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MECHANISM OF RESPIRATION-DRIVEN PROTON TRANSLOCATION IN THE INNER MITOCHONDRIAL MEMBRANE

KINETICS OF PROTON TRANSLOCATION AND ROLE OF CATIONS

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SUMMARY

The kinetics and mechanism of passive and active proton translocation in submitochondrial vesicles, obtained by sonication of beef heart mitochondria, have been studied.

Analysis of the anaerobic release of the protons taken up by submitochondrial particles in the respiring steady state shows that proton diffusion consists of two parallel, apparent first-order processes: a fast reaction which, on the basis of its kinetic properties and response to cations and various effectors, is considered to consist of a proton/monovalent cation exchange; and a slow process which, on analogous grounds, is considered as a single electrogenic flux.

The study of the various parameters of the respiration-linked active proton translocation and of the accompanying migration of permeant anions and K^+ led to the following conclusions: (i) The oxidoreduction-linked proton translocation is electrogenic. (ii) Cation counterflow is not a necessary factor in the respiration-driven proton translocation. (iii) The membrane potential developed by active proton translocation exerts a coupling with respect to permeant cations and anions. (iv) The respiration-driven proton translocation is secondarily coupled, through the $\Delta\mu_H$ component of the electrochemical proton gradient and at the level of a proton–cation exchange system of the membrane, to the flow of K^+ and Na^+.

INTRODUCTION

The mechanism and function of oxidoreduction-linked proton translocation represent major problems of energy transduction associated with downhill electron and hydrogen transfer in mitochondria, as well as in chloroplasts and bacterial membranes (see refs 1 and 2 for review). Available evidence shows that the respiration-driven proton translocation in mitochondria consists of a vectorial transmembrane flux. In isolated mitochondria respiration causes proton release into the suspending

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

medium[3,4]; this appears to be accompanied by intramitochondrial alkalinization[5-8]. In vesicles of the inner membrane, obtained by sonication of mitochondria[9,10], respiration gives rise to proton disappearance from the medium[11-14]. Different experimental approaches show that the inner membrane has an inner side, on which coupling factors and the active sites of NADH and succinate dehydrogenase, and possibly cytochrome a_3, are located, and an outer side where cytochromes *c* and *a* are found[15-17]. The two sides appear to be separated by a permeability barrier. In sonic submitrochondrial particles the inner side faces the medium, the outer side the interior of the vesicles[9,15-19]. The components of the two sides move as a whole without any apparent change in their relative positions[15,17].

The present studies have been carried out in submitochondrial vesicles obtained by sonication of beef heart mitochondria in the presence of Mg–ATP[9] or EDTA[10]. The use of these particles allows direct analysis of proton translocation in the inner mitochondrial membrane without interferences from the outer mitochondrial membrane and matrix solutes which can give rise to secondary fluxes[20]. Kinetic analysis demonstrates that passive proton diffusion in submitochondrial particles consists of two parallel fluxes: a proton–monovalent cation exchange and a noncoupled proton flow. Evidence is presented showing that the respiration-driven proton translocation consists of a single, electrogenic flow, mediated by system(s) separate from the cation carriers of the membrane. The oxidoreduction-linked, active translocation of protons is secondarily coupled, at the level of the proton–cation exchange system of the membrane, to the flow of K^+ and Na^+. Part of these investigations has been previously communicated[21,22].

METHODS

Submitochondrial particles

Mg–ATP- and EDTA-submitochondrial particles were prepared as described by Löw and Vallin[9] and Lee and Ernster[10], respectively. Heavy beef heart mitochondria[9], stored for 1–10 days at −10 °C, were suspended at a concentration of 20 mg protein/ml in 0.25 M sucrose, containing 15 mM $MgCl_2$ and 1 mM ATP (final pH 7.0) or 4 mM EDTA (pH 8.5), and were then exposed to ultrasonic energy for 60 s at 0 °C (Ultrasonic-Branson Sonifer, Model W 185; output, 70 W).

Radioisotopic analysis of water compartmentation and anion distribution

To determine water compartmentation, submitochondrial particles were suspended in 0.25 M sucrose, containing [^{14}C]dextran (mol. wt 60000–90000) and ^3H_2O. After centrifugation, the total water of the particle pellet which was exchangeable with ^3H_2O and the dextran-accessible water were calculated from the ^3H and ^{14}C radioactivity in the supernatant and $HClO_4$ extracts of the pellet (Table I). Submitochondrial particles contained an internal aqueous phase amounting to about 2.5 μl/mg protein. Mg–ATP particles were also incubated, in aerobiosis, with [^{14}C]-succinate. In the presence of antimycin A, succinate freely permeated the internal aqueous phase of the submitochondrial particles (Table I). Respiration, when antimycin was omitted, did not affect the distribution of succinate between the internal and external phase. Similar results were obtained in EDTA particles. The distribution of [^{14}C]thiocyanate was measured using the same procedure.

TABLE I

WATER COMPARTMENTATION AND [^{14}C]SUCCINATE DISTRIBUTION IN Mg–ATP PARTICLES

Submitochondrial particles (0.8 mg protein/ml) were incubated for 5 min in a reaction mixture containing 0.25 M sucrose, 10 mM potassium succinate, ^3H_2O and [^{14}C]dextran or [^{14}C]succinate. Final volume 8 ml. Final pH 7.0. Temperature 30 °C. Water is referred to 1 mg particle protein.

	cpm [^{14}C]*dextran* / *cpm* ^3H_2O	*μl water calculated from*		*cpm* [^{14}C]*succinate* / *cpm* ^3H_2O
		^3H_2O	[^{14}C]*Dextran*	
Supernatant	0.24			0.14
Pellet	0.12	6.50	4	0.13
Pellet + antimycin, 1 μg/ml	0.12	6.60	4	0.14

Electrometric recording of respiration and ion translocation

Submitochondrial particles, 1–3 mg protein/ml, were incubated in a standard reaction medium containing 0.25 M sucrose and 0.2 mg purified catalase/ml. Other additions are given in the legends to the figures and tables. Incubation was carried out in stoppered conical vessels under a constant stream of N_2. The vessels were surrounded by a glass jacket thermostated to ± 0.01 °C. The suspension was vigorously stirred by a magnetic stirrer. The pH of the suspension and the concentration of oxygen, K^+ and SCN^- were monitored by electrodes inserted through holes in the stopper. Once the particle suspension was made anaerobic by succinate oxidation, respiration was repetitively activated by additions of 1–5 μl/ml 0.3–3% H_2O_2. The addition of catalase alone to succinate-supplemented anaerobic particles, in the absence or presence of antimycin A, did not give rise to any detectable oxygen production (*cf.* ref. 23). Oxygen was, on the other hand, instantaneously (<20 ms) produced, under the experimental conditions used, upon addition of H_2O_2 to the catalase-supplemented particles. The catalase reaction was not rate limiting and enabled us to repetitively bring the system into the aerobic steady state without any significant dilution of the particle suspension.

Electrodes and electronic circuitry

Respiration was monitored polarographically by a Clark oxygen electrode (Yellow Springs Instruments Co. Inc., Model 5331). The voltage supply and the amplifier were those recommended by the makers of the electrode. The amplified output current was fed into a two-channel recorder (Honeywell, Model Electronik 194). The pH was measured potentiometrically using 50–100-MΩ glass electrodes (Ingold KG, Frankfurt/Main, Germany) and a KCl–agar bridge connected to a calomel electrode, or a Beckman combination electrode, No. 39030. The response time of the glass electrode, measured with a Roughton-type continuous flow pH meter[24], was, for a ΔpH as large as 0.5 unit, around 20 ms. The glass electrode was connected to a Keithley, Negative Capacitance Electrometer, Model 605, and from this to the Honeywell recorder. The overall response time of the pH recording system was about 100 ms at 30 °C. The sensitivity of the recorder could be adjusted to give a 5-cm deflection per mV, the speed could reach 2.5 cm/s. Potential changes were

quantitated as proton equivalents by double titration with standard solutions of KOH and HCl. The stabilized electronic equipment used allowed the pH to be measured with a precision of 0.001 pH unit. K^+ was monitored potentiometrically with a Beckman Cationic electrode, No. 39047, connected to an electronic circuit analogous to that used for measuring the pH; the response time was of the same order of that obtained for pH measurement. When measuring $[K^+]$, the pH was maintained at a level 3–4 units higher than the pK^+. Under the conditions used, the response of the cationic electrode to the pH changes studied was negligible. SCN^- was monitored with a selective, membrane electrode ("Sens-Ion", Amel, Milano, Italy) connected to the electronic circuitry described above. The SCN^- electrode showed a slower response than the glass electrode. The response of the cationic electrode and of the SCN^- electrode were calibrated by addition of known standards of KCl and KSCN, respectively.

Mathematical analysis

Electrometric measurements were processed with the aid of an Hewlett–Packard 9810A calculator. For the kinetic analysis of the proton flow, the calculator was directly connected to a Hewlett–Packard 9862A Calculator Plotter. The potentiometric traces of the anaerobic proton diffusion from submitochondrial particles were converted into proton equivalents and, where shown (see under Results), treated by the double exponential Eqn 1

$$(\Delta H^+)_{t=\infty} - (\Delta H^+)_t = (\Delta H_1^+)\, e^{-k_1 t} + (\Delta H_2^+)\, e^{-k_2 t} \qquad (1)$$

where $(\Delta H^+)_{t=\infty}$ stands for the amount of protons taken up by respiring particles at the steady state; $(\Delta H^+)_t$ represents the amount of protons retained by the particles at time t during the phase of anaerobic release; and $(\Delta H^+{}_1)$, $(\Delta H^+{}_2)$, k_1 and k_2 represent the amount of protons translocated by two parallel reactions and their respective first-order velocity constants.

Analysis of endogenous cations

Submitochondrial particles were mineralized with a sulpho–nitroperchloric melange[25]. Particles equivalent to 200 mg protein were reduced to 10 ml of a clear saline solution. Mg^{2+} was determined by atomic absorption, Ca^{2+} by flame emission with a Zeiss spectrophotometer. Mg–ATP particles contained 50 nmoles Mg^{2+} and 10 nmoles Ca^{2+} per mg protein; EDTA particles contained 2.5 nmoles Mg^{2+} and 15 nmoles Ca^{2+}. Thanks are due to Dr G. Nuovo for his help with the cation analysis.

Respiratory control

The respiratory control ratio of the Mg–ATP particles used in the present investigation was 2.5–3 with an NADH-regenerating system and about 2 with succinate. The same values were obtained in EDTA particles, supplemented with 1 μg oligomycin/mg protein[10]. The phosphorylating capacity of the Mg–ATP particles[26] and of the EDTA particles, the latter treated with low concentrations of oligomycin, was comparable to that reported by other authors[18,27].

RESULTS

Respiration-driven translocation of protons and electrolytes in submitochondrial particles

It was previously found that salts of monovalent cations enhance the initial rate and the extent of the respiration-driven proton uptake in EDTA- and Mg–ATP-submitochondrial particles[13,14]. The stimulation was directly related to the permeability of artificial phospholipid membranes and mitochondrial membranes to the anionic species, with the following affinity order: tetraphenylboron$^- > SCN^- > I^- > NO^-_3 > Cl^-$. At saturation, however, salts of a given cation, say K^+, brought the rate and the extent of the respiration-driven proton uptake to approximately the same level. This is exemplified by traces d–f of Fig. 1, which refer to cycles of proton translocation induced by oxygenation of anaerobic Mg–ATP particles, supplemented with succinate as respiratory substrate and saturating concentrations of KI, KSCN or KNO_3 (see ref. 14). These salts gave a 3–4-fold stimulation of the aerobic proton uptake. KCl at a concentration of 50 mM caused about 100% stimulation of proton translocation. The addition of valinomycin, in the presence of KCl, raised the proton uptake to the level reached with KSCN, KI and KNO_3, but had practically no effect when added in the presence of saturating concentrations of these salts.

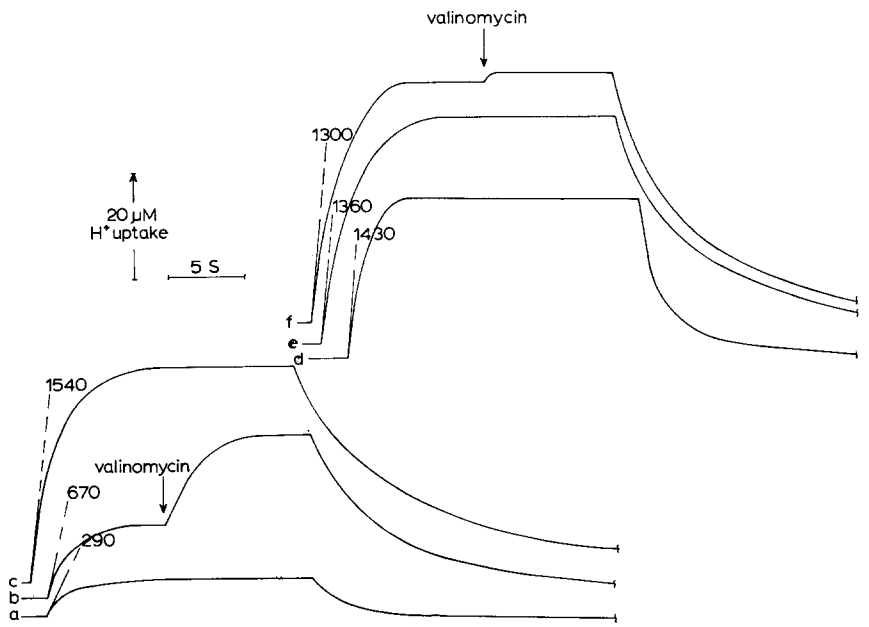


Fig. 1. Effect of potassium salts and valinomycin on the respiration-driven proton translocation in Mg–ATP particles. Submitochondrial particles (2.3 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 15 mM potassium succinate and: Expt b, 50 mM KCl; Expt c, KCl *plus* 0.2 µg/ml valinomycin; Expt d, 15 mM KSCN; Expt e, 40 mM KI; Expt. f, 50 mM KNO_3. It should be noted that 15 mM KSCN caused 20% inhibition of oxygen uptake. Final vol. 1.5 ml. Final pH 7.0. Temperature 30 °C. For other details see under Methods. The net initial rates of H^+ uptake, given in the figure, are expressed as ngions H^+/min per mg protein.

Fig. 2 compares the effect of KNO_3 and $NaNO_3$ on the respiration-driven proton uptake by Mg–ATP particles. The extent of H^+ uptake increased with the concentration of the two salts. However, with $NaNO_3$ the extent of H^+ uptake was, over the whole concentration range, about half that reached with KNO_3.

The experiment of Fig. 3 illustrates the movement of SCN^-, during a respiratory pulse of Mg–ATP particles. SCN^- was taken up by the particles along with protons during the aerobic phase and rereleased with anaerobiosis (*cf.* refs 20, 21 and 28). The rate of SCN^- uptake was apparently lower than that of H^+ uptake, however, this might be partly due to the SCN^- electrode which responds slower than the glass electrode. Fig. 4 shows that, on increasing the concentration of added KSCN, the respiration-driven SCN^- uptake by Mg–ATP particles increased and this was paralleled by an enhancement of the aerobic H^+ uptake. However, especially at

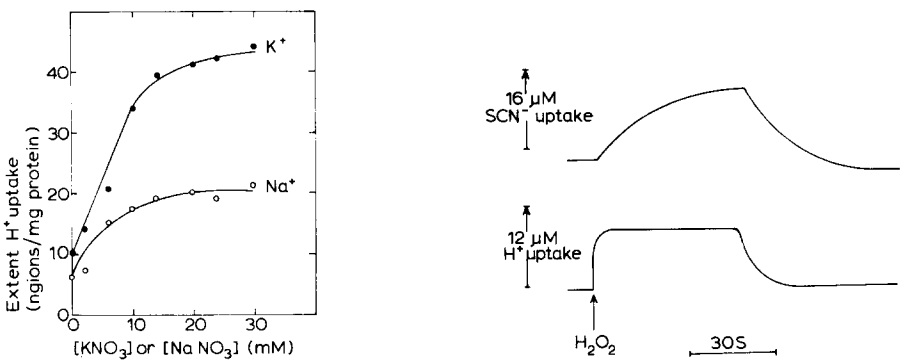


Fig. 2. Effect of KNO_3 and $NaNO_3$ on the respiration-driven proton translocation in Mg–ATP particles. Submitochondrial particles (1.7 mg protein/ml) were incubated in the standard reaction mixture containing, in addition: 15 mM potassium succinate or 15 mM sodium succinate and KNO_3 or $NaNO_3$ at the concentration given in the figure. Final vol. 1.5 ml. Final pH 7.0. Temperature 25 °C.

Fig. 3. Respiration-induced proton and thiocyanate translocation in Mg–ATP particles. Submitochondrial particles (1.0 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 15 mM potassium succinate, 1 mM KSCN. Final vol. 5 ml. Final pH 7.0. Temperature 30 °C. H^+ and SCN^- were monitored simultaneously as described under Methods.

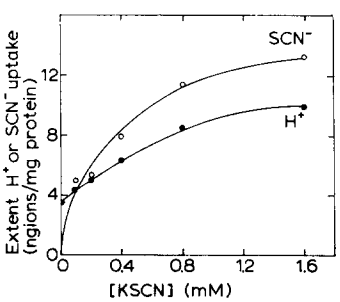


Fig. 4. Effect of KSCN concentration on the extent of respiration-driven H^+ and SCN^- uptake by Mg–ATP particles. The experimental conditions are those given in the legend to Fig. 3. For other details see under Methods.

higher concentrations of KSCN, the amount of anion taken up by the particles was significantly higher than that of H^+. Thus, other positively charged ion(s) should be taken up by the particles in order to maintain electrical neutrality. It should be recalled that succinate, which acted in our system both as respiratory substrate and buffer, freely permeated the particles during the anaerobic phase and that respiration did not cause any detectable extra uptake of [^{14}C]succinate by the particles (see Table I).

It has been reported that respiration induces K^+ uptake by submitochondrial sonic particles[20,27,30]. Fig. 5 shows that, in the absence of permeating anions, K^+ uptake by respiring Mg–ATP particles was negligible with respect to proton uptake. Tris–nitrate stimulated H^+ uptake and even more markedly stimulated K^+ uptake (*cf.* refs 29 and 30). However, also in the presence of this salt, the initial rate and

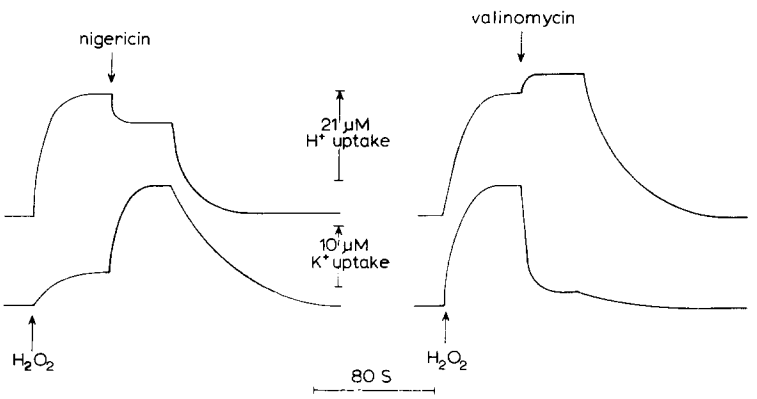


Fig. 5. Respiration-induced H^+ and K^+ translocation in Mg–ATP particles. Effect of nitrate. Submitochondrial particles (2.6 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 20 mM Tris–succinate, 500 μM K^+. Final vol. 8 ml. Final pH 7.0. Temperature 30 °C. Tris–nitrate was added at a final concentration of 30 mM. K^+ and H^+ were monitored simultaneously as described under Methods. The net initial rates of H^+ and K^+ uptake, given in the figure, are expressed as ngions per min mg protein.

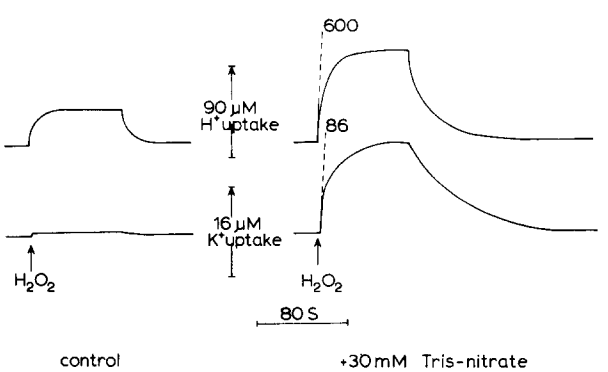


Fig. 6. Effect of ionophores on H^+ and K^+ translocation in Mg–ATP particles. Submitochondrial particles (1.1 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 15 mM Tris–succinate, 500 μM K^+, 5 mM Tris–nitrate and, where added, 0.25 μg/ml nigericin and 0.5 μg/ml valinomycin. Final vol. 8 ml. Final pH 7.0. Temperature 30 °C. For other details see under Methods.

the extent of K^+ uptake were considerably lower than those of H^+ uptake. Fig. 6 shows that the addition, in the aerobic steady state, of nigericin to Mg–ATP particles, supplemented with 5 mM Tris–nitrate, induced a marked further uptake of K^+. In contrast, however, with the results using NO_3^-, the nigericin-induced K^+ uptake was accompanied by discharge of H^+ from the particles. Valinomycin, added to respiring particles in the presence of 5 mM NO_3^- and nigericin, caused discharge of K^+ (*cf.* refs 29 and 30). The valinomycin-induced discharge of K^+ was accompanied by extra H^+ uptake by the particles.

In Fig. 7 the effect of the external K^+ concentration on the extent of the aerobic K^+ and H^+ uptake by Mg–ATP particles is presented. K^+ uptake increased with K^+ concentration and this was accompanied by decrease of H^+ uptake.

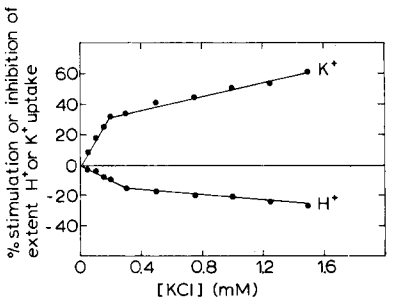


Fig. 7. Effect of external K^+ concentration on the respiration-driven H^+ and K^+ uptake by Mg–ATP particles. Submitochondrial particles (1.0 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 15 mM Tris–succinate; 5 mM Tris–nitrate and KCl at the concentrations indicated in the figure. Final vol. 6 ml. Final pH 7.0. Temperature 30 °C.

TABLE II

EFFECT OF THE BUFFERING POWER AND FCCP ON THE RESPIRATION-DRIVEN H^+ AND K^+ UPTAKE BY SUBMITOCHONDRIAL PARTICLES

Submitochondrial particles (2.6 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: Expt a, 20 mM Tris–succinate, 500 μM K^+ and 50 mM Tris–nitrate; Expt b, 20 mM choline succinate, 500 μM K^+ and 50 mM choline nitrate. For both experiments the final volume was 8 ml. Final pH 7.0. Temperature 30 °C. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Additions	*Extent uptake (ngions/mg protein)*			
	Expt a Tris medium		*Expt b Choline medium*	
	H^+	K^+	H^+	K^+
None	33.0	5.0	27.4	15.6
0.5 μM FCCP	25.5	2.7	16.0	5.7
1 μM FCCP	13.5	0.4	13.6	0.7
Buffering power (μgions H^+/pH unit)	143		16	

Table II illustrates the effect of the buffering power of the system on the aerobic K^+ and H^+ uptake by submitochondrial particles. In a choline medium, with a buffering power about ten times lower than that of the respective Tris medium, the extent of the aerobic K^+ uptake was 3-fold higher than in the Tris medium. The extent of H^+ uptake was, on the contrary, slightly higher in the Tris medium than in the choline medium. The experiment of Table II also shows that the aerobic K^+ uptake was practically suppressed by 1 μM FCCP. At this concentration the uncoupler caused about 50% decrease of the extent of H^+ uptake (see also ref. 22).

Table III shows the effect of valinomycin *plus* K^+ on the respiration-driven $S^{14}CN^-$ uptake by Mg–ATP particles. Respiration caused active uptake of $S^{14}CN^-$. The respiration-dependent uptake of SCN^- was suppressed by valinomycin: the antibiotic reduced the $S^{14}CN^-$ uptake to the level observed when respiration was inhibited by antimycin A.

TABLE III

RESPIRATION-LINKED $S^{14}CN^-$ UPTAKE BY SUBMITOCHONDRIAL PARTICLES

The reaction medium contained: 0.25 M sucrose, 15 mM potassium succinate, 10 mM $KS^{14}CN$ and 5 mg particle protein. Final vol. 5 ml. Final pH 7.0. After 2 min incubation at 30 °C, the particles were rapidly separated from the medium by centrifugation.

Additions	*Amount in the vesicles of $S^{14}CN^-$ (nmoles)*
None	174
30 mM KCl + 0.2 μg/ml valinomycin	110
1 μg/ml antimycin	109

Kinetics of the anaerobic proton release

Kinetic analysis of the anaerobic release of the protons taken up by the particles during the respiratory phase (see Fig. 1) allows direct insight into the mechanism of passive H^+ diffusion in submitochondrial particles. There are no mixing problems and the improved electronic circuitry used had an adequate recording time (see under Methods). In Fig. 8 are presented the semilogarithmic, first-order plots of the anaerobic H^+ release from Mg–ATP particles after an oxygen pulse with succinate at 30 °C. The kinetics of H^+ release show a clear initial upward deviation from a linear first-order plot (Expt a). The biphasic nature of H^+ release was even more marked when the respiration-induced cycle of H^+ translocation took place in the presence of valinomycin *plus* K^+ (Expt b). Expt c shows that the interruption of the electron flow by antimycin A caused a proton release which was kinetically similar to that occurring with anaerobiosis. It might be mentioned that the biphasic nature of H^+ release was not evident in previous measurements where the process was followed only at 20–25 °C and the system used to record pH changes had a slower response time (ref. 12; see also ref. 31).

The most simple kinetic model which might apply to the biphasic kinetics of proton release is that of two parallel first-order processes with different intrinsic velocities[32], as described by Eqn 1 (see under Methods). By applying Eqn 1 the biphasic kinetics could, in fact, be resolved into two linear first-order plots with

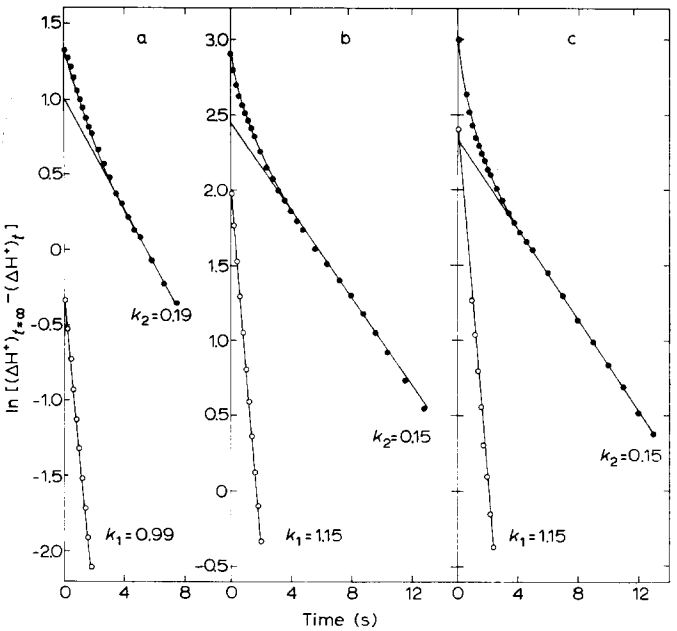


Fig. 8. Kinetics of the release of protons taken up by Mg–ATP particles during respiration. Submitochondrial particles (1.2 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 15 mM potassium succinate and Expts b and c, 30 mM KCl and 0.13 μg/ml valinomycin. In Expt c, antimycin was added in the aerobic steady state at a concentration of 1 μg/mg protein. Final vol. 1.5 ml. Final pH 7.0. Temperature 30 °C.

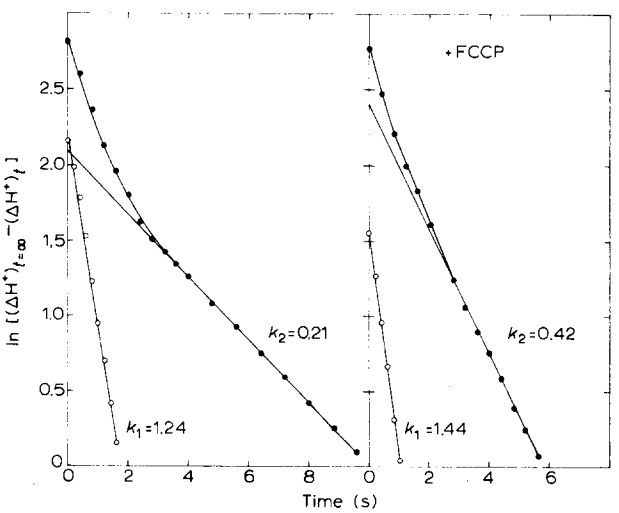


Fig. 9. Effect of FCCP on the kinetics of the anaerobic proton release from Mg–ATP particles. Submitochondrial particles: 1.1 mg protein/ml. Additions to the basic reaction mixture: 15 mM Na–succinate and 15 mM NaSCN. Final vol. 1.5 ml. Final pH 7.5. Temperature 25 °C. FCCP was added in the anaerobic state at a final concentration of 0.1 μM.

different slopes. The H^+ decay can, therefore, be considered as consisting, under the prevailing conditions, of a fast (1) and a slow (2) process. The slopes of the two lines give the respective velocity constants and the intercepts with the ordinate give the amounts of protons translocated by the two processes. Valinomycin *plus* K^+ caused a decrease of k_2 and of the amount of protons translocated by Reaction 2 but a slight enhancement of k_1. The constants k_1 and k_2 were, in the presence of antimycin A, equal to those calculated for the proton release caused by oxygen exhaustion.

The respiration-driven proton uptake by submitochondrial particles was not affected by 0.1 μM FCCP (see also ref. 22), neither did the uncoupler abolish the biphasic nature of the anaerobic proton release (Fig. 9). FCCP caused a marked increase of k_2, but had practically no effect on k_1.

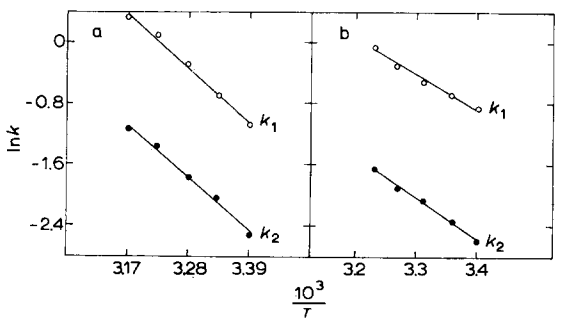


Fig. 10. Arrhenius plots for the proton diffusion reactions in Mg–ATP particles. Submitochondrial particles, Expt a: 1.4 mg/ml; Expt. b: 1.3 mg/ml. Additions to the basic reaction mixture: 15 mM potassium succinate and, Expt a, 30 mM KCl *plus* 0.1 μg/ml valinomycin; Expt b, 30 mM KI. Final vol. 1.5 ml. Final pH 7.5.

The Arrhenius plots of Fig. 10 illustrate the temperature dependence of the anaerobic proton diffusion in the presence of KI or of valinomycin *plus* KCI. The plots give, in the 20–41 °C range, straight lines for both the proton-diffusion processes. From the slopes of the lines, activation energies of 9.2 kcal/mole for Reaction 1 and 10.4 kcal/mole for Reaction 2 could be calculated in the system with KI. In the presence of valinomycin the activation energies of Reactions 1 and 2 were 12.9 and 12.5 kcal/mole, respectively. The activation energies of the two proton-diffusion processes are equivalent to the breaking of 3–4 hydrogen bonds[33]. Thus simple proton diffusion through water-filled pores of the membrane would be excluded for both processes. It might be mentioned that the activation energies for the proton-diffusion reactions are close to the activation energy for the valinomycin-mediated energy-linked K^+ uptake by intact mitochondria[34]. In Fig. 11 the kinetics of the anaerobic proton release in a K^+ and Na^+ medium are compared. Proton diffusion was markedly faster in the Na^+ than in the K^+ medium. Analysis of the biphasic proton release showed, however, that k_1 but not k_2 was significantly higher with Na^+ than with K^+.

Sensitivity of proton translocation to inhibitors of cation translocation

Massari and Azzone[8], and Scarpa and Azzone[35] have reported that the aerobic uptake by isolated mitochondria of Ca^{2+} and of K^+ in the presence of valinomycin

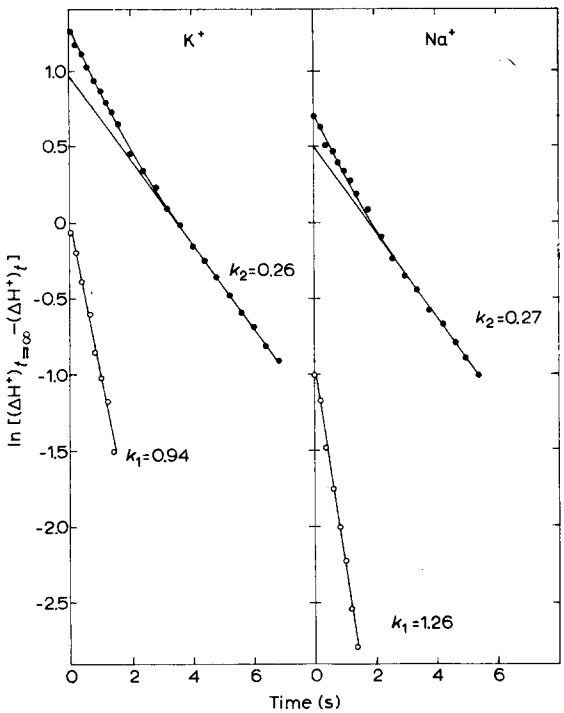


Fig. 11. Effect of monovalent cations on the kinetic parameters of the anaerobic proton release from Mg–ATP particles. Submitochondrial particles: 1.3 mg protein/ml. Additions to the basic reaction mixture: 15 mM potassium succinate or sodium succinate. Final vol. 1.5 ml. Final pH 7.5. Temperature 30 °C.

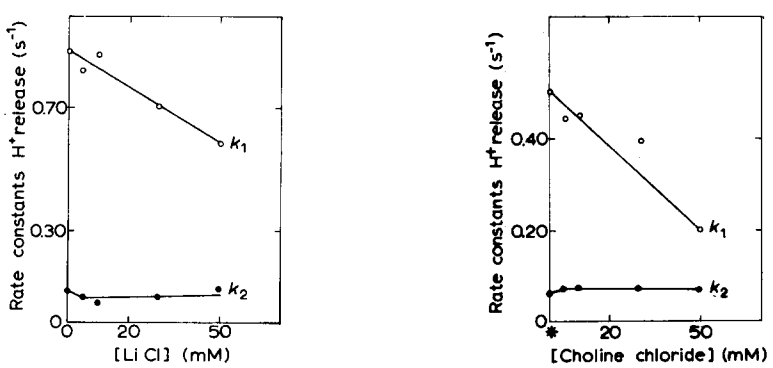


Fig. 12. Effect of LiCl on the passive proton translocation in Mg–ATP particles. Submitochondrial particles (2.0 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 10 mM potassium succinate, 1 mM K-EDTA and LiCl at the concentrations indicated in the figure. Final vol. 2 ml. Final pH 7.5. Temperature 30 °C.

Fig. 13. Effect of choline-chloride on the passive proton translocation in Mg–ATP particles. Submitochondrial particles (2.0 mg protein/ml) were incubated in a reaction mixture containing, in addition to the basic components: 10 mM potassium succinate, 1 mM K-EDTA, 1 μg/ml valinomycin and choline-chloride at the concentrations indicated in the figure. Final vol. 2 ml. Final pH 7.5. Temperature 25 °C.

is inhibited by impermeant cations. In particular, Li^+ was found to be a competitive inhibitor of aerobic K^+ uptake by mitochondria[8]. LiCl up to a concentration of 50 mM did not inhibit the respiration-driven proton uptake by Mg–ATP particles, but rather it gave a slight enhancement (about 15% at 50 mM LiCl) of both the initial rate and the extent of H^+ uptake. This stimulation was due to inhibition of the proton back flow. Fig. 12 shows that LiCl gave, in fact, a marked inhibition of the fast Reaction 1 of the anaerobic proton diffusion. It was, however, without effect on the slow component (2) of the proton diffusion. Choline chloride, in the absence (not shown) and in the presence of valinomycin, had (Fig. 13) effects similar to those exhibited by LiCl.

Dibucaine is a positively charged local anaesthetic which binds to membrane phospholipids[36,37]. Dibucaine inhibits spontaneous and valinomycin-mediated diffusion of K^+ across the mitochondrial membrane[38] and artificial phospholipids membranes[39]. It is a competitive inhibitor of respiration-driven K^+ uptake by valinomycin-treated mitochondria with a K_i of 50 μM[38]. In Fig. 14 the effect of dibucaine on the various parameters of the respiration-induced proton translocation in Mg–ATP particles is illustrated. In a K^+ medium dibucaine, in the concentration range of 50–300 μM, caused a marked inhibition of the initial rate of the aerobic proton uptake by the particles. In a choline medium the rate of proton uptake was smaller than that in a K^+ medium. In this case dibucaine stimulated the initial rate of proton uptake. Dibucaine depressed the extent of the aerobic proton uptake both in the K^+ and choline medium. The depression of the extent of H^+ uptake was, at least in part, due to stimulation of the proton back flow. In fact, dibucaine caused a marked decrease of the $t_{1/2}$ of the anaerobic proton decay in both the K^+ and choline medium.

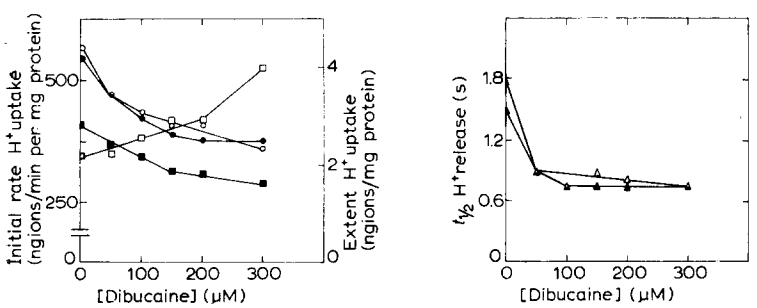


Fig. 14. Effect of dibucaine on the respiration-induced proton translocation in Mg–ATP particles. Submitochondrial particles: 1.6 mg protein/ml. Additions to the basic reaction mixture: 15 mM potassium succinate or 15 mM choline succinate and dibucaine at the concentrations given in the figure. Final vol. 1.5 ml. Final pH 8.0. Temperature 30 °C. □—□, initial rate proton uptake; ○—○, extent proton uptake; △—△, $t_{1/2}$ proton release. Open symbols refer to the choline medium, closed symbols to the K^+ medium.

In Table IV the effect of EDTA and EGTA on the respiration-driven proton translocation in Mg–ATP particles supplemented with 5 mM KSCN is shown. 3 mM EDTA had practically no effect on respiration and aerobic proton uptake. The chelating agent caused, however, an increase of the initial rate of the anaerobic proton release. The result was that the steady-state H^+/O quotient (measured as the ratio between the initial rate of the anaerobic H^+ release and the steady-state respiratory

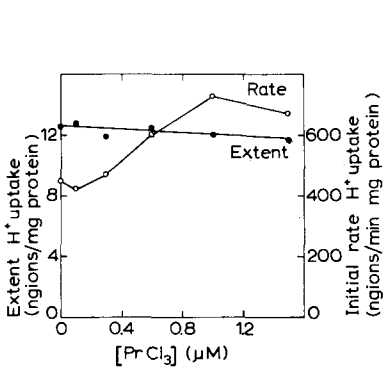
TABLE IV

EFFECT OF EDTA, EGTA AND DIBUCAINE ON PROTON TRANSLOCATION IN Mg–ATP PARTICLES

Submitochondrial particles: 1.6 mg protein/ml. The reaction mixture contained, in addition to the basic components: 15 mM choline succinate, 5 mM KSCN and, where indicated, 3 mM choline EDTA, 3 mM choline EGTA and 300 μM dibucaine. Final vol. 1.5 ml. Final pH 7.0. Temperature 30 °C For other details see under Methods.

Additions	*H^+ uptake extent (ngions/mg protein)*	*H^+ release initial rate (ngions/min per mg protein)*	*Steady-state respiratory rate (ngatoms/min per mg protein)*	*Steady-state H^+/O*
–	14.6	642	1159	0.55
EDTA	19.5	732	1116	0.66
EDTA + EGTA	16.6	652	1055	0.62
EDTA + EGTA + dibucaine	8.3	1041	852	1.22

rate) was increased by EDTA. The addition of EGTA together with EDTA had no further effect on the respiration-driven proton turnover. This was, on the other hand, significantly increased when dibucaine was added in the presence of the two chelating agents. Fig. 15 shows that praseodymium, at concentrations which completely block the energy-linked Ca^{2+} uptake by isolated mitochondria[40], did not cause any depression, rather it enhanced the initial rate of the respiration-linked proton uptake by Mg–ATP particles supplemented with dibucaine and thiocyanate. The lanthanide had practically no effect on the extent of proton uptake.



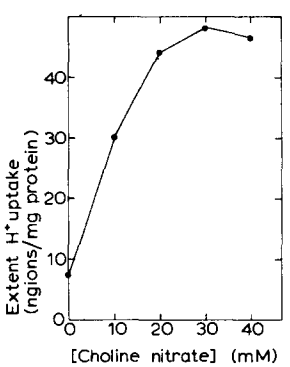


Fig. 15. Effect of praseodymium on the respiration-driven H^+ uptake by Mg–ATP particles. Submitochondrial particles: 1.8 mg protein/ml. Additions to the basic reaction mixture: 15 mM choline succinate, 5 mM KSCN, 300 μM dibucaine and $PrCl_3$ at the concentrations given in the figure. Final vol. 1.5 ml. Final pH 7.0. Temperature 30 °C.

Fig. 16. Stimulation by choline nitrate of respiration-linked proton uptake by EDTA particles. EDTA particles: 1.5 mg protein/ml. Additions to the basic reaction mixture: choline succinate 15 mM, 2 μg/ml oligomycin and choline nitrate at the concentrations given in the figure. Final vol. 1.5 ml. Final pH 7.5. Temperature 30 °C.

Proton translocation in EDTA particles

In EDTA particles oligomycin inhibits the passive proton diffusion and enhances aerobic proton uptake[13,20]. In the presence of this antibiotic the activity of the respiration-linked proton translocation was equivalent to that of Mg–ATP particles. In EDTA particles the process was stimulated to the same extent, as in Mg–ATP particles, by valinomycin *plus* K^+ and salts of permeant anions. In these particles the anaerobic proton release also consisted of two parallel processes. Fig. 16 illustrates the stimulatory effect of choline nitrate on the respiration-driven proton uptake by EDTA particles. It can be seen that, at saturating concentrations of nitrate, respiring EDTA particles took up about 50 ngions H^+/mg protein.

DISCUSSION

Proton extrusion from respiring mitochondria is associated, by a stoicheiometric relation, with the uptake of Ca^{2+} of or K^+ in the presence of valinomycin[41]. Energy-linked proton ejection from mitochondria is also observed in the absence of valinomycin and added Ca^{2+} (refs 3 and 4); in this case, however, the process is depressed by chelating agents[4,42]. These and related observations are explained in terms of three mechanisms: (i) primary uphill electrogenic proton translocation, secondarily compensated by passive electrophoretic migration of cations[19,43]; (ii) free-diffusional flow of protons in the electric field generated across the membrane by an electrogenic $\sim$ -driven cation pump [34,44]; (iii) $\sim$ -driven electroneutral proton–cation exchange mediated by a proton–cation carrier in the membrane[8]. As an essential prerequisite for mechanisms (ii) and (iii), but not for (i), the respiration-driven proton current should be compulsorily linked to cation counterflow. To discriminate between these models we have analyzed the relationship between proton and cation flow in the absence and presence of metabolism.

Passive proton diffusion

Analysis of the kinetics of the anaerobic release of the protons taken up by respiring submitochondrial particles shows that proton diffusion consists of two parallel, apparent first-order net processes: a fast Reaction 1, which is completed within a few seconds, and a slow Reaction 2, which continues until all the protons taken up by the particles have been released back into the suspending medium. k_1 depends upon the cationic species, being greater with Na^+ than with K^+. The addition to a K^+ medium of the impermeant cations Li^+ or choline causes a strong decrease of k_1. k_1 is practically unaffected by FCCP or by inhibition of electron flow by antimycin. k_2 is practically independent of the cationic species, but it is greatly increased by FCCP. Antimycin has no effect on k_2; however, this constant is significantly lowered by the addition of valinomycin in the presence of K^+. It is concluded that the fast process of proton diffusion in sonic submitochondrial particles consists of a proton–cation exchange at the level of a carrier in the membrane (see also refs 42 and 45). The slow process of proton diffusion, on the other hand, appears to consist of a single electrogenic current. A membrane potential positive inside the particles, acts as driving force for proton translocation along this pathway. The process can, therefore, be depressed when the $\Delta\psi$ is dissipated by valinomycin *plus* K^+ (see below).

Active proton translocation

Recently, it has been found that both in mitochondria and submitochondrial particles the energy-linked proton translocation can be accompanied by migration of a variety of organic and inorganic permeant anions in the same direction of protons and of organic lipophilic cations in the opposite direction[20,21,28,46]. Valinomycin *plus* K^+ and salts of permeant anions cause a large increase of the initial rate and the extent of the respiration-driven proton uptake by sonic submitochondrial particles[13,14]. At saturation, salts of rapidly permeating anions are as effective as valinomycin *plus* K^+ in promoting the respiration-driven proton translocation (Fig. 1). The addition of valinomycin to respiring submitochondrial particles causes, as in intact mitochondria, K^+ translocation in the direction opposite to that of protons (Fig. 6). There is a direct relationship between anion accumulation by respiring particles and stimulation of proton uptake (Fig. 2). Furthermore, permeant anions, unlike valinomycin, promote K^+ translocation in the same direction of protons (Fig. 5). The conclusion that can be drawn from these observations is that co-migration of permeant anions can completely replace the promoting effect exerted on the oxidoreduction-linked proton current by valinomycin-mediated K^+ counterflow.

That K^+ counterflow is not essential for the respiration-driven proton uptake by submitochondrial particles is also shown by the fact that dibucaine reduces the initial rate of the aerobic proton uptake in a K^+ medium but stimulates it in a choline medium (Fig. 14). In a K^+ medium proton influx is compensated, evidently, at least in part, by K^+ counterflow. Dibucaine inhibits K^+ efflux[38] and as a consequence H^+ uptake is depressed. This is not the case with choline which is impermeant to mitochondrial and artificial phospholipid membranes.

Submitochondrial particles contain significant amounts of divalent cations (see under Methods and ref. 12). Hence, it has been proposed that the respiration-driven proton uptake by submitochondrial particles is secondary to energy-linked release of endogenous cations from the vesicles (12; see also ref. 47). Attempts to detect energy-linked extrusion of divalent cations from sonic submitochondrial particles have so far, however, given negative results[12,48]. Mg–ATP particles contain an amount of Ca^{2+} and Mg^{2+} which, if mobile, could account for charge compensation of proton uptake by respiring particles. However, the combined addition of excess EDTA and EGTA had, in the presence of dibucaine and thiocyanate, no inhibitory effect on the respiration-linked proton translocation (see Table IV), neither was the process inhibited, under the same conditions, by concentrations of Pr^{3+} which completely block aerobic Ca^{2+} translocation in mitochondria (Fig. 15). EDTA particles contain 2.5 nmoles Mg^{2+} and 15 nmoles endogenous Ca^{2+} per mg protein. However, in the absence of added permeant cations choline nitrate (+ oligomycin) brought the extent of the aerobic H^+ uptake by these particles form 3–5 ngions up to 50 ngions/mg protein (Fig. 16). Thus, even if migration of endogenous divalent cations can partly compensate the respiration-linked proton translocation, divalent cation counterflow, like monovalent cation counterflux, is not compulsory to the respiration-linked proton current.

These results led us to conclude that the respiration-linked proton translocation across the inner mitochondrial membrane consists of a single, electrogenic flow.

Electrogenic proton pumping will be rapidly limited, once a small amount of proton has been translocated, by the ensuing membrane potential. According to

Mitchell[19] the net translocation of 1 ngion H^+/mg protein would develop a membrane potential of 250 mV. The potential acts as a force on all ionic components of the system and thus drives migration in the electric field across the membrane of any permeant ion[49]. Dissipation of $\Delta\psi$ by ion equilibration results, in turn, in a stimulation of the primary electrogenic flux. As predicted by this mechanism, it is experimentally found that respiration-driven anion and cation translocation are mutually competitive. Further compelling support in favour of an electrogenic proton translocation comes from the recent finding[50] that valinomycin *plus* K^+ enhances by 3–4-fold the rate of respiratory carrier oxidation in the anaerobic–aerobic transition of submitochondrial particles, but leaves the steady-state respiration unaffected. Dissipation of $\Delta\psi$ by valinomycin-mediated migration of K^+ speeds up proton translocation and electron flow until the ensuing extra $\Delta\mu_H$ replaces the dissipated $\Delta\psi$; then electron flow subsides to control value.

K^+ translocation

In sonic submitochondrial particles respiration in the absence of ionophores induces K^+ translocation in the same direction of protons. Of the two fluxes it is the proton current which is primarily linked to electron flow. In the absence of permeating anions the proton pump is fairly active; K^+ uptake is hardly appreciable. Also, in the presence of an excess of nitrate, which gave a marked stimulation of aerobic K^+ uptake, the initial rate of K^+ translocation remained one order of magnitude lower than that of proton uptake. The aerobic K^+ uptake took place at the expense of the aerobic proton gradient (Fig. 7). Furthermore, respiration-driven K^+ uptake was almost completely suppressed by FCCP at a concentration which caused 50% decrease of the extent of the aerobic proton uptake (Table II).

The stimulation of the aerobic K^+ uptake by permeating anions provides evidence that the driving force for cation uptake is the $\Delta\mu_H$ component of the aerobic $\Delta\tilde{\mu}_H$. This is further supported by the observation that the aerobic K^+ uptake is inversely related to the buffering power of the system (Table II). The aerobic K^+ uptake by sonic particles is mediated, evidently, by the proton–cation exchange system of the membrane which catalyzes the fast Reaction 1 of proton diffusion in anaerobiosis. In fact, Li^+ or choline which caused inhibition of the anaerobic proton–cation exchange, resulted in an enhancement of aerobic proton uptake. Proton uptake was markedly depressed when K^+ in the suspending medium was replaced by Na^+ which exchanges faster with protons (see Fig. 11). In aerobiosis the proton–cation exchange system of the mitochondrial membrane clearly operates parallel with the respiration-linked proton translocator.

The present data on proton and cation flows across the inner mitochondrial membrane might be related to the molecular architecture of the membrane, as now seen from various experimental approaches. Freeze–etching electron microscopy[51,52] (see also ref. 53), as well as digestion of the mitochondrial membrane with phospholipases[15], indicate that large areas of the membrane are constituted by phospholipid bilayers. Proteins would be embedded in the lipid matrix giving a mosaic structure[54]. The similarity of the effects of valinomycin and local anaesthetics on K^+ diffusion in mitochondrial membranes and artificial phospholipid bilayers makes it conceivable that spontaneous and valinomycin-mediated diffusion of K^+ in the mitochondrial membrane occurs across the lipid bilayer regions. This flux will be governed by the

permeability coefficients of the cation in the hydrophobic region[55] and by the surface potential (Z potential) determined by the polar phospholipid heads[56]. A valinomycin–K^+ complex facilitates the diffusion of K^+ in the hydrophobic region. Positively charged anaesthetics, on the other hand, interacting with phospholipid polar heads induce a positive change in the Z potential, decrease the K^+ concentration at the interface and inhibit its diffusion across the membrane.

Dibucaine, on the contrary, does not inhibit the respiration-driven proton translocation; neither is this process inhibited by Li^+ and choline, which inhibit the proton–monovalent cation exchange. The aerobic proton current is also uninhibited by lanthanides which are potent inhibitors of the mitochondrial Ca^{2+} pump[40]. Thus the oxidoreduction-linked proton translocation is mediated by system(s) different from those involved in cation translocation.

The only inhibitors known at present for the respiration-driven proton translocation are the inhibitors of the respiratory chain. This favours those mechanisms postulating a primary coupling between redox reactions and vectorial proton translocation at the level of the same components of the respiratory chain[19,22].

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