they indicate that the number of protons involved in one turnover of the ATPase complex is greater than two.

This research was supported in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).


Proton Pump and Membrane Potential in Liver Mitochondria

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The great merit of the chemiosmotic hypothesis has been that of focusing the attention on the role of $H^+$ in energy transduction. However, the molecular details of this process are far from being understood. Mitchell (1966, 1976) has proposed that both ATP synthesis and cation and anion transport are driven by a primary $\Delta \mu_H^+$ (electrochemical proton-activity difference) of some 230mV. In mitochondria more than 90% of $\Delta \mu_H^+$ is assumed as $\Delta \psi$ (membrane potential) (Mitchell, 1976).

An obligatory role of $\Delta \mu_H^+$, and therefore of $\Delta \psi$, in ATP synthesis leads to the relationship: $\Delta G_p$ (phosphate potential)/$\Delta \mu_{H^+} = n$, where $n$ is the number of $H^+$ ions required for the synthesis of one molecule of ATP. If $n$ is fixed, whether two or four, the relation $\Delta G_p/\Delta \mu_{H^+}$ leads to the prediction of an osmotic threshold, i.e. the minimal value of $\Delta \mu_{H^+}$ below which ATP synthesis is abolished. The value of the osmotic threshold depends on the value of $n$. With a $\Delta G_p$ of 5.60mV, a value of $n$ equal to two implies an osmotic threshold of 280mV; for $n$ equals four, the osmotic threshold is 140mV. We have tested the existence of the osmotic threshold by measuring $\Delta G_p$ and $\Delta \mu_{H^+}$ at various uncoupler concentrations. $\Delta \mu_{H^+}$ has been determined on the Ca$^{2+}$ distribution in the presence of 10mm-Tris acetate. $\Delta G_p$ is found to decrease only slightly in the range of $\Delta \mu_{H^+}$ values 170–30mV and then drastically below 30mV. The $\Delta G_p/\Delta \mu_{H^+}$ ratio is about 3 at high $\Delta \mu_{H^+}$ while it increases hyperbolically parallel to the decline of $\Delta \mu_{H^+}$ tending to infinity when $\Delta \mu_{H^+}$ tends to zero. Similar results have been obtained also with submitochondrial particles, when the decrease of $\Delta \mu_{H^+}$ is induced by NH$_4$NO$_3$. Since only a limited number of charges can be transported per site, if the relation between $\Delta G_p$ and $\Delta \mu_{H^+}$ is hyperbolic, $\Delta \mu_{H^+}$ is not likely to be an obligatory intermediate in ATP synthesis.

As to the role of $\Delta \psi$ in ion transport, a physiological argument opposes the view of a primary respiratory-chain-generated $\Delta \psi$ of 200mV (Mitchell, 1976); 200mV implies an accumulation ratio of about $10^7$ for Ca$^{2+}$. Since Ca$^{2+}$ concentrations in the matrix

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is about 100 \mu M, Ca^{2+} concentrations in the cytosol should be lower than 100 \mu M. This is far too low for the activation of actinomycin during muscle contraction (Azzone et al., 1975).

A primary \Delta \psi of 200 mV should lead to a steady-state accumulation ratio of all permeant cations as predicted by the Nernst equation. However, this is not observed. The values of \Delta \psi, calculated in the presence of 10 mM-acetate, obtained from K^+ (plus valinomycin), univalent organic cations, Ca^{2+}, Mn^{2+} and Sr^{2+} accumulation ratios are not identical. Rather they vary from about 196 mV with K^+ at low K^+ concentrations, to 150 mV with K^+ at 500 \mu M-K^+, to 143 mV with triphenylmethylphosphonium ions, to 130 mV with Ca^{2+}, to 109–91 mV with Mn^{2+} and to 95 mV with Sr^{2+} (Azzone et al., 1977). Further, the values of \Delta \psi are affected by the cation permeability: the presence of Mg^{2+} decreases \Delta \psi calculated from the Ca^{2+} distribution, whereas valinomycin increases \Delta \psi calculated from the K^+ distribution.

Valinomycin-treated steady-state mitochondria maintain a K^+ concentration gradient without energy expenditure (Mitchell & Moyle, 1969; Azzone & Massari, 1971, 1973). It is generally assumed that this is due to the cation electrochemical gradient being zero. However, this is not proven. It has been reported that inhibitors of the bivalent-cation carrier such as La^{3+} or Ruthenium Red, cause Ca^{2+} efflux from steady-state mitochondria (Sordahl, 1973; Stucki & Ineichen, 1974). The observation has been explained by assuming that mitochondria possess an antiporter for bivalent cations that catalyses a continuous \Delta \psi-driven cation efflux. We have tested this hypothesis by measuring the rate of penetration of acetate salts of bivalent cations into anaerobic mitochondria in the presence of increasing concentrations of ionophore X537A. The rate of bivalent-cation influx increases proportionally to the ionophore concentration, but becomes zero when no ionophore is added. An alternative hypothesis is that the cation electrochemical potential in steady state is not zero. Cations tend to leave mitochondria at a rate proportional to the size of the cation gradient and to the permeability of the membrane for H^+ (acting as counter-ions). The hypothesis implies that, in steady state, the cation-gradient-driven cation efflux is continuously compensated by a cation re-uptake (through the pump) in the absence, but not in the presence, of Ruthenium Red. This hypothesis is supported by the following experiment. In the presence of uncouplers a lower steady-state cation uptake is observed. However, Ruthenium Red causes a faster cation efflux. Thus an increase of electrical H^+ permeability due to uncouplers causes a parallel increase of the rate of cation efflux. In the case of the electrogenic pump an increased H^+ influx via uncoupler should be compensated by an increased respiratory rate rather than by an increased cation efflux.

The strongest argument in favour of a \Delta \psi driving electrophoretically cation uptake and anion extrusion is the accumulation of K^+ in valinomycin-treated mitochondria. The argument is strengthened by the observation that lipid-soluble cations and anions are also taken up or extruded. There are, however, two types of energy-linked ion fluxes that are anomalous in respect to this picture: (a) the uptake of Cl^- and (b) the extrusion of univalent and bivalent cations (Azzone et al., 1975, 1976).

Consider first the uptake of Cl^- in intact mitochondria Cl^- is not taken up, because Cl^- permeability is low and the proton pump drives Cl^- extrusion. However, induction of Cl^- uptake may be obtained in two cases: (a) in the presence of trialkyltins, which catalyse OH^-/Cl^- exchange and therefore render Cl^- uptake similar to that of weak acids; (b) in presence of excess of permeant cations and absence of weak acids. It may be that the excess of permeant cations and the absence of weak acids causes saturation of the proton pump by cation transport and electroneutral coupling between H^+ and Cl^- influx.

Consider then the extrusion of cations. In intact mitochondria cations are not extruded because the proton pump drives uptake. However, cation extrusion has been observed in swollen mitochondria (Azzi & Azzone, 1967; Brierley, 1970; Azzone et al., 1976) and in brown-adipose-tissue mitochondria (Nicholls, 1974). How is the extrusion
Fig. 1. Electrical fluxes coupled through short-range interactions

Reaction (1) in the primary H⁺-ion extrusion which is coupled to electrical cation and anion fluxes through short-range interactions. Reaction (2) is the leak of H⁺ ions, either natural or induced by carbonyl cyanide p-trifluoromethoxyphenylhydrazone. The electrical H⁺-ion influx is coupled through short-range interactions to the electrical anion influx and cation efflux. Reaction (3) is the electroneutral uptake of anions. Reaction (4) is the H⁺/bivalent cation exchange via the electroneutral bivalent cation ionophores/X537A or A23187. Cat⁺⁺ represents a uni- or bi-valent cation.

of cations interpreted? The electrogenic proton-pump prediction is that of a ΔpH-driven electroneutral exchange via endogenous or exogenous ionophores. The proton-driven cation–anion pump prediction is also that of a ΔpH-driven electroneutral exchange, but via a tight coupling between the electrical fluxes of H⁺ and of cations (Fig. 1). The proton-driven cation–anion pump is supported by three observations: (a) rates of endogenous H⁺/K⁺ or H⁺/bivalent-cation antiporters are negligible in respect to observed extrusion rates; (b) extrusion of bivalent cations is sensitive to Ruthenium Red and La³⁺ (which are inhibitors of the native electrical carrier), addition of electroneutral bivalent cation ionophores restores the Ruthenium Red-inhibited extrusion; (c) fluxes of lipid-soluble organic cations are electrical by definition, yet all organic cations are actively extruded.
The term 'gate' indicates the membrane region where the permeability for H$^+$ is controlled. The proton fluxes are assumed as tightly coupled to cation or anion fluxes. The H$^+$ channel can be opened by 'energy', membrane stretching or high pH. Cat$^{(+)}$ represents a uni- or bi-valent cation.

The electroneutral proton pump (Massari & Azzone, 1970a,b) was based on a proton carrier and tight coupling between H$^+$ and cation fluxes. The chemiosmotic mechanism (Mitchell, 1966) envisages a H$^+$ channel as part of the adenosine triphosphatase. Fig. 2 shows a mitochondrial membrane with H$^+$ channels; the channels catalyse H$^+$ transport under both passive and active conditions. The H$^+$ channel is seen as possessing gates at the matrix and cytosol interphase. The gate is opened either passively by membrane damage or actively by 'energy' (provided by the respiratory chain or the adenosine triphosphatase). The H$^+$ flux through the channel is coupled through short-range electrostatic interaction to cation and anion fluxes. This renders the fluxes electroneutral. However, the proton fluxes may be either active or passive. The former leads to formation of ΔpH and the latter to consumption of ΔpH.

References:
Proton Pumps in Bacterial Photosynthesis

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We have previously suggested that the cyclic electron-transport chain in *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* is arranged as two proton pumps operating in series, one pump being associated with the photochemical reaction centre and its immediate secondary donor (cytochrome *c*₂) and acceptor (ubiquinone), and the other with the 'dark' electron transport through which the cycle is completed, from ubiquinone back to cytochrome *c*₂ (Jackson & Crofts, 1971; Jackson & Dutton, 1973; Crofts *et al.*, 1971). Evidence for these two distinct sites has come from several lines of investigation, including kinetic studies of flash-induced redox changes, pH changes, and changes in membrane electrical gradient as indicated by the carotenoid change, and especially the way in which these parameters change as the ambient redox potential is changed (Jackson & Dutton, 1973; Cogdell *et al.*, 1972; Dutton & Jackson, 1972; Evans & Crofts, 1974; Crofts *et al.*, 1975; Crofts, 1974; Prince & Dutton, 1975). In the present paper we discuss extensions of this work in which aspects of both proton pumps are examined further.

Reconstitution of the proton-pumping activity of purified photochemical reaction centres from *Rhodopseudomonas sphaeroides*

Reaction centres prepared from cells of either wild-type or the R26 mutant of *Rhodopseudomonas sphaeroides* by (NN-dimethyl-laurylamine oxide) extraction (Clayton & Wang, 1971) have been incorporated into liposomes by a cholate-dialysis technique (Kagawa & Racker, 1971). The orientation of the reaction centres appeared to depend upon the concentration ratio of protein to lipid, as has been observed independently by Reiss-Husson (1976). The orientation could be tested by observing the reactivity of P870 or cytochrome *c* on illumination of the liposomes in the absence or presence of added reduced mammalian cytochrome *c*, together with either 1,4-naphthaquinone or 1,4-naphthaquinonesulphonate, before and after addition of excess of cholate.

In liposomes with a protein/lipid ratio of 1:30 (w/w), approx. 60% of reaction centres were arranged so that externally-added reduced cytochrome *c* could reduce P870⁺ following activation by a saturating (5 μs) flash (Fig. 1a). On continuous illumination the extent of external cytochrome *c* oxidation observed in the presence of added 1,4-naphthaquinone was much greater than that with 1,4-naphthaquinonesulphonate (Fig. 1b), indicating that the reaction sites of the primary donor and acceptor were on opposite sides of the liposome membrane. In these liposomes continuous illumination induced a pH change, measured by glass electrode or pH indicator, showing extrusion of protons (Fig. 1c). The pH changes were reversible, greatly stimulated by valinomycin (markedly when K⁺, Rb⁺ or Cs⁺ were present), and inhibited by carbonyl cyanide

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