion, the present investigation shows that mitochondria isolated from Ehrlich ascites tumour cells with the use of digitonin are energetically fully competent. Their respiratory control, membrane potential and proton permeability are comparable to those of liver mitochondria. Mitochondria in situ, in fully permeabilized cells, exhibit the same nonlinear flux/force relationship as isolated mitochondria.

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