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LOCALIZED COUPLING IN OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA FROM JERUSALEM ARTICHOKE *(HELIANTHUS TUBEROSUS)*

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Delocalized chemiosmotic coupling of oxidative phosphorylation requires that a single-value correlation exists between the extent of $\Delta\tilde{\mu}_{H^+}$ and the kinetic parameters of respiration and ATP synthesis. This expectation was tested experimentally in nigericin-treated plant mitochondria in single combined experiments, in which simultaneously respiration (in State 3 and in State 4) was measured polarographically, $F\Delta\psi$ (which under these conditions was equivalent to $\Delta\tilde{\mu}_{H^+}$) was evaluated potentiometrically from the uptake of tetraphenylphosphonium$^+$ and the rate of phosphorylation was estimated from the transient depolarization of mitochondria during State 4–State 3–State 4 transitions. The steady-state rates of the different biochemical reactions were progressively inhibited by specific inhibitors active with different modalities on various steps of the energy-transducing process: succinate respiration was inhibited competitively with malonate or noncompetitively with antimycin A, or by limiting the rate of transport into the mitochondria of the respiratory substrate with phenylsuccinate; $\Delta\tilde{\mu}_{H^+}$ was dissipated by uncoupling with increasing concentrations of valinomycin; ADP phosphorylation was limited with oligomycin. The results indicate generally that when the rate of respiratory electron flow is decreased, a parallel inhibition of the rate of phosphorylation is also observed, while very limited effects can be detected on the extent of $\Delta\tilde{\mu}_{H^+}$. This behavior is in marked contrast to the effect of uncoupling where the decreased rate of ATP synthesis is clearly due to energy limitation. Extending previous observations in bacterial photosynthesis and in respiration by animal mitochondria and submitochondrial particles the results indicate, therefore, that respiration tightly controls the rate of ATP synthesis, with a mechanism largely independent of $\Delta\tilde{\mu}_{H^+}$. These data cannot be reconciled with a delocalized chemiosmotic coupling model.

Introduction

The relevance of protons at high electrochemical potential in the coupling of the exergonic redox processes of respiration and photosynthesis and of the endergonic phosphorylation of ADP, catalyzed by a membrane-bound ATP synthase, is a well established fact, demonstrated experimentally in many biological systems. According to a generally accepted model, ADP phosphorylation is driven by the flux of protons across the coupling membrane through a proton-conducting channel, the F_0 portion of the ATP synthase, and is catalyzed within an active site [1–4] located in the F_1 portion of this enzyme complex. Moreover, according to this model, the source of high-energy protons driving this processes is the bulk phase water at the face of the membrane opposite to F_1 and conse-

Abbreviations: TPP^+, tetraphenylphosphonium ion; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; $TPMP^+$, tetraphenylmethylphosphonium ion; $TBMA^+$, tribenzylmethylammonium ion.

quently the high-free-energy state of the membrane can be produced in many different ways, either physiologically relevant or artificially induced under laboratory conditions. Thus, the transmembrane electrochemical potential difference of protons (and consequently ATP formation) can be sustained by the respiratory or photosynthetic H^+-translocating electron transport [4,5], the bacterial H^+-translocating pyrophosphatase [6], bacteriorhodopsin in reconstituted proteoliposomes [7], artificial acid-base transitions [8,9], electric fields generated by ion diffusion potentials [10], or externally applied voltage differences [11]. The unifying interpretation of these phenomena is that under all conditions high-potential protons are asymmetrically formed at one side of the coupling membrane, by influencing either the proton activity or the electrostatic potential (or both) in the water phases, so that an exergonic H^+ flux across the F_0 channel, driving ADP phosphorylation, can be induced.

Given the exceedingly fast rate of diffusion of H^+ in water, high-potential protons are thought to be immediately delocalized and available for the coupling processes without any kinetic constraints; so the nature and location in the membrane of the primary H^+ pumps are immaterial to the activity of the ATP synthase. This situation has been clearly and formally defined by Caplan and Essig [12], and subsequently by Westerhoff and Van Dam [13], using nonequilibrium thermodynamics, by stating that the cross-coefficients between the affinity of the primary H^+ pumps and the rate of the ATP-forming reaction must be zero for pure chemiosmotic coupling. In a more intuitive way, it can be stated, according to the fully delocalized and reversible chemiosmotic coupling model, the kinetic behavior of ATP synthase must be determined by the concentration of substrates and products (ADP, ATP, Mg^{2+} and ionic environment, all parameters included in the free energy change for ATP formation) and by the extent of the transmembrane electrochemical potential difference of protons, measured by the electrochemical potentials of protons in the two aqueous phases at the two sides of the coupling membrane – a view that implies an equilibrium situation between bulk water and the F_0 channel or the ATP-releasing site on the opposite faces of the membrane. This kinetic behavior originates from the postulated reversibility of the ATP synthase and the full delocalization of protons within the aqueous compartment delimited by the coupling membrane, a compartment which defines the 'phosphorylation unit' in the coupling process.

These theoretical expectations have not been met by the results of many experiments performed on several biological systems. Early experiments in intact mitochondria have demonstrated that the rate of respiration under phosphorylating or nonphosphorylating conditions was correlated differently to the extent of $\Delta\tilde{\mu}_{H^+}$, an observation at odds with the views of delocalized coupling and suggesting a direct interaction of the respiratory chain and the ATP synthase [14,15]. Conversely, in bacterial photosynthetic systems, the rate of ATP formation was found to be directly correlated to the rate of the photosynthetic redox processes and the number of active oxidoreduction chains rather than with the extent of $\Delta\tilde{\mu}_{H^+}$ [16,17]. Similar data were subsequently obtained in submitochondrial particles [18]. On the other hand, a marked insensitivity of the extent of $\Delta\tilde{\mu}_{H^+}$ to the inhibition of electron transfer was found to be a general feature of energy-transducing membranes and was interpreted either as evidence for a nonohmic behavior of the coupling membrane [19] or as a 'slipping' of the oxidoreductive H^+ pumps [20]. The direct link between phosphorylation and oxidoreduction processes was also substantiated under presteady-state conditions of bacterial photosynthesis in flashing light [21]. In higher plant chloroplasts the time lag for the initiation of photophosphorylation in a dark-to-light transition was found to be essentially insensitive to the buffering of the thylakoid internal space also when the onset of the membrane potential was largely prevented by K^+ and valinomycin [22]; since this lag was interpreted as being the time required for the formation of a $\Delta\tilde{\mu}_{H^+}$ critical for driving ATP formation (see, however, Ref. 23), it was concluded that bulk phase $\Delta\tilde{\mu}_{H^+}$ was not fully relevant in the coupling process (see, however, Ref. 24 for steady-state photosynthesis).

All these results point to a relative independence of the activity of the ATP synthase from the values of the bulk phase protonic potentials and to a certain degree of control by the oxidoreductive

reactions on the process of ATP formation. Since the concept of the coupling includes all thermodynamic and kinetic parameters relating forces and flows, the above-mentioned observations indicate that the oxidoreduction reactions and ATP synthesis are directly coupled independently of the value of $\Delta\tilde{\mu}_{H^+}$, a conclusion invalidating one of the basic assumptions of the chemiosmotic model.

In this paper we examine the flow and force relationships during oxidative phosphorylation by whole mitochondria from Jerusalem artichoke tuber. The results obtained are very similar to those previously described in the steady state in other energy-transducing systems, and an interpretation is drawn coincident with the conclusions detailed above, thus extending the number of biological systems tested. While this work was in progress, very similar experiments although with different techniques were performed very accurately by Zoratti et al. [25] in rat liver mitochondria with results practically coincident with those presented here.

Materials and Methods

Preparation of mitochondria

Intact mitochondria were prepared from dormant tubers of Jerusalem artichoke (*Helianthus tuberosus*) as described by Møller et al. [26]; the final mitochondrial pellet contained 20–35 mg protein per ml, as determined with the biuret method.

Measurements of respiratory activities

Oxygen consumption, membrane potential and oxidative phosphorylation were evaluated simultaneously in a single experiment. Mitochondria (0.5–0.8 mg protein per assay) were added to a reaction mixture containing buffer (0.4 M sucrose, 5 mM Tes, 1 mg/ml bovine serum albumin, pH 7.2) supplemented with 10 mM KH_2PO_4, 20 mM KCl and 5.6 mM $MgCl_2$. The measurements were carried out at 28°C in a glass vessel (total volume 1.8 ml) in which oxygen consumption was measured polarographically with a Clark electrode (Yellow Springs Instruments, model 5331), and membrane potential with a membrane minielectrode reversible to TPP^+ [27,28]; the cuvette was connected through an agar-filled salt-bridge to a saturated KCl reference electrode (Radiometer, Copenhagen, model K401). The signals, suitably amplified, were fed to a dual-trace recorder (Linseis, Munich, model LS 24.90). Routinely the concentration of TPP^+ in the assay was kept at 5 μM in order to avoid the uncoupling effects by this lipophilic cation evident at higher concentrations; the calibration of the minielectrode was obtained with stepwise additions of TPP^+ directly to the assay. The traces of a routine assay are shown in Fig. 1: in a single experiment several additions of ADP could be made. For every addition a depression of TPP^+ uptake could be registered from which the values of $\Delta\psi$ in static head for phosphorylation (State 4) and under phosphorylating conditions (State 3) could be evaluated. The time coincidence between the change in $\Delta\psi$ and the change in respiratory rate was quite accurate [29]; for this reason, for a given amount of ADP added, the rate of phosphorylation could be readily calculated by the time elapsed in a State 3–State 4–State 3 cycle [30]. Generally, the first addition of ADP elicited a slower rate of respiration in State 3, as already described by Raison et al. [31] in several types of plant mitochondria; for this reason the measurements of $\Delta\psi$ and of the rate of oxygen consumption and ATP synthesis were always performed following the second addition of ADP. Therefore, every experiment refers to different samples of mitochondria operating under identical conditions.

On several occasions, and specifically for critical experimental conditions (e.g., extensively uncoupled mitochondria or drastically inhibited electron flow), the rate of phosphorylation was also tested by the incorporation of [^{32}P]phosphate, performed directly in the reaction vessel. The results agreed closely. The intramitochondrial osmotic volume was taken to be identical with the inulin-impermeable space, measured isotopically with [^{14}C]inulin and [^{3}H]H_2O using the centrifugation method. Under the conditions of our assay a volume of 0.9 μl/mg protein was measured. For the computation of $\Delta\psi$ no correction for energy-independent binding of TPP^+ was made, since the potentiometric traces showed that this binding was negligible in deenergized mitochondria (i.e., in the presence of 0.1 mM KCN and uncouplers).

Results

A typical experimental trace is shown in Fig. 1: after calibration of the electrode with TPP^+ (1–5 μM), mitochondria were added to the assay, and, after the exhaustion of the brief outburst of respiration of endogenous substrates, and parallel transient polarization of the membrane, succinate was added at saturating concentrations (15 mM). If respiratory inhibitors were to be added, the addition was made after the exhaustion of endogenous substrates and a preincubation of 3–5 min before the addition of succinate was allowed. When a steady-state respiratory rate, and a corresponding steady-state $\Delta\psi$ in State 4 were reached, additions of ADP were made to induce several transitions from State 4 to State 3. In all experiments 0.1 μM nigericin was added; this H^+/K^+ antiporter, in the presence of a high overall concentration of K^+ in the assay (30 mM), prevented the formation of a transmembrane pH difference, which would have been very small due to the presence of phosphate in the assay medium, and allowed therefore the estimation of the total $\Delta\tilde{\mu}_{H^+}$ from the uptake of TPP^+.

In principle, during an ADP pulse the ADP/ATP concentration ratio (and therefore the phosphate potential) and the value of $\Delta\psi$ change continuously. Calculations under the conditions of the assay, i.e., after the total consumption of the first addition of 100 μM ADP, indicate that for a 90% consumption of the second 100 μM ADP pulse, the value of ΔG_{ATP} increases by no more than 15%. The change in $\Delta\psi$ in a State 4–State 3 transition is per se only 10–15% of the total $\Delta\psi$; the polariza-

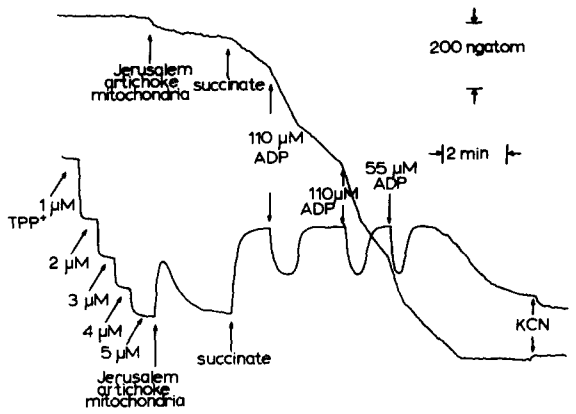

Fig. 1. Example of simultaneous recording of the respiratory rates and of the changes in TPP^+ concentrations in the assay medium during the various respiratory states. Every assay is preceded by a calibration of the ion-selective minielectrode with TPP^+ to give a final concentration of 5 μM. Assay medium contained: 0.4 M sucrose, 5 mM Tes, 1 mg/ml bovine serum albumin, 10 mM KH_2PO_4, 20 mM KCl, 5.6 mM $MgCl_2$, 100 nM nigericin and 5 μM TPP^+. Where indicated, mitochondrial suspension (20–35 mg protein/ml) was added to give a final concentration of 0.5–0.8 mg/ml. All the assays were carried out at 28°C and the final pH was 7.2. The respiratory rates and the degree of respiratory control were calculated according to the method of Estabrook [29] and Chance and Williams [30]. The phosphorylation rate was calculated as described in Materials and Methods.

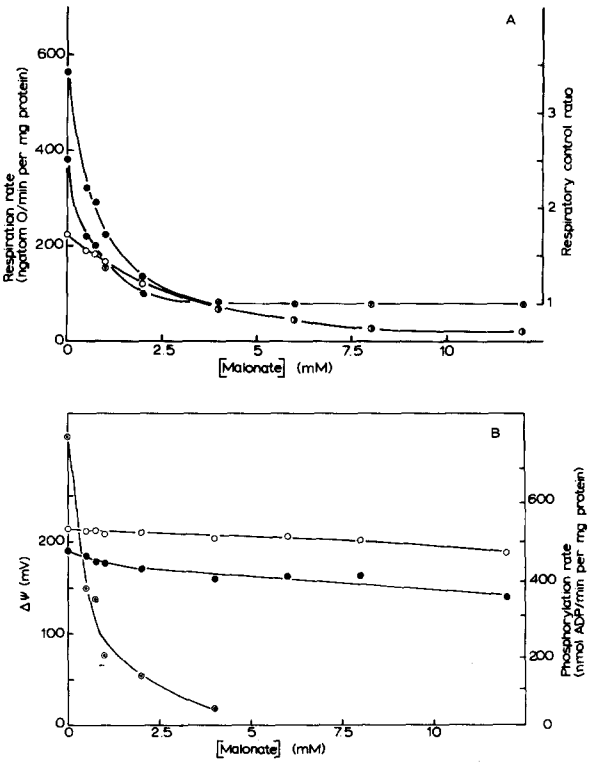

Fig. 2. Effects of inhibition by malonate on the respiration-linked functions of Jerusalem artichoke mitochondria. (A) Effects on the rate of respiration in State 3 (●) and State 4 (○), and on the respiratory control ratio (⊙). (B) Effects on the membrane potential ($\Delta\psi$) induced by State 3 (●) and State 4 (○) respiration, and on the rate of phosphorylation (⊙). For experimental conditions see Materials and Methods and Fig. 1. Mitochondria were incubated for 3 min in the presence of malonate before the addition of the substrate (15 mM succinate). ADP additions: 110 μM for every State 3 cycle.

tion and depolarization rates are prompt, and $\Delta\psi$ remains constant in State 3 for a time long enough to evaluate a true steady-state value. The approximations implicit in the method used for studying the correlation between $\Delta\psi$ and the rate of ATP synthesis are therefore acceptable and amply compensated by the advantages of monitoring these parameters continuously. The accurate proportionality found between the amount of ADP added and the time of depolarization observed supports this conclusion.

The effect of the inhibition by malonate on the respiration-induced polarization of the membrane and on oxidative phosphorylation is shown in Fig. 2A and B. Malonate, a structural analog of succinate, competes with this substrate, both in the transport process catalyzed by the dicarboxylate carrier and for the active site of succinate dehydrogenase. Its presence will therefore affect both the intramatrix concentration of succinate and the turnover of succinate dehydrogenase that will be statistically decreased. However, the values of the kinetic constants of the dicarboxylate carrier (K_m(succinate) = 0.62 mM [32], K_i(malonate) = 0.80 mM [32]) and of succinate dehydrogenase (K_m(succinate) = 0.23 mM [33], K_i(malonate) = 5 μM [33]) are such that the major effect has to be expected on the rate of succinate oxidation, while the intramitochondrial concentration of succinate, and therefore the affinity of succinate oxidation, is maintained rather constant. Inhibition of oxygen consumption by malonate has a very small effect on the extent of $\Delta\psi$ both in State 4 and in State 3, confirming a behavior already described in animal respiration. At 12 mM malonate, corresponding to a theoretical inhibition of succinate dehydrogenase to less than 3%, a decrease in $\Delta\psi$ in State 4 of 23 mV and of 49 mV in State 3 are observed as compared to the control values. By contrast, the rate of phosphorylation parallels closely the rate of respiration under phosphorylation conditions. Noticeably, moreover, the depolarization of the membrane during a State 4–State 3 transition takes place to a constant extent also when oxidative phosphorylation is inhibited to an undetectable rate (less than 3% for 12 mM malonate).

Very similar results are obtained if the entry of succinate in the mitochondria is inhibited by phenylsuccinate, a competitive inhibitor of the dicarboxylate carrier, which is not translocated into the matrix [34,35]. In this case the intramitochondrial concentration of succinate becomes limiting for respiration; this concentration decrease has a limited impact on the lagely positive affinity for succinate oxidation, which should be decreased maximally by about 10% for a 100-

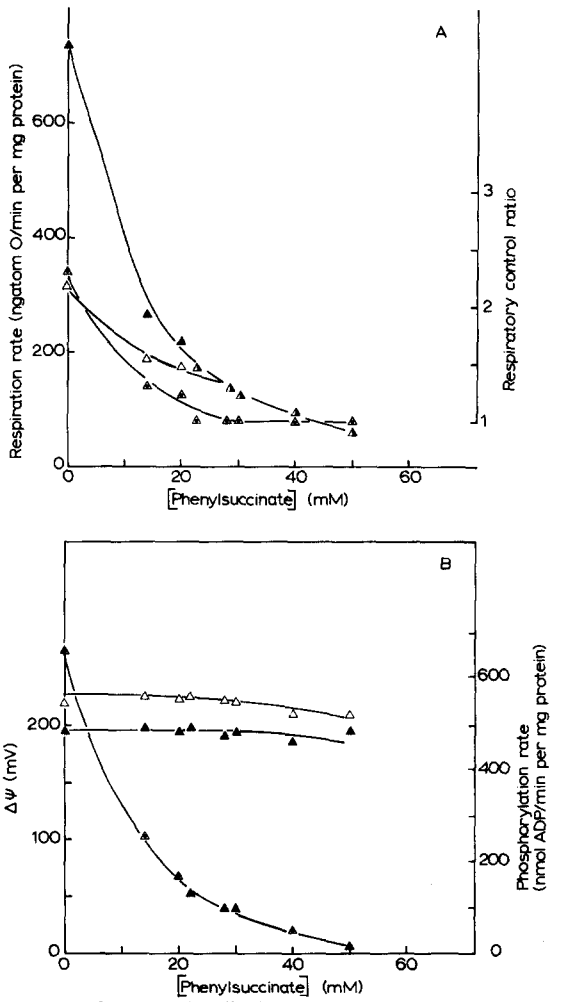

Fig. 3. Effects of inhibition by phenylsuccinate on the respiration-linked functions of Jerusalem artichoke mitochondria. (A) Effects on the rate of respiration in State 3 (▲) and in State 4 (△), and on the respiratory control ratio (▲). (B) Effects on the membrane potential ($\Delta\psi$) induced by State 3 (▲) and State 4 (△) respiration, and on the rate of phosphorylation (▲). For experimental conditions see Materials and Methods and Fig. 1. Mitochondria were incubated for 3 min in the presence of phenylsuccinate before the addition of the substrate (15 mM succinate). ADP additions: 83 μM for every State 3 cycle.

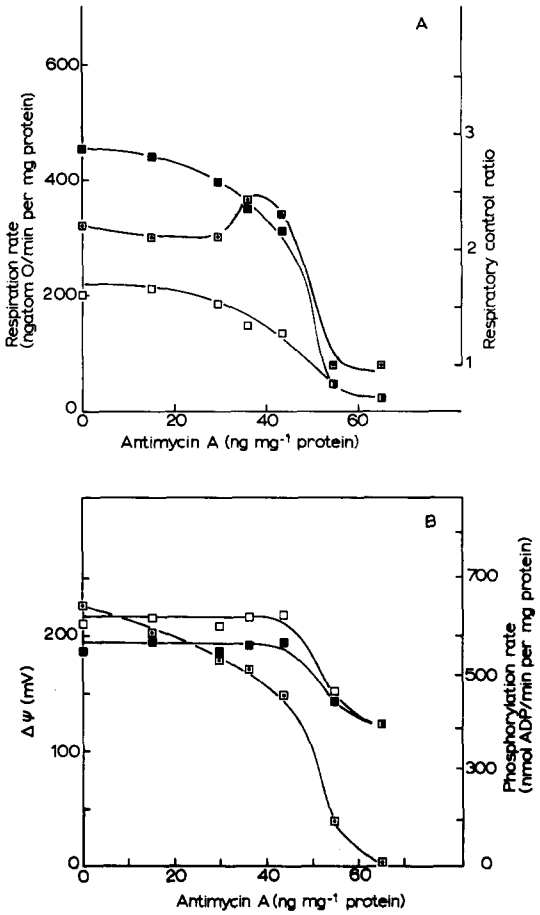

Fig. 4. Effects of inhibition by antimycin A on the respiration-linked functions of Jerusalem artichoke mitochondria. (A) Effects on the rate of respiration in State 3 (■) and in State 4 (□), and on the respiratory control ratio (⊡). (B) Effects on the membrane potential ($\Delta\psi$) induced by State 3 (■) and State 4 (□) respiration, and on the rate of phosphorylation (⊡). For experimental conditions see Materials and Methods and Fig. 1. Mitochondria were incubated for 5 min in the presence of antimycin A before the addition of the substrate (15 mM succinate). ADP additions: 83 μM for every State 3 cycle.

fold concentration decrease. Nevertheless, the comparative response of respiration, of the rate of oxidative phosphorylation and of $\Delta\psi$ to phenylsuccinate inhibition is very similar to that by malonate, presenting the same critical correspondence between rates of respiration and ATP formation, irrespective of the very limited depression of $\Delta\psi$ (Fig. 3A and B).

The titration of respiration with antimycin A is shown in Fig. 4A and B. This antibiotic binds very tightly to the receptor site in the ubiquinol-cytochrome *c* oxidoreductase complex and inactivates the single electron-transport chain. The titration curve of respiration is characteristically sigmoidal with a very sharp drop at about 50 ng/mg protein under our experimental conditions. The rate of respiration under phosphorylating conditions is slightly less inhibited than that in the absence of ADP, so that the respiratory control index goes through a maximum at substrating antimycin concentration. This behavior had been previously reported in rat liver mitochondria [20] and taken as evidence for slipping of the oxidoreductive proton pump. Again the titration of oxidative phosphorylation follows closely that of respiration in State 3; the sudden drop in activity at about 50 μM antibiotic is accompanied by a marked decrease in $\Delta\psi$, more evident than that for the inhibition by malonate or by phenylsuccinate. It is not clear if this response is due to a clearcut titration of all electron-transport chains or to a parallel uncoupling action of antimycin A, as suggested for many respiratory systems. Noticeably in rat liver mitochondria the formation of $\Delta\psi$ in State 3 or in State 4 is much more resistant to antimycin inhibition than in plant mitochondria, behaving in a fashion very similar to that described for malonate inhibition [25].

Drastically different is the effect of progressive uncoupling. In our experiments (Fig. 5A and B) the uncoupling was promoted by limiting concentrations of valinomycin, which, in association with the nigericin always present in all assays at saturating concentrations, catalyzes a dissipative circuiting of K^+ of intensity increasing with the concentration of the ionophore. Under our experimental conditions, valinomycin was added after the addition of succinate and the attainment of a steady-state $\Delta\psi$ and a constant respiratory rate under nonphosphorylating conditions, in order to avoid excessive swelling and consequent damage of the mitochondria during the course of the measurements.

Under these conditions, upon addition of valinomycin, a sudden depolarization is observed (Fig. 5), due to the initial electrogenic flux of K^+; within 1 min, however, a new steady-state condition of respiration is attained, characterized by a

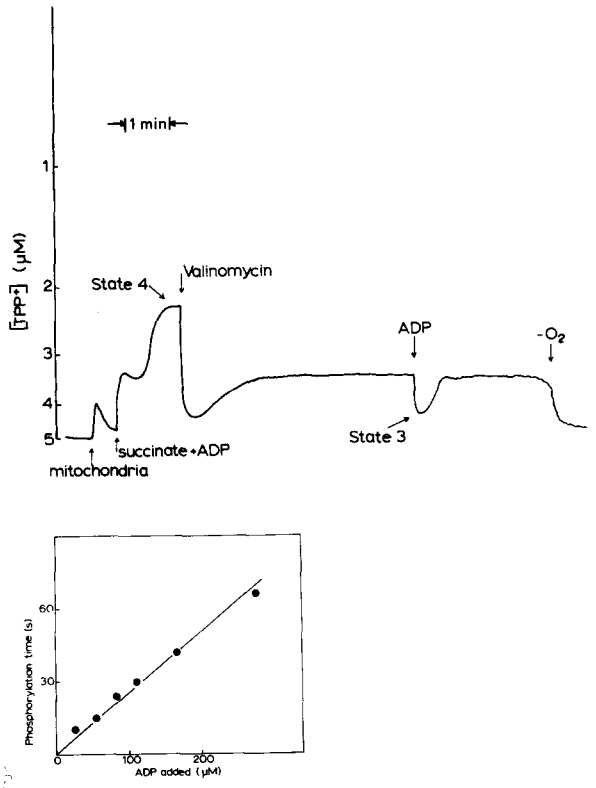

Fig. 5. Time course of $\Delta\psi$ upon addition of limiting concentrations of valinomycin (0.005 μM) in the presence of 30 mM K^+ and 0.1 μM nigericine, and State 4–State 3 transition in mitochondria partially uncoupled by a steady dissipative circuiting of K^+. In the inset the linearity of the phosphorylation time with ADP concentration is shown, demonstrating that a steady state of oxidative phosphorylation, respiratory rate in State 3 (not shown), and $\Delta\psi$ were truly attained under these conditions.

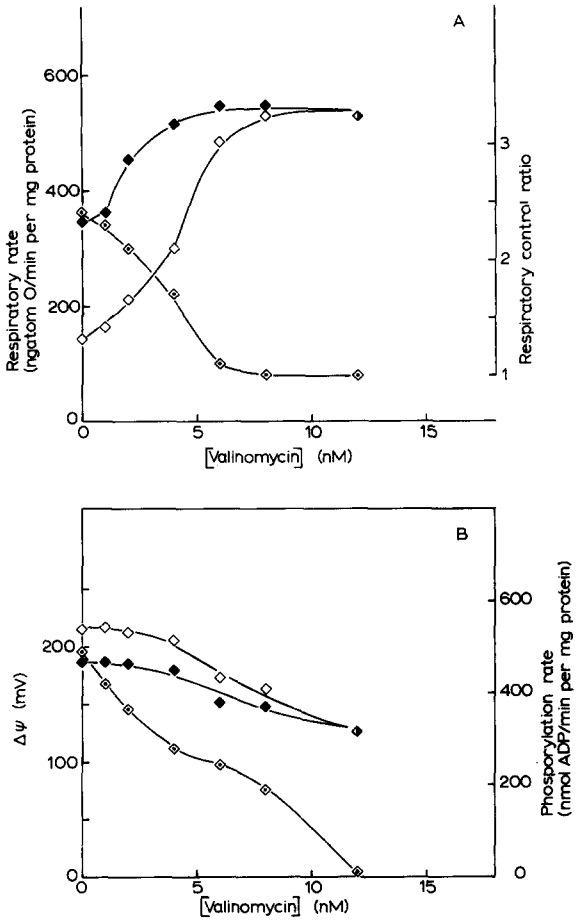

Fig. 6. Effects of uncoupling by nigericin (100 nM) and limiting concentrations of valinomycin on the respiration-linked functions of Jerusalem artichoke mitochondria. (A) Effects on the rate respiration in State 3 (◆) and in State 4 (◇), and on the respiratory control ratio (◈). (B) Effects on the membrane potential ($\Delta\psi$) induced by State 3 (◆) and State 4 (◇) respiration, and on the phosphorylation rate (◈). For experimental conditions see Materials and Methods and Fig. 1. Substrate: 15 mM succinate. Valinomycin was added after the exhaustion of the first ADP addition (83 μM).

lower $\Delta\psi$, in which part of the respiratory energy is dissipated by a futile K^+ cycle catalyzed by the association of the two ionophores; additions of ADP cause a State 4–State 3 transition, similar to that observed in the above-described experiments. The dependence of respiration and oxidative phosphorylation, and of $\Delta\psi$ under steady-state phosphorylating and nonphosphorylating conditions are shown in Fig. 6A and B. Our experimental conditions induced a stimulation of State 4 respiration and loss of respiratory control, progressively increasing with the concentration of valinomycin added; the rate of respiration in State 3 was also stimulated significantly, indicating that the ATP formation is rate limiting in our preparations of Jerusalem artichoke mitochondria. As expected, the value of $\Delta\psi$ was decreased by valinomycin and the rate of ATP formation progressively inhibited. The correlation between the extent of $\Delta\psi$ and rate of oxidative phosphorylation was, however, significantly different from that of the previous experiments; a significant rate of ATP formation was found at about 150 mV in

contrast with the data of Figs. 2–4. Remarkably, inhibition of oxidative phosphorylation was also observed at very low valinomycin concentrations (5 nM) when the decrease in $\Delta\psi$ was negligible, suggesting that the inhibition might not be a direct consequence of the depolarization of the mitochondrial membrane also in the case of uncoupling by ionophores. The effects of other uncouplers were not tested, given their interferences with the TPP^+ electrode.

Finally, the consequences of the inhibition of ATP synthesis by oligomycin are shown in Fig. 7A and B. As expected, this antibiotic depresses respiration under phosphorylating conditions, with a sigmoidal concentration dependence, but does not alter respiration in State 4. Parallel to the inhibition of oxidative phosphorylation, a decrease in ADP-induced depolarization is observed; the $\Delta\psi$ drop is completely abolished in good correlation with the decrease in the respiratory control index and inhibition of ATP formation. These experiments therefore offer an experimental demonstration of the phenomenological correlation between the drop of $\Delta\psi$ during a State 4–State 3 transition and the process of phosphorylation. Very similar results have been obtained by Zoratti et al. [25] in rat liver mitochondria.

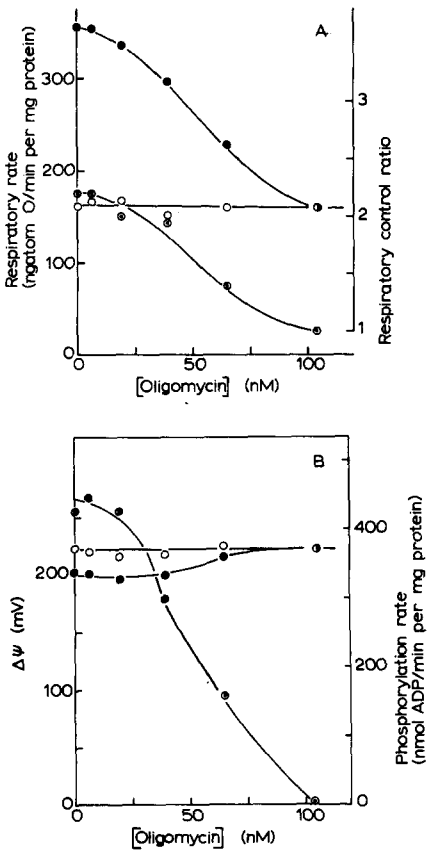

Fig. 7. Effects of inhibition by oligomycin on the respiration-linked functions of Jerusalem artichoke mitochondria. (A) Effects on the rate of respiration in State 3 (●) and in State 4 (○), and on the respiratory control ratio (⊙). (B) Effects on the membrane potential ($\Delta\psi$) induced by State 3 (●) and State 4 (○) respiration, and on the rate of phosphorylation (⊙). For experimental conditions see Materials and Methods and Fig. 1. Substrate: 15 mM succinate. Oligomycin was added after the exhaustion of the first ADP addition (83 μM).

Discussion

In this experimental work the estimation of the membrane potential has been made from the distribution of TPP^+; no correction for binding was made, since no evidence for it was provided by the TPP^+ electrode response in Jerusalem artichoke mitochondria. Reports of passive binding in TPP^+ (or of the related lipophilic cations $TPMP^+$ and $TBMA^+$) have been reported for rat liver mitochondria; this passive partition causes an overestimation of the membrane potential, as compared to K^+ distribution in the presence of valinomycin, and corrections have been proposed [25,28,36]. This phenomenon, which varies in mitochondria prepared from different sources, seems to be negligible in plant mitochondria. The omission of corrections for binding, however, does not invalidate the conclusion of the present work, since the correction would have possibly decreased the estimated $\Delta\psi$ by a fixed value, not altering therefore the pattern of the experimental data points (Figs. 2–7).

According to a delocalized chemiosmotic model, the steady-state value of $\Delta\tilde{\mu}_{H^+}$ should reflect a steady-state balance of the outward proton flux driven by the respiratory chain and the inward proton flux due to the ATPase catalysis and the passive proton leaks. Thus, if the value of $\Delta\tilde{\mu}_{H^+}$ remains constant under nonphosphorylating conditions upon inhibition of respiration in spite of the decreased proton-extrusion rate, an unabated initial rate of ATP formation should be observed

upon ADP addition, which should rapidly decrease $\Delta\tilde{\mu}_{H^+}$ to a lower steady-state value. Therefore, a larger depolarization is expected in a State 4–State 3 transition in respiration-inhibited mitochondria.

The behavior of Jerusalem artichoke mitochondria is absolutely inconsistent with this model. The value of $\Delta\tilde{\mu}_{H^+}$ remains constant or decreases slightly upon inhibition of respiration by more than 80% (Fig. 8). This observation has been made in bacterial chromatophores, mammalian mitochondria, submitochondrial particles and bacterial membrane fragments; its interpretation has been amply discussed [18–20,37]. In this context it must only be observed that the same resistance of $\Delta\psi$ to inhibition of respiration is seen also under phosphorylating conditions so that a constant extent of depolarization upon ADP addition is observed when respiration is progressively inhibited. The relevance of this potential drop to the mechanism of ATP formation is, on the other hand, conclusively demonstrated by its inhibition with oligomycin. The possibility that depolarization is not due to the protonic currents of the mechanism of ATP synthesis, but rather to the electrogenic ADP-ATP exchange catalyzed by the adenine nucleotide translocator, is amply discussed in Ref. 25. These results indicate that the inward and outward protonic currents must be always balanced during inhibition of the respiratory chain, so that in the presteady state upon ADP addition inward flow never greatly exceeds the outward flow. The observation that the decrease in the rate of phosphorylation parallels faithfully the rate of electron flow (Fig. 9) supports this view and suggests, according to a revised chemiosmotic model, that the activity of the single ATP synthetase complexes is strictly related to the number of electron-transport chains operative under a given set of conditions. This conclusion had been previously reached for bacterial chromatophores [16] and demonstrated in single-turnover resolution [17,21]. The recent experiments performed by Zoratti et al. [25] in rat liver mitochondria contemporarily to the present ones are in perfect agreement with this view.

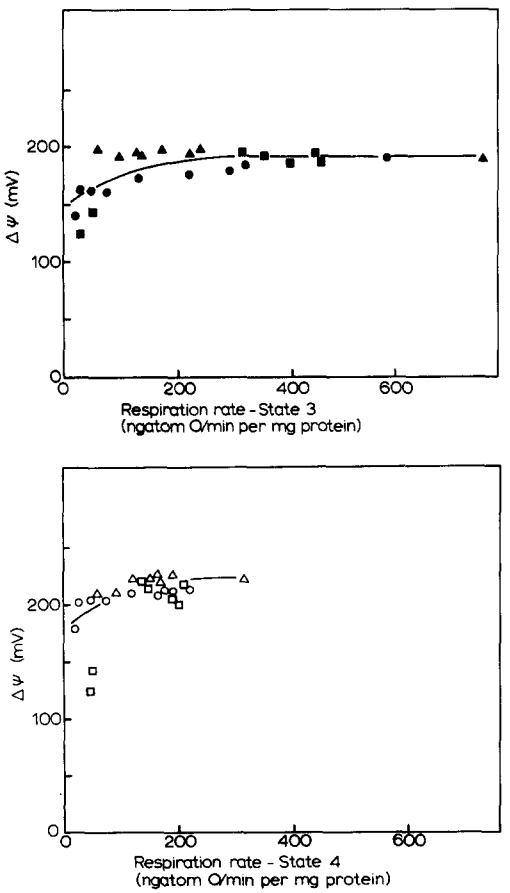

Fig. 8. Correlation between the rates of succinate oxidation and the values of the membrane potential ($\Delta\psi$) in State 3 (closed symbols) and State 4 (open symbols) in respiration-inhibited mitochondria from Jerusalem artichoke. Inhibitors: malonate (●,○), phenylsuccinate (▲,△), antimycin A (■,□). Data from Figs. 2–4.

The alternative conclusion, that the protonic electrochemical potential difference, as evaluated by the ion-distribution technique or by other methods, is not relevant as a driving force for ATP synthesis, seems to these authors hardly reconcilable with independent experimental evidence of chemiosmotic coupling (cf. Refs. 5–11).

The constancy of the ATP/$2e^-$ ratio when the rate of respiration is extensively inhibited is also hardly compatible with the expectation of the delocalized chemiosmotic model, according to which the passive diffusion of H^+ through the membrane leaks should compete with the chemiosmotic utilization of protons by the ATP synthetase, the greater is the inhibition of the input redox pump. On the contrary, this constancy of

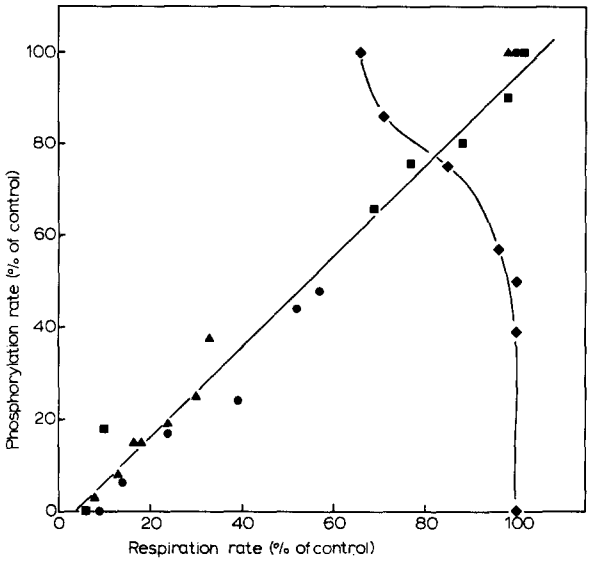

Fig. 9. Correlation between the rates of succinate oxidation and of phosphorylation in State 3 in respiration-inhibited mitochondria from Jerusalem artichoke. Inhibitors: malonate (●), phenylsuccinate (▲), antimycin A (■), valinomycin (◆). Data from Figs. 2–5.

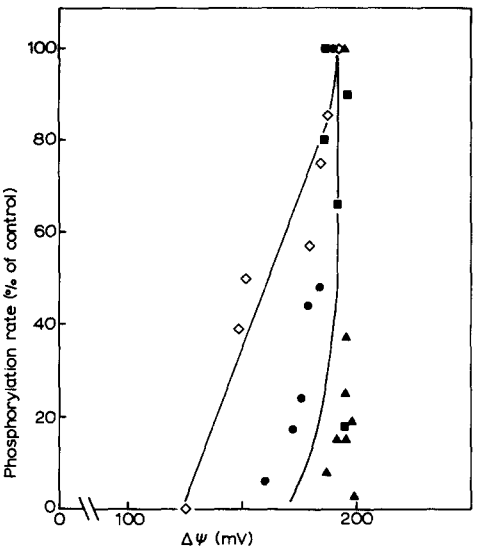

Fig. 10. Correlation between the values of membrane potential in State 3 and the rate of phosphorylation in respiration-inhibited mitochondria from Jerusalem artichoke. Inhibitors: malonate (●), phenylsuccinate (▲), antimycin A (■) and valinomycin (◇). Data from Figs. 2–5.

the ATP/$2e^-$ ratio is altered only by the onset of uncoupling conditions.

On the basis of the above relationships between $\Delta\tilde{\mu}_{H^+}$, and the rates of respiration and of phosphorylation, it has to be expected that large variations of the rate of ATP formation are experimentally observed also when a constant $\Delta\tilde{\mu}_{H^+}$ is maintained, provided that one perturbs the rate of electron transfer (Fig. 10). This behavior is identical with that previously reported for bacterial chromatophores inhibited by antimycin A or activated by actinic light of strongly limiting intensity [16]. Surprisingly, this behavior is also observed when mitochondria are partially uncoupled by nigericin and subsaturating concentrations of valinomycin (cf. also Ref. 38). This observation indicates that the dissipative K^+ circulation catalyzed by the synergistic action of these two ionophores could influence the coupling between ATP formation and respiration even before large dissipative fluxes would affect the bulk phase difference of $\Delta\psi$.

As a whole these experiments present conclusive kinetic evidence of the strict correlation between electron-transfer and ATP-forming reactions in the respiratory activity of Jerusalem artichoke mitochondria. The data extend to another system observations made in parallel in mammalian mitochondria and previously well documented in bacterial photosynthetic vesicles and respiratory fragments from mitochondria or aerobic bacteria. This kinetic behavior can be considered therefore as a general one for all energy-transducing membrane systems, either in eukaryotic or prokaryotic organisms, with the only possible exception being high plant chloroplasts for which an univocal correlation between the rate of ATP formation and ΔpH was measured [24]. In this system, on the other hand, the delocalization of the coupling is strongly suggested by the stacked organization of the grana lamellae, which forces a displacement of the ATPase complexes away from the intramembrane redox chains. Also, in chloroplasts serious discrepancies with chemiosmotic behavior have been documented, especially under presteady-state conditions [22,39].

The localized interaction between the redox events and ATP formation was also implicitly suggested by double inhibitor titration experiments pioneered by Baum et al. in mitochondria

[40,41], and recently extended to bacterial chromatophores [42,43], which demonstrated an additive effect of energy-transfer and electron-transfer inhibitors, also when supplemented at subsaturating concentrations.

Significant discrepancies and the lack of a correlation between $\Delta\tilde{\mu}_{H^+}$ and the free energy change for ATP formation have been also observed in State 4 in mitochondria and bacteria under different experimental conditions (i.e., change in osmolarity, induction of passive fluxes of ions or of weak acids, addition of uncouplers [36,44,45]), indicating a variability of the force ratio at static head for ATP synthesis or a lack of equilibration of ΔG_{ATP} vs. $\Delta\tilde{\mu}_{H^+}$ incompatible with a basic tenet of the chemiosmotic theory.

The mechanistic interpretation of the present data, as already stated by us previously for bacterial chromatophores [16,17,21], and extensively discussed recently by Zoratti et al. [25] for rat liver mitochondria (cf. also Ref. 46), must be sought either in a intramembrane protonic coupling between individual redox chains and ATP synthetases or in an activation mechanism of the ATP synthetases by the redox reactions. Direct evidence for such mechanisms which is useful for the choice between these two alternatives cannot, however, be obtained with kinetic or thermodynamic approaches.

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References

1 Mitchell, P. (1961) Nature 191, 144–148
2 Mitchell, P. (1966) Biol. Rev. 14, 455–502
3 Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399–430
4 Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E.C. (1977) Annu. Rev. Biochem. 46, 955–1026
5 Mitchell, P. and Moyle, J. (1968) Eur. J. Biochem. 4, 530–539
6 Keister, D.L. and Minton, N.J. (1971) Arch. Biochem. Biophys. 147, 330–339
7 Sone, N., Yoshida, M. Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 2956–2960
8 Jagendorf, A.T. and Uribe, E. (1966) Proc. Natl. Acad. Sci. U.S.A., 55, 170–177
9 Thayer, W.S. and Hinkle, P.C. (1975) J. Biol. Chem. 250, 5336–5342
10 Witt, H.T., Schlodder, E. and Gräber, P. (1976) FEBS Lett. 59, 272–276
11 Schuldiner, S., Rottenberg, H. and Avron, M. (1973) Eur. J. Biochem. 39, 455, 462
12 Caplan, S.R. and Essig, A. (1969) Proc. Natl. Acad. Sci. U.S.A. 64, 211–218
13 Westerhoff, H.V. and Van Dam, K. (1979) Curr. Top. Bioenerg. 9, 1–62
14 Padan, E. and Rottemberg, H. (1973) Eur. J. Biochem. 40, 431–437
15 Azzone, G.F., Pozzan, T., Massari, S. and Bragadin, M. (1978) Biochim. Biophys. Acta 501, 296–306
16 Baccarini Melandri, A., Casadio, R. and Melandri, B.A. (1977) Eur. J. Biochem. 78, 389–402
17 Venturoli, G. and Melandri, B.A. (1982) Biochim. Biophys. Acta 680, 8–16
18 Sorgato, M.C., Branca, D. and Ferguson, S.J. (1980) Biochem. J. 188, 945–948
19 Nicholls, D. (1974) Eur. J. Biochem. 50, 305–315
20 Pietrobon, D., Azzone, G.F. and Walz, D. (1981) Eur. J. Biochem. 177, 389–394
21 Melandri, B.A., Venturoli, G., De Santis, A. and Baccarini Melandri, A. (1980) Biochim. Biophys. Acta 592, 38–52
22 Ort, D.R., Dilley, R.D. and Good, N.E. (1976) Biochim. Biophys. Acta 449., 108–124
23 Harris, D.A. and Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87–102
24 McCarty, R.E. and Porstis, A.R., Jr. (1976) Biochemistry 15, 5110–5114
25 Zoratti, M., Pietrobon, D. and Azzone, G.F. (1982) Eur. Biochem. 126, 443–451
26 Møller, I.M., Chow, W.S., Palmer, J.M. and Barber, J. (1981) Biochem. J. 193, 37–46
27 Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membrane Biol. 49, 105–121
28 Casadio, R., Venturoli, G. and Melandri, B.A. (1981) Photobiochem. Photobiophys. 2, 245–253
29 Estabrook, R. (1967) Methods Enzymol. 10, 41–47
30 Chance, B. and Williams, G.R. (1955) Nature 175, 1120–1121
31 Raison, J.K., Lyons, J.M. and Campbell, L.C. (1973) J. Bioenerg. 4, 397–408
32 De Santis, A., Stipani, I. and Genchi, G. (1977) Boll. Soc. Ital. Biol. Sper. 53, 1414–1417
33 King, T.E. (1967) Methods Enzymol. 10, 216–224
34 Chappel, J.B. and Robinson, B.H. (1968) in Metabolic Roles of Citrate (Goodwin, T.W., ed.), pp. 123–131, Academic Press, New York
35 Quagliariello, E., Palmieri, F., Prezioso, D. and Klingenberg, M. (1969) FEBS Lett. 4, 251–254
36 Wilson, D.F. and Forman, N.G. (1982) Biochemistry 21, 1438–1444

37 Jackson, J.B. (1982) FEBS Lett. 139, 139–143
38 Zoratti, M., Pietrobon, D., Conover, T. and Azzone, G.F. (1981) in Vectorial Reactions and Ion Transport in Mitochondria and Bacteria (Palmieri, F., Quagliariello, E., Siliprandi, N. and Slater, E.D., eds.), pp. 331–338, Elsevier/North-Holland, Amsterdam
39 Graan, T., Flores, S. and Ort, D.R. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 25–34, Elsevier/North-Holland, New York
40 Baum, H., Hall, G.S. and Nalder, J. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C.S., eds.), pp. 747–755, Adriatica Editrice, Bari
41 Baum, H. (1977) in The Molecular Biology of Membranes (Flaischer, S., Hatefi, Y., McLennan, D.H. and Tzagoloff, A., eds.), pp. 243–262, Plenum Press, New York
42 Hitchens, G.D. and Kell, D.B. (1982) Biochem. J. 206, 351–357
43 Hitchens, G.D. and Kell, D.B. (1982) Biosci. Rep. 2, 743–749
44 Azzone, G.D., Massari, S. and Pozzan, T. (1978) Biochim. Biophys. Acta 501, 307–316
45 Westerhoff, H.V., Simonetti, A.L.M., De Jange, P.C., Van der Zende, W.J., Van der Bend, R.L. and Van Dam, K. (1981) in Vectorial Reactions and Ion Transport in Mitochondria and Bacteria (Palmieri, F., Quagliari
46 Westerhoff, H.V., Simonetti, A.L.M. and Van DAm, K. (1981) Biochem. J. 200, 193–202