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Proton Translation in Submitochondrial Vesicles

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Abstract

An investigation of proton translocation in submitochondrial vesicles from rat liver has been made under simple experimental conditions. Choline chloride was used both as the oxidizable substrate and the ionic medium for the measurement of activity during oxygen pulse experiments:

(1) The passive permeability measured from the decay of proton efflux after an oxygen pulse could be described by a first-order equation. An H^+/O ratio of 2·5 was obtained for choline oxidation in the presence of oligomycin and/or $MgCl_2$. Oligomycin decreased the passive proton permeability and respiration, concomitant with an increase in proton uptake. Respiratory control was directly related to the passive proton permeability and inversely related to the magnitude of the proton gradient. The decreased respiration and passive permeability reflecting respiratory control is most evident in the pH range 5·8–7·5.

(2) Preparation of submitochondrial vesicles in the presence of EDTA resulted in proton production during an oxygen pulse given at alkaline pH. Cytochrome *c* enhanced proton uptake by approximately 1 H^+/cytochrome *c*, but only in the presence of Triton X-100. These results are indicative of the asymmetric behavior of the coupling membrane and provide direct evidence of the participation of electron transport components in proton translocation.

Introduction

More definite studies on the relation between H^+ translocation and structural changes in mitochondria are required to clarify the basic issues of energy coupling mechanisms. Since submitochondrial vesicles (SMV) contain a high proportion of membrane and are largely free of matrix enzymes, they represent a more resolved system for the analysis of H^+ translocation and structural changes. Lee and Ernster[1] were among the first to indicate that oligomycin could be used in SMV to partially restore coupling to non-phosphorylating sonic preparations. Mitchell and Moyle[2] showed that SMV from heart mitochondria were capable of an inefficient O_2-dependent proton uptake and that the vesiculated membrane was partially impermeable to protons. They also observed enhancement of proton uptake by oligomycin. Tager, Papa, Guerrieri, Groot and Quagliariello[3] have confirmed these results of Mitchell and Moyle and have additionally shown a decrease in the passive proton permeability of the vesicles caused by oligomycin. In bacterial chromatophores a similar result has been obtained by von Stedingk and Baltscheffsky[4] and Scholes, Mitchell and Moyle.[5]

Chance and Mela[6] suggested that the uptake of H^+ in SMV was probably in response

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to a commensurate movement of divalent cations. Coupling activity in SMV and chromatophores was later shown to be modified by other monovalent cations by Papa, Guerrieri, Tager and Quagliariello,[7] Montal, Chance, Lee and Azzi[8] and Cockrell and Racker,[9] and Jackson, Crofts and von Stedingk.[10]

Packer and Utsumi[11] showed a definite relationship between proton translocation and rat-liver mitochondrial structure by showing that during aging under anaerobic conditions three fairly distinct phases could be distinguished: In phase I intact mitochondria have limited permeability to protons and show O_2-dependent proton uptake. In phase II there is an increased permeability to protons with a maximal permeability found after aging *in vitro* under anaerobic conditions for about 20 min, or without aging, by minimal Triton X-100 treatment. O_2-dependent proton production increased reaching the stoichiometry reported by Mitchell and Moyle[12] after the membrane structure was altered. In phase III, when no proton barrier exists, a small O_2-dependent proton uptake due to oxido-reduction components, which consume protons in reaction, was observed; energy coupling was lost in these extensively damaged membranes. The analogous phase of proton translocation in SMV should be phase II where the permeability of H^+ is already increased.

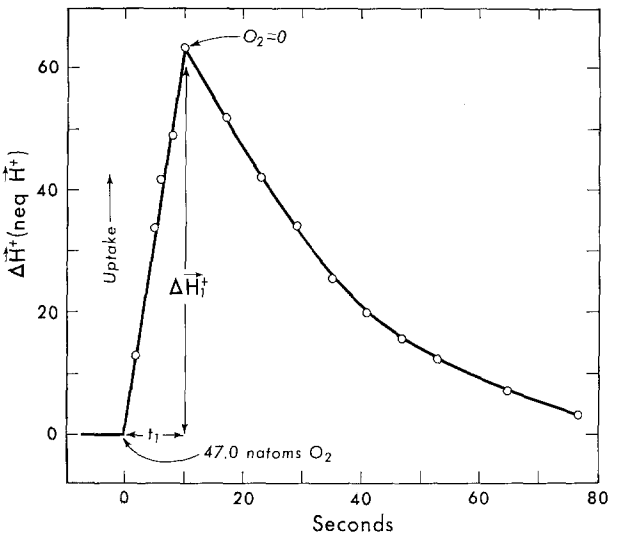

Figure 1. Kinetics of proton movements during an oxygen pulse experiment. Conditions: 0·15 M choline chloride at pH 6·10; 15·3 mg protein; 0·39 μg oligomycin/mg protein; 25°C. t_1 and H_1 refer to equations (2) and (4). Other conditions as in Methods.

Materials and Methods

Preparations. Rat liver mitochondria, from male Sprague-Dawley albino rats starved overnight for 16 h, were isolated in 0·33 M sucrose, 4·0 mM Tris(pH 7·4) and 1·0 mM EDTA according to Packer, Utsumi and Mustafa.[13] For preparation of SMV the final mitochondrial pellet from four livers was taken up into 10 ml of isolation medium and slowly suspended into 60 ml glass distilled water. After 5 min the suspension was sonicated for 1 min with a Branson Sonifier (model S-75) at 4–4·5 amps, centrifuged for 10 min at 14,500 g and the supernatant drawn off into cold ultracentrifuge tubes. SMV were collected by centrifugation for 40 min at 104,000 g and resuspended in 3–4 ml of glass distilled water. In EDTA treated preparations (EDTA-SMV) 1·0 mM EDTA was added to the mitochondrial suspension in glass distilled water before sonication. Protein concentrations were determined by the Folin procedure of Lowry, Rosebrough, Farr and Randall.[14]

Bivalent cations. Total Ca^{2+} and Mg^{2+} were estimated by the method of Brierley and Knight.[15] The free Ca^{2+} and Mg^{2+} was determined after treating SMV in distilled water with 1% lanthanum chloride in 5% HCl.

Proton translocation. Proton flux measurements in SMV were determined with a combination type glass electrode in a semienclosed cuvette of approximately 6–6·5 ml similar to that used by Packer and Utsumi.[11] A Clark-type oxygen electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, U.S.A.) and the glass electrode were inserted through fitted holes in an adjustable Teflon stopper covering the cuvette. Small entry portals in the Teflon stopper were used to make additions and to gas the contents. Calibration was made by additions of anaerobic 10 mM HCl and 0·2% choline hydroxide from Manometric micrometer glass syringes. Temperature was controlled at 25 ± 0·5°C by water circulation around the cuvette.

Oxygen pulse experiments were according to Mitchell and Moyle[12] and Packer and Utsumi[11] using a basic reaction medium of unbuffered 0·15 M choline chloride. Calibrated amounts of aerobic medium were introduced to an anaerobic suspension of SMV and proton movements followed to a sensitivity of 0·0005 pH units with a model 26 Expandomatic Radiometer pH meter and G-14 mV Varian Recorder. When dilution effects were to be avoided, catalase (Sigma)and H_2O_2 were used to generate a calibrated amount of oxygen.[11]

Calculation of proton translocation parameters. Proton movements during an oxygen pulse are illustrated in Fig. 1, showing an uptake followed by a passive efflux when the added oxygen is exhausted. Passive efflux, active uptake rate and steady state pH gradients were calculated as follows.

According to Mitchell and Moyle[16] the rate of passive flux of protons across the inner mitochondrial membrane, or from the SMV, depends upon the gradient established during respiration. Thus,

$$dH^+/dt = K\Delta H^+ \qquad (1)$$

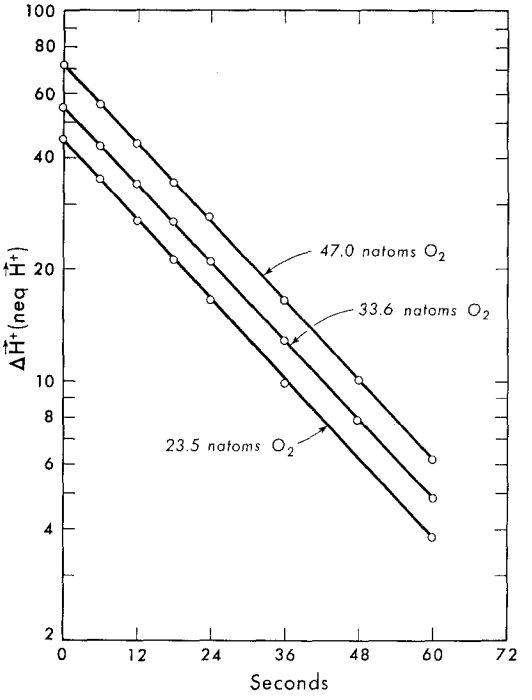

Figure 2. Dependence of the passive efflux of protons from SMV upon the oxygen-induced pH gradient. Conditions as in Fig. 1. Proton gradient was varied by introducing differing amounts of oxygen into the reaction mixture.

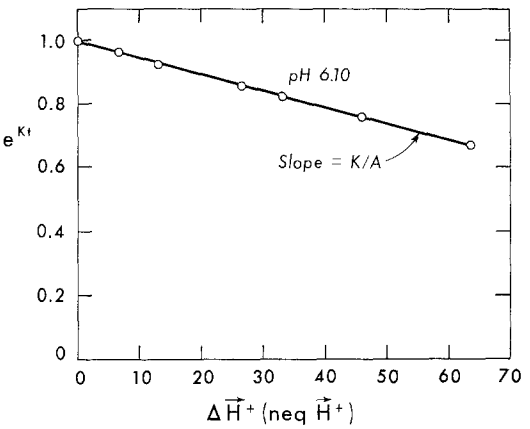

Figure 3. Measurement of the active influx of protons into SMV. The curve shows the relation between active proton influx to the exponential increase in the passive efflux of protons during an oxygen pulse. Conditions as in Fig. 1. Explanation in text.

where K is the passive proton diffusion coefficient and ΔH^+ the established gradient. Integration gives

$$\log(\Delta H^+) = K\Delta t/2{\cdot}3 + \log(\Delta H_i^+) \quad (2)$$

where ΔH_i^+ is the value of the pH gradient as the SMV became anaerobic and Δt is the interval of time after oxygen exhaustion. A plot of $\log \Delta H^+$ against Δt yields a straight line (Fig. 2) with an intercept of $\log(\Delta H_i^+)$ and a slope of $K/2{\cdot}3$. In Fig. 2, the initial gradient of protons ΔH_i^+ was varied by increasing the magnitude of the oxygen pulse. It is seen that the initial rate of proton efflux from SMV is dependent upon the established pH gradient, but that the diffusion coefficient, or slope of the line, is unaffected by a change in the gradient. This first-order relationship has been found to hold under the experimental conditions used between pH 5·3 and 7·8.

The active uptake of protons in SMV during an oxygen pulse must be corrected for the outward passive diffusion of protons, since it has been established that the higher the gradient formed across the membrane, the faster the protons leak out. The rate of proton uptake depends both on the rate of production (A) and on the rate of passive efflux ($K\Delta H^+$). Thus,

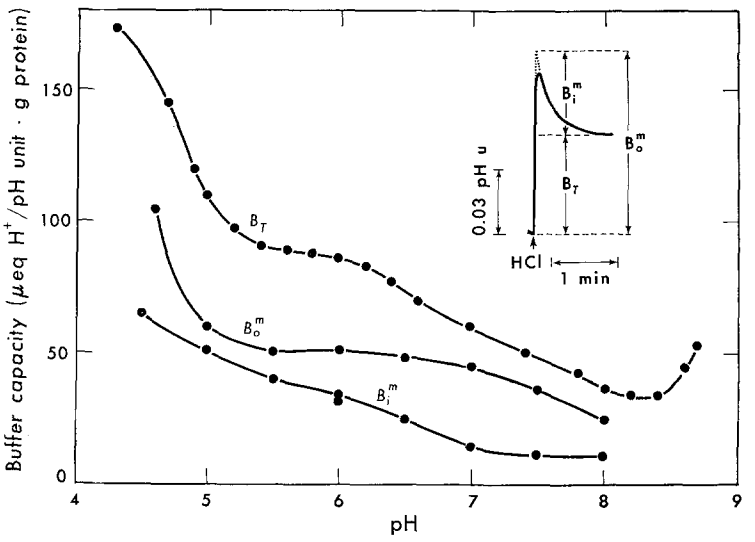

Figure 4. Estimation of buffer capacity of the SMV membrane as a function of external pH. Conditions: 0·15 M choline chloride; 15·8 mg protein; 25°C. Anaerobic pulses of HCl were applied to a thoroughly anaerobic suspension of SMV. The change in external pH was not allowed to exceed 0·05 pH unit during each acid pulse. Determinations of B_T, B_o^m, and B_i^m are explained in the text.

$$dH^+/dt = A + K\Delta H^+ \quad (3)$$

Integrating, we obtain

$$e^{kt} = K/A(\Delta H^+) + 1 \quad (4)$$

and a plot of e^{kt} against ΔH^+ will yield a straight line with an intercept of 1 and a slope of K/A (Fig. 3).

When active and passive flux of protons during an oxygen pulse become equal, a steady state ensues. In this condition, from equation (3), we have the relationship

$$-A/K = \Delta H_{ss}^+ \quad (5)$$

where ΔH_{ss}^+ is the steady-state value for the pH gradient formed across the SMV membrane.

Thus, by monitoring the magnitude and kinetics of proton movements, it is possible

to follow the proton permeability of the SMV membrane [equation (2)], the rate of proton uptake [equation (4)] and the efficiency of proton uptake (given by the H^+/O ratio).

ANS fluorescence was detected as described by Packer, Donovan and Wrigglesworth.[25] Front-face fluorescence was detected between 455–578 nm at an angle of 20 degrees to the incident beam of 290–404 nm.

Results

Buffer Capacity

To determine the permeability of SMV to protons and changes in concentration during experiments, it was first necessary to determine the buffer capacity. This was established as shown in Fig. 4, where the total buffering capacity (B_T) represents the difference between the magnitude of the initial pH change following an acid pulse (B_o^m) and the internal buffering (B_i^m) obtained from the equilibration of the pH change following distribution of protons across the SMV membrane. Figure 4 (insert) shows the kinetics of proton equilibration during an HCl pulse experiment. The pH dependence of the buffering capacity is also shown in Fig. 4. It provides the basis for calculations of proton activity.

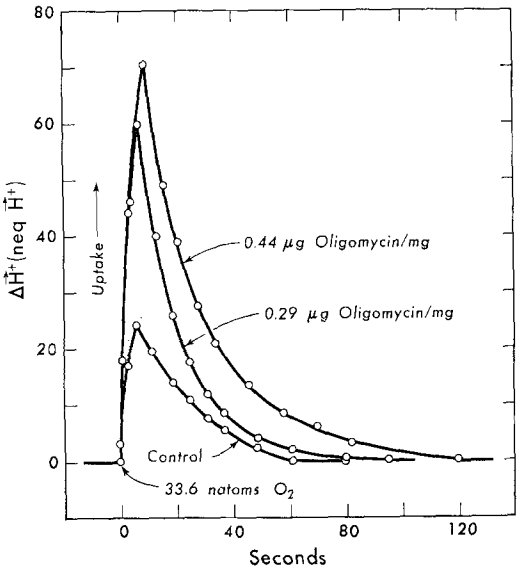

Figure 5. Enhancement of O_2 dependent H^+ uptake by oligomycin. Conditions: 0·15 M choline chloride at pH 6·10; 13·8 mg protein.

The Effect of Oligocymin on Proton Translocation

The presence of oligomycin can enhance oxygen-dependent proton translocation,[2, 3, 6] and therefore a study of the effect of oligomycin on this activity was made. The results in Fig. 5 and Table I show that approximately 1 μg oligomycin/mg protein maximally stimulate proton uptake activity. At higher concentrations oligomycin decreased respiratory control. Analysis of the kinetics of passive proton loss, as shown in Table I, reveals that the presence of oligomycin causes an inhibition of respiratory control and a decreased passive proton permeability. Table I also shows that the stimulation of respiration by oligomycin could be reversed by uncouplers,[1] suggesting respiratory control in SMV.

pH Dependence of Proton Translocation Activity

Determination of the ratio H^+/O provides a measure of the efficiency of proton translocation and a maximum of 2·3 was found at pH 5·8 for choline oxidation, as shown in Fig. 6A. Values of 3·8 are seen in aged liver mitochondria under similar test conditions.[18]

TABLE I. Relationship between respiratory control and proton translocation. Conditions: 0·15 M choline chloride; 10·8 mg protein; pH 6·10 where pH is not indicated. 47 natoms O_2 were used to initiate proton uptake. All experiments were in the presence of 0·75 μg oligomycin/mg protein, except for the control and where the concentration of oligomycin is given

Conditions	K* (min^{-1})	A* (μ eq H^+/g.min)	ΔH_{ss}^+* (μ eq H^+/g)	RCI†	H^+/O
Control	12·4	15·1	1·22	1·0	0·50
Oligomycin (μg/mg)					
0·25	9·7	16·2	1·67	1·98	0·80
0·50	6·0	19·2	3·20	2·48	1·28
0·75	3·6	22·4	6·22	2·83	1·64
1·00	2·5	23·8	9·55	3·00	1·72
Dinitrophenol (μM)					
50	7·8	39·4	5·05	0·77	1·34
100	11·9	27·5	2·31	0·54	0·95
150	13·1	16·5	1·26	0·46	0·41
pH					
7·5	11·5	15·8	1·37	1·00	0·37
7·0	6·0	19·4	3·24	1·38	0·81
6·5	3·6	21·2	5·90	2·40	1·15
5·8	2·9	31·6	10·9	2·80	2·20
Gramicidin (0·5 μg/mg)	12·3	30·0	2·44	0·64	0·82
Nigericin ($1·8 \times 10^{-8}$ M)	3·5	12·1	3·45	0·77	1·09
5 mM KCl	3·4	9·40	2·76	0·70	0·71
10 mM KCl	3·3	6·8	2·07	0·64	0·52
Valinomycin (75 μg/mg)	3·6	23·7	6·60	0·94	1·75
Valinomycin plus nigericin	39·7	39·8	4·10	0·71	1·21

* K is the passive proton coefficient; A is the active proton uptake rate; and ΔH_{ss}^+ is the steady-state gradient as in methods.

† RCI, the respiratory control index, is the ratio of the initial respiration divided by the rate after treatment, under the indicated conditions.

Of particular interest is the finding that choline respiration in the pH range of 5·8 to 7·5 shows an inverse relation to the magnitude of H^+/O ratio. Thus, the efficiency of proton translocation diminishes as respiration increases. Table I and Fig. 6B further show that the calculated proton gradient formed in the steady state also reflects this inverse relationship to respiration, showing a close relationship between the magnitude of the proton gradient and respiratory activity as first predicted by Mitchell.[19]

The permeability of SMV to protons also changes as a function of pH, as shown in Fig. 7. The passive proton efflux given by the diffusion coefficient [equation (2)] has a similar pH dependence to respiration. The rate of active proton uptake follows the H^+/O ratio.

Energy Coupling and Respiratory Control

Further evidence of energy coupling was obtained by testing DNP, gramicidin, nigericin and valinomycin, as shown in Table I. Increasing concentrations of DNP directly enhance passive proton permeability. It was found that the magnitude of proton

uptake decreases with DNP concentration. Gramicidin and nigericin also decreased proton uptake during oxygen pulse experiments. On a concentration basis, gramicidin was about 100 times more effective than DNP in increasing proton permeability.

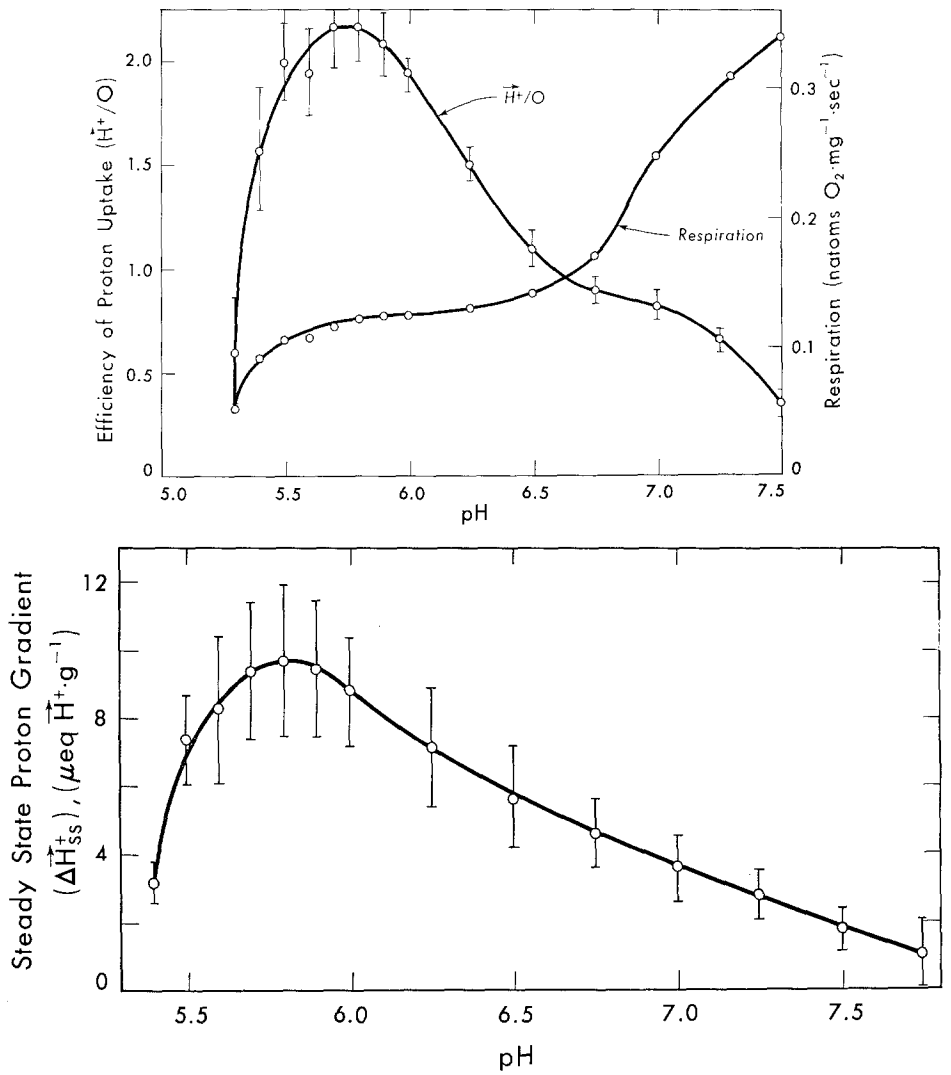

Figure 6. pH Dependence of H^+/O ratio, respiration and steady-state gradient of SMV. Conditions: 0·15 M choline chloride; 0·42 μg oligomycin/mg protein; at indicated pH. Standard errors of measurements taken from four SMV preparations are given; ΔH^+_{ss} calculated according to equation (5).

Nigericin slightly decreased the active proton uptake, but its inhibitory action was markedly accentuated if monovalent ions, such as K^+, were added. In other experiments it has been established that Li^+, Na^+, and NH_4^+ enhance the inhibitory action of nigericin. The action of nigericin is also potentiated in the presence of valinomycin. Since a threefold increase in the passive permeability is observed under these conditions, the results indicate that respiratory control is affected by proton and other monovalent cation

gradients established across the membrane of SMV, and that a linear relation between passive proton permeability and respiration rate can exist.

Effects of Cytochrome c

The activity of electron transport is involved in proton translocation. Since the formation of a proton gradient in turn may modify the decay of redox changes, it was of interest to design some experiments to test more directly the involvement of an electron transport component. The effect of additions of cytochrome *c* on active proton uptake following an oxygen pulse is shown in Fig. 8, where it can be seen that only a small increment in the magnitude of the proton uptake is brought about by the added cytochrome *c* in SMV. It seemed likely that this could result from the known inability of the cytochrome *c* molecule to penetrate SMV and from an asymmetric localization of cytochrome *c* action as reported by Lee and Carlson.[20] To test this assumption, SMV were treated with a level of Triton X-100 that increased the proton permeability, but which did not completely abolish the oxygen - dependent proton uptake. Under these conditions, the proton uptake is enhanced to the extent of 1·2 H^+/cytochrome *c* added (Fig. 8). Also, the passive proton permeability decreases in a linear manner with cytochrome *c* concentration. Since respiration was not appreciably affected by the presence of cytochrome *c*, either in the absence or presence of Triton, it seems likely that it participates directly in proton translocation. The presence of oligomycin or DNP had little effect on the proton uptake induced by cytochrome *c* in Triton-treated SMV.

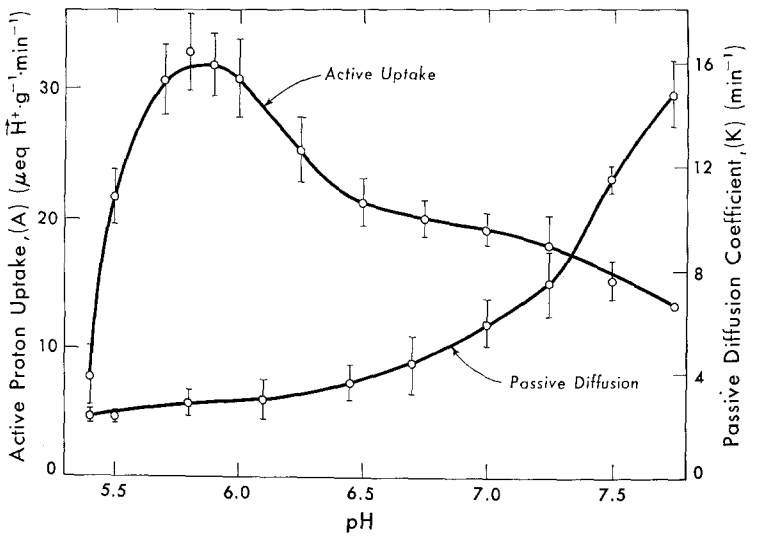

Figure 7. pH Dependence of the kinetics of proton uptake and loss of protons in SMV. Conditions: as in Fig. 6. *K* and *A* were calculated according to equations (2) and (4) respectively.

Effects of Bivalent Cations and EDTA Treatment

Oxygen-dependent H^+ translocation is being studied in SMV in a simple ionic environment mainly composed of choline chloride, H^+ and OH^-. It would be of interest to know what ions move to maintain electric neutrality during proton translocation. In mitochondria, bivalent cations readily exchange for protons[21] in amounts commensurate with the magnitude of proton translocation. Table II shows that free calcium and magnesium in SMV represent a substantial proportion of the total bivalent cations present. Moreover, their concentration is approximately 13 μmoles/g protein, while the

magnitude of proton uptake generally is 8–12 μmoles/g protein. Thus, the bivalent cations are present in amounts commensurate with the magnitude of the proton uptake. Hence, it is possible that endogenous free bivalent cations may exchange across SMV membranes to account for the maintenance of electric neutrality. Even in SMV prepared in the presence of EDTA a considerable amount of the bivalent cations are retained.

These results indicated it was of interest to study further the effect of added bivalent cations on proton translocation. Table III shows that calcium was largely without effect, whereas magnesium did increase the magnitude of the H^+/O and the active and steady-state levels of proton uptake. The effect of magnesium was biphasic in that concentrations greater than 4 mM resulted in an inhibition of proton uptake. Similar biphasic effects of magnesium have been

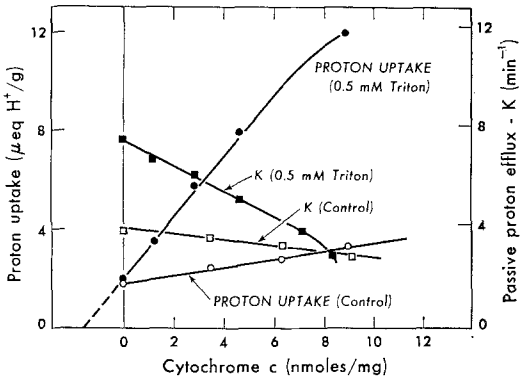

Figure 8. The effect of cytochrome *c* on proton uptake in SMV. Conditions: 0·15 M choline chloride, pH 6·1; 10·2 mg protein. Oxygen pulses of 47 natoms O_2 were used.

Table II. Estimation of calcium and Magnesium in SMV. Standard errors are given from six different preparations. Conditions for estimation are as in Methods

	Usual Preparation (μmoles/g protein)			EDTA Preparation (μmoles/g protein)
	Total	Free	Bound	Total
Calcium	4·21 ±1·15	1·96 ±0·34	2·25	4·53 ±0·97
Magnesium	13·62 ±2·40	9·75 ±1·95	3·87	9·70 ±1·90

TABLE III. Effect of calcium and magnesium on proton translocation in SMV. Conditions: 0·15 M choline chloride; 0·75 μg oligomycin/mg protein; 13·2 mg protein

Conditions	H^+/O	$K(min^{-1})$	$A(\mu$ eq $H^+/g.min)$	$\Delta H^+_{ss}(\mu$ eq $H^+/g)$
pH 6·70				
Control	1·52	5·4	20·2	3·75
1 mM $CaCl_2$	1·49	5·4	20·2	3·75
1 mM $MgCl_2$	1·61	5·9	25·6	4·35
3·5 mM $MgCl_2$	1·79	6·1	31·6	5·20
pH 5·85				
Control	1·80	3·6	29·3	8·37
1 mM $MgCl_2$	2·35	3·5	36·4	10·39
3·5 mM $MgCl_2$	2·82	3·5	44·1	12·60

reported by Lee and Ernster[1] on oxidative phosphorylation in beef heart SMV. Since magnesium did not decrease the passive proton permeability, it seems likely that these effects on oxidative phosphorylation result from the action of magnesium on energy coupling found here. Figure 9 shows that the addition of EDTA to a usual preparation of SMV causes a large decrease in proton uptake. An increase of respiration was observed under these conditions, in agreement with earlier respiration studies of Gregg and Lehninger.[22]

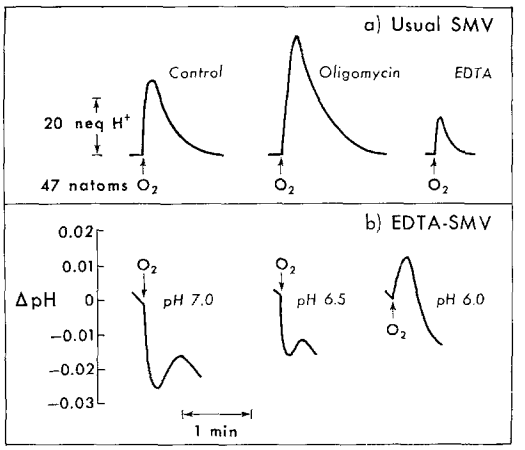

Figure 9. Effect of EDTA on proton translocation during an oxygen pulse and during sonication. Conditions as in Fig. 8. (A) Usual preparation of SMV; 0·75 μg oligomycin/mg protein and 0·5 mM EDTA added where indicated. (B) EDTA prepared SMV; no further additions were made except for oxygen.

It is not possible to determine from these experiments whether the enhanced proton uptake by magnesium results from an increased exchange of a charged counter-ion, or as a consequence of some other effect which magnesium may have upon the membrane. To assess the importance of bivalent cations further, experiments were performed as in Fig. 9 on SMV prepared in the presence of EDTA (see Methods and ref. 23). When proton translocation was tested in the presence of EDTA-SMV, an actual reversal of the direction of uptake was observed during an oxygen pulse. The proton production occurred in the pH range between 6·3 and above. If oligomycin is added in this pH range, oxygen-dependent proton translocation is changed again in the direction of uptake.

Further evidence that physical changes in the SMV membrane occur as a consequence of removal of bivalent cations in EDTA-SMV preparations was obtained from studies of ANS fluorescence upon addition of oxygen, a response that is enhanced in the presence of oligomycin, and which is inhibited by DNP (Fig. 10). In EDTA-SMV preparations, the response in ANS fluorescence is in the opposite direction upon oxygenation when experiments are performed in the alkaline pH range, but not in the acid pH range. The latter result confirms the findings of Azzi *et al.*[24] and Azzi.[25] It is also shown in this experiment that the addition of HCl to anaerobic SMV increases ANS fluor-

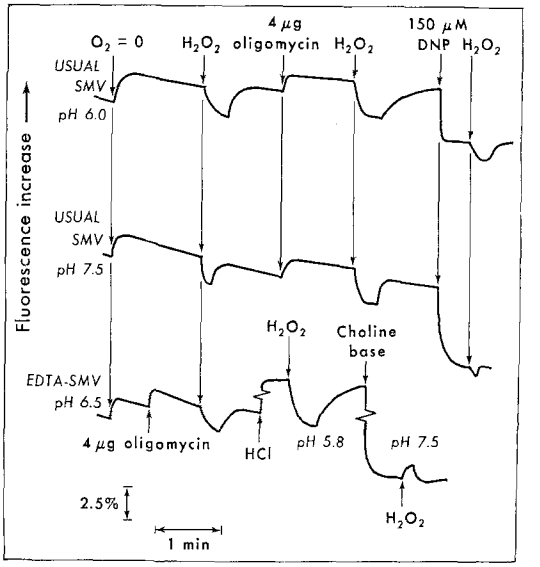

Figure 10. ANS fluorescence intensity changes during an oxygen pulse. Conditions: 0·15 M choline chloride; 1·0 mg protein/ml; 100 μg ANS. Excitation and emission as in Methods. The equivalent of 100 natoms O_2 were added as hydrogen peroxide in the presence of 100 units catalase.

escence while choline hydroxide causes a decrease in fluorescence similar to the results reported by Wrigglesworth and Packer.[26]

Discussion

Energy Coupling

The present investigation seems to provide a basis for understanding energy coupling and respiratory control in SMV in terms of ion gradients. A direct relationship between respiration and passive proton permeability has established the basic parameters that control respiration. Various treatments which affect proton permeability, such as the presence of oligomycin, the action of chemical uncouplers, ion transport-inducing antibiotics and changes in pH are consistent with the idea that ion gradients control respiration. Rumberg, Reinwald, Schröder and Siggel[27] have made similar findings indicating that the control of electron flow in chloroplasts is controlled by the passive efflux of protons across grana thylakoid membranes. Thus, a striking correlation exists between SMV and grana where electron flow in both systems results in proton uptake and where in both instances proton gradients and passive proton efflux control electron flow. These conclusions are in agreement with the chemiosmotic hypothesis[12] and similar conclusions reached by Papa *et al.*,[7] Cockrell and Racker[9] and Montal *et al.*[8] with SMV and by Jackson *et al.*[10] in studies with chromatophores.

Evidence for Asymmetry of Membrane Functions

The asymmetric functional organization of the inner mitochondrial membrane is generally recognized, evidence being revealed in this investigation by ionic effects and the action of cytochrome *c* upon proton translocation in SMV. Greville[28] in particular has recently reviewed the anisotropic effects of cytochrome *c*. Carafoli and Muscatello[29] have shown that added cytochrome *c* is not readily oxidized by SMV except in the presence of a detergent or if SMV are prepared in the presence of cytochrome *c* where it is trapped inside. Thus, the action of cytochrome *c* is at the outer surface of the inner membrane of intact mitochondria and at the inner surface of the SMV membrane. It further appears from this investigation that the oxidation of cytochrome *c* stimulated by detergents can be coupled to proton translocation. An asymmetric position of the cytochrome *c* molecule in the membrane seems potentially capable of providing a mechanism for anisotropic proton translocation. Extension of these studies to other redox components of the electron transport chain could provide a basis for explaining the observed stoichiometry of oxygen-dependent proton gradients.

The action of magnesium and EDTA on proton translocation in SMV also shows the "sidedness of the membrane". In SMV preparations supplemented with magnesium the H^+/O ratio is enhanced and the passive proton permeability is slightly decreased at pH 5·85. In EDTA treated preparations, where the magnesium content is reduced, the passive proton permeability increases somewhat and the active proton uptake is decreased. More extensive removal of bivalent magnesium ions occurs in EDTA-SMV preparations, where more than 25% of the total magnesium is lost, and it is observed that the passive proton permeability is greatly increased. Under these circumstances the direction of proton translocation is actually reversed so that proton production occurs during an oxygen pulse. In this case proton gradients across the membrane are either reversed in direction or cannot be established due to the large increase in passive

proton permeability. The small oxygen-dependent proton production may reflect the direct anisotropic release of protons by respiratory components (opposite to that seen with cytochrome *c* effects above). These results may indirectly provide a measure of the balance of the proton exchanging respiratory components. Further evidence for a residual redox proton absorbing component was also obtained in SMV treated with uncouplers and Triton X-100. Here SMV was rendered completely permeable to protons and a residual proton uptake was observed. The magntiude of this pool of endogenous redox components varied between 1·6–2·7 μeq H^+/g protein.

Five different treatments of SMV have been observed to change the kinetics of H^+ translocation during an oxygen pulse. The addition of oligomycin, dinitrophenol, nigericin and in other experiments glutaraldehyde and Triton X-100, cause changes in H^+ translocation and also all cause alterations in either the magnitude or the kinetics of cytochrome oxidation-reduction as observed by changes in optical transmission (unpublished results). All of these treatments are known to affect the proton translocation across the SMV membrane, but by different mechanisms. They bear a striking relationship to similar findings by Schliephake, Junge and Witt,[30] Rumberg *et al.*,[27] and Rumberg and Siggel[31] in grana thylakoids and by Fleischman and Clayton[32] and Jackson and Crofts[33] in chromatophores where it has been established that the physical state of the membrane, as it affects ion permeability, modifies the reactivity of oxidation-reduction pigments within the membrane.

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References

1. C.-P. Lee and L. Ernster in: J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (eds.), *Regulation of Metabolic Processes in Mitochondria*, Elsevier, Amsterdam, (1966) p. 218.
2. P. Mitchell and J. Moyle, *Nature*, **208** (1965) 1205.
3. J. M. Tager, S. Papa, F. Guerrieri, G. S. P. Groot, and E. Quagliariello, *Proc. Biochem. Soc.*, **35** (1969) (abstr.).
4. L.-V. von Stedingk and H. Baltscheffsky, *Arch. Biochem. Biophys.*, **117** (1966) 400.
5. P. Scholes, P. Mitchell, and J. Moyle, *European J. Biochem.*, **8** (1969) 450.
6. B. Chance and L. Mela, *J. Biol. Chem.*, **242** (1967) 830.
7. S. Papa, F. Guerrieri, J. M. Tager, and E. Quagliariello, *Biochim. Biophys. Acta*, **172** (1969) 184.
8. M. Montal, B. Chance, C.-P. Lee and A. Azzi, *Biochem. Biophys. Res. Commun.*, **34** (1969) 104.
9. R. S. Cockrell and E. Racker, *Biochem. Biophys. Res. Commun.*, **35** (1969) 414.
10. J. B. Jackson, A. R. Crofts, and L.-V. von Stedingk, *European J. Biochem.*, **6** (1968) 41.
11. L. Packer and K. Utsumi, *Arch. Biochem. Biophys.*, **131** (1969) 386.
12. P. Mitchell and J. Moyle, *Biochem. J.*, **105** (1967) 1147.
13. L. Packer, K. Utsumi, and M. G. Mustafa, *Arch. Biochem. Biophys.*, **117** (1966) 381.
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193** (1951) 265.
15. G. P. Brierley and V. A. Knight, *Biochemistry*, **6** (1967) 3892.
16. P. Mitchell and J. Moyle, *Biochem. J.*, **104** (1967) 588.
17. L. Packer, M. P. Donovan, and J. M. Wrigglesworth, *Biochem. Biophys. Res. Commun.*, **35** (1969) 832.
18. K. Utsumi, unpublished observations.
19. P. Mitchell, Research Report, Glynn Research Ltd., Bodmin, Cornwall, England (1966).
20. C.-P. Lee and K. Carlson, *Fed. Proc.*, **27** (1968) 828.
21. A. L. Lehninger, E. Carafoli, and C. S. Rossi, *Advan. Enzymol.*, **29** (1967) 259.
22. C. T. Gregg and A. L. Lehninger, *Biochim. Biophys. Acta*, **78** (1963) 127.
23. R. A. Beyer, in: R. W. Estabrook and M. E. Pullman (eds.) *Methods in Enzymology*, Vol. X, Academic Press, New York, (1967) p. 186.

24. A. Azzi, B. Chance, G. K. Radda, and C.-P. Lee, *Proc. Natl. Acad. Sci. U.S.*, **62** (1969) 612.
25. A. Azzi, *Biochem. Biophys. Res. Commun.*, **37** (1969) 254.
26. J. M. Wrigglesworth and L. Packer, *Arch. Biochem. Biophys.*, (1970) in press.
27. B. Rumberg, E. Reinwald, H. Schroder, and U. Siggel, *Naturwissenschaften*, **55** (1968) 77.
28. G. D. Greville, in: D. Rao Sanadi (ed.), *Current Topics in Bioenergetics*, Vol. III, Academic Press, New York, (1969) p. 1.
29. E. Carafoli and V. Muscatello, *Abstr. 5th Meeting Fed. European Biochem. Soc.*, Prague (1968) p. 65.
30. W. Schliephake, W. Junge, and H. T. Witt, *Z. Naturforschung*, **23b** (1968) 1571.
31. B. Rumberg and U. Siggel, *Naturwissenschaften*, **56** (1969) 130.
32. D. E. Fleischman and R. K. Clayton, *The Effect of Photosynthetic Uncouplers and Electron Transport Inhibitor upon Spectral Shifts and Delayed Luminescence of Photosynthetic Bacteria* (1970) to be published.
33. J. B. Jackson and A. R. Crofts, *FEBS Letters*, **4** (1969) 185.