Effect of Ion Conductance Changes in the Mitochondrial Membrane on the Kinetics of Respiratory Carriers†

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ABSTRACT: The response of respiratory carriers to ion-conductance changes, induced in the mitochondrial membrane by antibiotics, has been investigated and compared to the effect these substances have on the respiration-linked proton pump. Oligomycin (at a concentration of 0.1–1 μg/mg of particle protein) caused a marked stimulation of the respiration-driven proton uptake by EDTA submitochondrial particles and inhibition of the subsequent anaerobic proton release. This was accompanied by inhibition of respiration at the steady state and by a marked decrease of the rate of oxidation of respiratory carriers in the anaerobic–aerobic transition. Valinomycin caused a three- to fourfold stimulation of the respiration-driven proton uptake by oligomycin-treated particles and an equally marked enhancement of the rate of oxidation of flavoproteins, *b* cytochromes, and ubiquinone but had no effect on the steady-state respiratory rate. These data provide circumstantial evidence in favor of the electrogenic nature of the proton pump. Conversion of transmembrane ΔpH into $\Delta\psi$, brought about by nigericin plus K^+, was without significant effect on the steady-state respiration and the kinetics of respiratory carriers. Of the various respiratory carriers examined cytochrome b_{566} was the component to be most profoundly affected by oligomycin and valinomycin.

A topic central to the problem of energy transfer in mitochondria is that of the energy-linked proton pump. The proton pumping activity is very closely associated with the electron-transport system in the mitochondrial membrane. Respiration-driven proton pump is fully active in submitochondrial particles, which have little if any phosphorylating capacity (Lee and Ernster, 1966; Papa *et al.*, 1970a), and is particularly resistant to uncouplers of oxidative phosphorylation (Mitchell and Moyle, 1967; S. Papa, F. Guerrieri, and D. Larosa, 1972, unpublished data). Studies in intact mitochondria (Mitchell and Moyle, 1967), submitochondrial particles (Papa *et al.*, 1970b; *cf.* Montal *et al.*, 1970; Grinius *et al.*, 1970), chromatophores (Jackson *et al.*, 1968), and chloroplasts (Von Stedingk, 1970) have provided data which support the view (Mitchell, 1966) that electron-flow-driven proton translocation is electrogenic (see however Massari and Azzone, 1970; Slater, 1971; Karlish and Avron, 1971). Available evidence indicates that proton translocation is coupled to oxidoreductions through exchange of primary bonds and to cation and substrate–anion translocation at the level of separate carriers in the mitochondrial membrane (Papa, 1970, 1971). It is, however, unknown whether coupling between electron and proton flow is performed by the same components of the respiratory chain or if other molecular entities are involved. According to Mitchell (Mitchell, 1966) oxidoreduction-linked proton translocation is the consequence of the sequence and spatial arrangement in the mitochondrial membrane of the respiratory carriers. On the other hand, it is known that protonation and deprotonation of groups in the apoprotein can accompany redox transitions of the heme of cytochromes (Straub and Colpa-Boonstra, 1962; Urban and Klingenberg, 1969; Czerlinsky and Dar, 1971).

The activity of the proton pump can be affected by antibiotics of the valinomycin type, which make the mitochondrial membrane selectively permeable to K^+ (Chappell and Crofts, 1966; Pressman *et al.*, 1967), and of the nigericin type, which induce specific proton–cation exchange diffusion (Pressman *et al.*, 1967). Ion redistribution across the membrane caused by these agents alters the relative contribution of the electrical and chemical components of the thermodynamic potential difference of protons across the membrane established by the proton pump (Mitchell and Moyle, 1969; Crofts and Jackson, 1970). In this paper the effect of these perturbations on the kinetics of respiratory carriers in the transition from the resting anaerobic state to the aerobic active state in beef heart submitochondrial particles is described and compared to the influence these situations have on the respiratory chain at the steady state and on the various parameters of the respiration-induced proton translocation. These studies provide circumstantial evidence for the electrogenic nature of the oxidoreduction-driven proton pump. The relevance of the results obtained to the oxidoreductase proton translocator concept of Mitchell (1966) is also discussed. Kinetics data point to a specific interrelationship between one of the *b* cytochromes of the respiratory chain and the proton pump.

Experimental Procedure

Submitochondrial particles (EDTA particles) were prepared by sonication of "heavy" beef heart mitochondria in the presence of EDTA (Lee and Ernster, 1968). Oxygen consumption was measured polarographically with a Clark electrode. Proton translocation was studied by monitoring potentiometrically the pH of the particle suspension with a fast responding, low-resistance glass electrode (Ingold) (response time about 50 msec) connected to a negative capacitance electrometer (Keitley, Model 605) and a recorder (Honeywell, Model 194). The potential changes were quantitated in terms

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TABLE I: Effect of Oligomycin, Valinomycin, and Nigericin on Respiration-Induced Proton Translocation in EDTA Particles.[a]

Additions	Proton Uptake		Anaerobic Proton Release		Steady-State Respiratory Rate (ng-atom of O min^{-1}/mg of Protein)	Steady-State H^+/O
	Net Initial Rate (ng-ion of H^+ min^{-1}/mg of Protein)	Extent (ng-ion of H^+/mg of Protein)	Initial Rate (ng-ion of H^+ min^{-1}/mg of Protein)	$t_{1/2}$ (sec)		
	625	4.0	250	0.84	580	0.43
Oligomycin	940	14.2	190	5.62	400	0.47
Oligomycin, valinomycin	3180	38.4	319	8.60	400	0.80
Oligomycin, valinomycin, nigericin	1580	4.9	583	0.48	510	1.14

[a] The reaction mixture (final volume 1.5 ml; pH 7) contained: 250 mM sucrose, 10 mM potassium succinate, 20 mM KCl, 0.4 mg of purified catalase (from beef liver, Boehringer), and 1.5 mg of particle protein. The incubation was carried out at 25° under nitrogen atmosphere and rapid stirring. Respiration was started by adding 10 μl of 0.2% H_2O_2 to anaerobic particles. Additions were: 3.0 μg of oligomycin, 0.75 μg of valinomycin, and 0.75 μg of nigericin. For other details, see Methods.

of proton equivalents by double titration with standard solutions of KOH and HCl. The electronic equipment used allowed the pH to be measured with a precision of 0.001 pH unit (Papa *et al.*, 1970b). Kinetic studies of respiratory-chain components were performed using a regenerative, stopped-flow apparatus (Chance *et al.*, 1967) in combination with a double dual-wavelength spectrophotometer. The proper wavelengths were obtained with four interference filters located in quadrature (90° phase separation) on a 60-Hz spinning disk (B. Chance, N. Graham, and V. Legallais, 1972, unpublished data). This apparatus made possible continuous readout of two pairs of wavelengths, so that the absorptions of two respiratory carriers could be measured simultaneously and without interference. Narrow bandwidth filters (1–3 nm) were obtained from Omega Optical Co. Mixing time and absorption changes were displayed on a four-channel storage oscilloscope. EDTA particles were made anaerobic by the addition of succinate, and the flux was adjusted by suitable addition of malonate. Oxygen-saturated medium was delivered from the side syringe giving 1.25% dilution per discharge of the flow apparatus. The reaction mixture and other experimental details are given in the legends to tables and figures.

Results

Effect of Oligomycin and Ionophores on Proton Translocation and Kinetics of Respiratory Carriers.

In Table I the effect of oligomycin, valinomycin, and nigericin on various parameters of the respiration-driven proton translocation in EDTA particles is shown. EDTA particles consist of vesicles of the inner mitochondrial membrane turned *inside out* (Lee and Ernster, 1966), and the polarity of the proton pump is inverted with respect to intact mitochondria (Papa *et al.*, 1970a; Montal *et al.*, 1970). Respiration was activated by oxygen pulses of the particles made anaerobic by the addition of succinate. Oligomycin gave a 3.5-fold increase of the extent but only a 50% stimulation of the net initial rate of the respiration-driven proton uptake. The initial rate of the anaerobic release of the amount of protons taken up by the particles in the aerobic steady state was 26% inhibited by oligomycin The $t_{1/2}$ of this process was increased by oligomycin from 0.84 to 5.62 sec. Thus the enhancement of the net proton uptake given by oligomycin is due to inhibition of the passive back-diffusion of protons (see Papa *et al.*, 1970a; Papa, 1971). The depression of the proton diffusion caused by oligomycin was accompanied by inhibition of the steady-state respiration (*cf.* Lee and Ernster, 1966, 1968).

Valinomycin, in the presence of an excess of K^+, caused more than threefold increase of the net initial rate of proton uptake. Also the extent of proton uptake was considerably increased but less then the initial rate. Respiration was not affected by valinomycin (see also Montal *et al.*, 1970). Valinomycin raised the $t_{1/2}$ of the anaerobic proton release from 5.62 to 8.60 sec. There was, however, a 68% increase of the initial rate of proton release. Reasons to believe that this increase of the rate of proton release is, in large part, apparent have been presented elsewhere (Papa *et al.*, 1970b; Papa, 1971).

Nigericin, added in the presence of valinomycin and K^+, accelerated the anaerobic proton release. This was accompanied by stimulation of respiration and decrease of the extent of proton uptake. Also the net initial rate of proton uptake decreased, but less than the extent. These facts indicate a real increase of the proton turnover at the steady state. In the absence of valinomycin (not shown) nigericin reduced to a minimum the extent of proton uptake, but had no significant effect on the respiratory rate (see also Montal *et al.*, 1970).

In Table II the effect of oligomycin and ionophores on the kinetics of the oxidation of cytochrome *b* in the transition from anaerobiosis to aerobiosis of succinate-supplemented submitochondrial particles is presented. Malonate was present at an appropriate concentration so that the rate of reduction of respiratory carriers by succinate was negligible with respect to their oxidation. Oligomycin caused a marked depression of the rate of cytochrome *b* oxidation; also the extent of oxidation was lowered but to a smaller extent. Valinomycin, added in the presence of oligomycin and K^+, returned the rate of cytochrome *b* oxidation to the value found in the absence of oligomycin but did not affect the extent of cytochrome *b* oxidation. The addition of nigericin in the presence of valinomycin plus K^+ caused some increase of the extent of

TABLE II: Effect of Oligomycin, Ionophores, and FCCP on Cytochrome *b* Oxidation in the Anaerobic–Aerobic Transition of EDTA Particles.[a]

Additions	Cytochrome *b* Oxidation (560–575 nm)	
	$t_{1/2}$ (msec)	Extent (nmoles/mg of Protein)
	122	0.140
Oligomycin	445	0.117
Oligomycin, valinomycin	115	0.115
Oligomycin, valinomycin, and nigericin	127	0.121
Oligomycin, valinomycin, nigericin, and FCCP	120	0.121
Oligomycin, nigericin	420	0.120

[a] The reaction mixture (final volume, 20 ml; final pH, 7) contained: 250 mM sucrose, 10 mM potassium succinate, 5 mM potassium malonate, 20 mM KCl, 1.5 mg/ml of particle protein. Additions were: 2 μg/ml of oligomycin, 0.5 μg/ml of valinomycin, 0.5 μg/ml of nigericin, 1 μM FCCP. Incubation temperature was 20°. When the particle suspension became anaerobic, 0.25 ml of oxygen-saturated medium was delivered from the side syringe into the reaction mixture. For other details, see Methods.

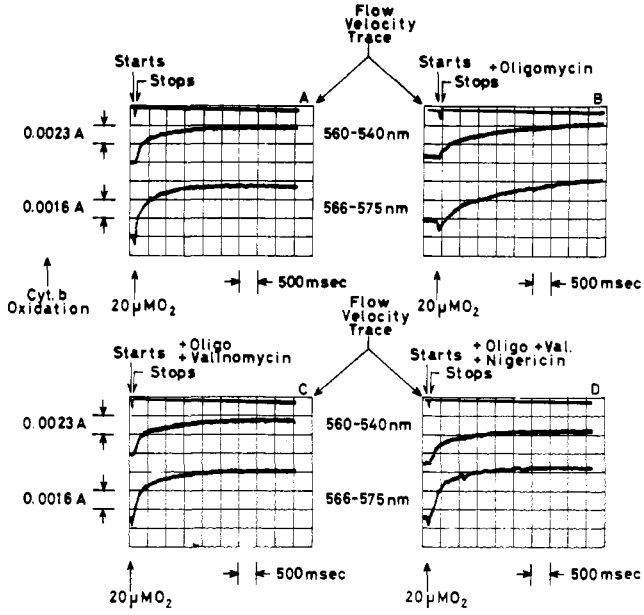

FIGURE 1: Kinetics of cytochrome *b* oxidation induced by oxygen pulses of anaerobic EDTA particles. The experimental conditions and procedure were those described in the footnote to Table II. Additions were: 2 μg/ml of oligomycin, 0.5 μg/ml of valinomycin, 0.5 μg/ml of nigericin.

cytochrome *b* oxidation but had no effect on the rate of oxidation. Further addition of 1 μM FCCP[1] was without any effect on the oxidation of cytochrome *b*. In the absence of valinomycin, nigericin plus K^+ did not affect the oxidation of cytochrome *b*.

Potentiometric (Wilson and Dutton, 1970; Dutton *et al.*, 1970) as well as kinetic (Chance *et al.*, 1970) and spectral studies (Chance *et al.*, 1970; Chance, 1958; Chance and Schoener, 1966; Chance *et al.*, 1970; Slater *et al.*, 1970; Sato *et al.*, 1971; Brandon *et al.*, 1972) have provided evidence for the existence of two distinct species of *b* cytochromes in pigeon heart, beef heart, and rat liver mitochondria: one having an absorption maximum in the α region near 560 (b_K), the other near 566 (b_T). In the experiment of Figure 1 the kinetics of the oxidation of cytochrome *b* was studied by monitoring simultaneously the absorbance decrease at the wavelength couples 560–540 and 566–575 nm. In control experiments it was found that the absorbance increase, measured upon addition of an excess of dithionite to a fully oxidized respiratory chain (aerobic state in the presence of 10 mM succinate and 50 mM malonate), was at 560–540 nm equal to that measured at 560–575 nm and 1.3-fold higher than that at 566–575. The traces at 566–575 nm show that the absorbance decrease at 566 nm caused by rapid oxygenation of succinate-supplemented anaerobic particles was preceded by a small absorbance increase (see also Figure 3), which was particularly clear in the presence of oligomycin (see Erecinska *et al.*, 1972). Such an absorbance increase was not apparent at 560–540 nm. In the absence of oligomycin (Figure 1A) the rate of the absorbance decrease was at 560–540 nm slightly higher than at 566–575, the $t_{1/2}$ being 190 and 224 msec, respectively. Oligomycin (Figure 1B) slowed down the rate of the absorbance decrease; the inhibition at 566–575 nm being much more marked than at 560–540 nm ($t_{1/2}$ 850 and 580 msec, respectively). At both the wavelength couples valinomycin plus K^+ eliminated the inhibition of the absorbance decrease caused by oligomycin (Figure 1C). Further addition of nigericin (Figure 1D) had no appreciable effect on the rate of the absorbance decrease.

In Figure 2 the absorbance decreases, measured at 560–540 nm and 566–575 nm during the anaerobic–aerobic transition of the particles, are plotted according to the first-order equation. Figures 2A and 2B illustrate the kinetics of the absorbance decrease in the absence of oligomycin. The process was, at both the wavelength couples, biphasic. By applying the double exponential equation the biphasic kinetics could be resolved into two apparent first-order processes. This situation is that which commonly arises when variation of a parameter is caused by parallel first-order reactions of two distinct substances, having different velocity constants (Frost and Pearson, 1961). The slope of the two lines gives the respective velocity constants, the intercepts with the ordinate the *per cent* of the two species present at zero time. Thus the overall absorbance decrease corresponds to the oxidation of two chemically distinct species of *b* cytochromes (eq 1, where

$$\ln (\Delta E_{t=0} - \Delta E_t) = \ln (\Delta Eb_1^{2+} e^{-k_1 t} + \Delta Eb_2^{2+} e^{-k_2 t}) \quad (1)$$

E represents the absorbance experimentally measured). A fast-reacting *b* ferrocytochrome ($t_{1/2}$, 70 msec at 560 nm, 110 msec at 566 nm), which contributed to the absorbance decrease by 71% at 560 nm and 65% at 566 nm, and a slowly reacting *b* ferrocytochrome ($t_{1/2}$, 390 msec at 560 nm and 470 msec at 566 nm), contributing to the absorbance decrease by 29 and 35%, respectively. Oligomycin reduced the velocity constant of the oxidation of the slow cytochrome *b* to one-fourth of the control value, that of the fast to about one-half (Figures 2C and

[1] Abbreviation used is: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

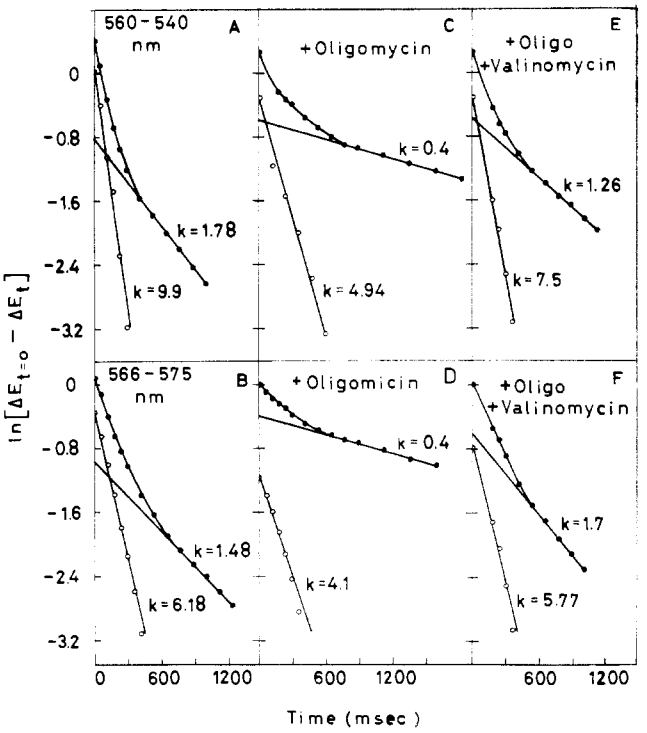

FIGURE 2: First-order plot of the absorbance decrease at 560–540 and 566–575 nm in the anaerobic–aerobic transition of succinate-supplemented EDTA particles. The experimental procedure, the experimental conditions, and the additions are those given in the legend to Figure 1. The extinction (E) is given in arbitrary units.

2D). In the presence of oligomycin the contribution of the slow cytochrome *b* to the overall absorbance decrease raised from 29 to 42% at 560 nm and from 35 to 56% at 566 nm. Valinomycin plus K^+ increased by 210–320% the velocity constant of the oxidation of the slow component but only by 40–50% the constant of the oxidation of the fast-reacting cytochrome *b* (see Figures 2E and 2F). Valinomycin, however,

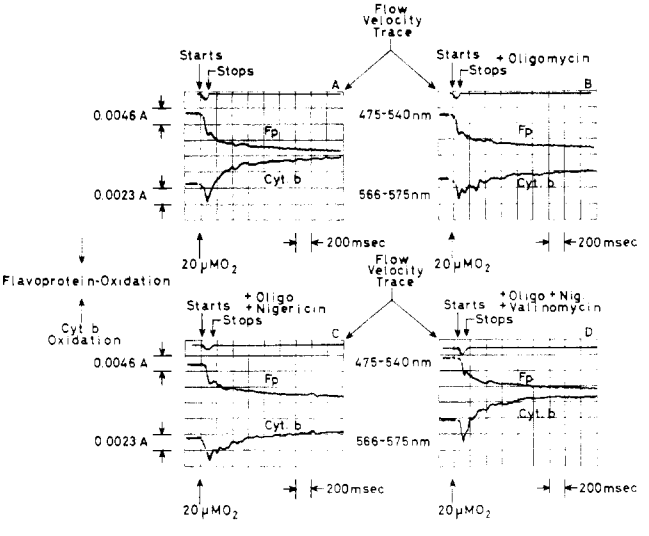

FIGURE 3: Simultaneous recordings of the oxidation of flavoproteins and cytochrome *b* induced by oxygen pulses of anaerobic EDTA particles. The experimental conditions and procedure were those described in the footnote to Table II. Additions were: 2 μg/ml of oligomycin, 0.5 μg/ml of nigericin, 0.5 μg/ml of valinomycin.

TABLE III: Effect of Oligomycin and Ionophores on the Oxidation of Flavoproteins and Cytochrome *b*.[a]

Expt	Additions	Flavoprotein Oxidation (475–540 nm) $t_{1/2}$ (msec)	Cytochrome *b* Oxidation (566–575 nm) $t_{1/2}$ (msec)
1		235	200
	Oligomycin	450	800
	Oligomycin, nigericin	450	800
	Oligomycin, nigericin, valinomycin	230	190
2		250	220
	Oligomycin	548	940
	Oligomycin, valinomycin	320	240

[a] The experimental conditions and procedure were those described in the footnote to Table II. Additions were: 2 μg/ml of oligomycin, 0.5 μg/ml of nigericin, 0.5 μg/ml of valinomycin. For other details, see Methods.

did not change the percentage contribution of the two *b* cytochromes to the overall absorbance changes. Nigericin, added in the presence of valinomycin plus K^+ or in the presence of K^+ alone had no significant effect on the two velocity constants (not shown). Data quantitatively similar to those reported for the wavelength pair 560–540 nm were obtained at 560–575 nm.

The experiment of Figure 3 compares the kinetics of the oxidation of *b* cytochromes, followed at 566–575 nm, with that of flavoproteins (475–540 nm). The absorbance recordings at 566–575 nm of Figure 1 indicated that the oxygen-induced oxidation of *b* cytochromes was preceded by a fast, small reduction. Figure 3 gives a better resolution of the reduction of cytochrome *b* induced by oxygenation of anaerobic succinate-supplemented particles. The reduction was practically completed during the mixing time. When the flow stopped, oxidation of cytochrome *b* ensued. The flavoprotein trace shows that simultaneously with the reduction of cytochrome *b* there occurred a rapid oxidation of flavoproteins, followed, when the flow stopped, by a second slower but much larger oxidative phase. Since the abrupt transient reduction of cytochrome *b* is not appreciable at 560–575 nm or 560–540 nm the compound which was reduced must be the slowly reacting species of *b* cytochromes. Oligomycin, nigericin plus K^+, or nigericin plus valinomycin had no appreciable effect on the initial reduction of cytochrome *b*, neither did they affect the accompanying fast phase of flavoprotein oxidation. On the other hand, the second, slow phase, of flavoprotein oxidation showed a similar sensitivity to these substances as already observed for cytochrome *b* (Figure 3 and Table III). Oligomycin caused a marked depression of this phase of flavoprotein oxidation. Nigericin plus K^+ had no effect on the rate of flavoprotein oxidation, but this was greatly increased by the combined addition of nigericin and valinomycin. Valinomycin relieved, also in the absence of nigericin, the inhibition of flavoprotein oxidation caused by oligomycin (Table III). However these effects on flavoprotein oxidation were less marked than those observed for *b* cytochromes at 566–575 nm.

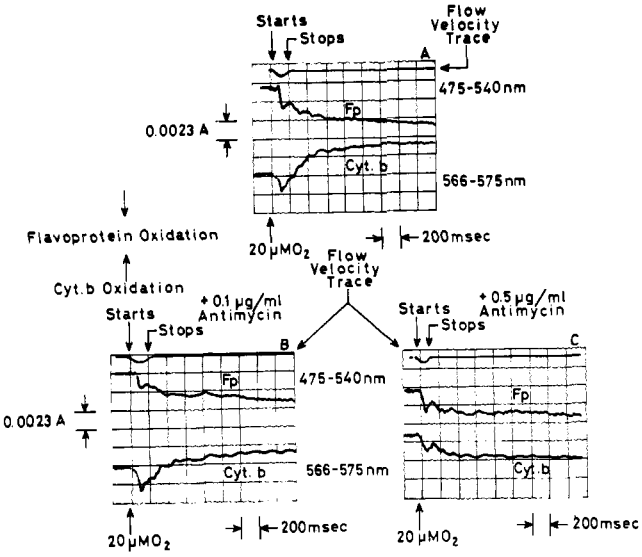

FIGURE 4: Effect of antimycin A on the oxidation of flavoproteins and cytochrome *b* induced by oxygen pulses of anaerobic EDTA particles. The reaction mixture contained, in addition to the components indicated in the footnote to Table II, 2 μg/ml of oligomycin, 0.5 μg/ml of valinomycin, and 0.5 μg/ml of nigericin.

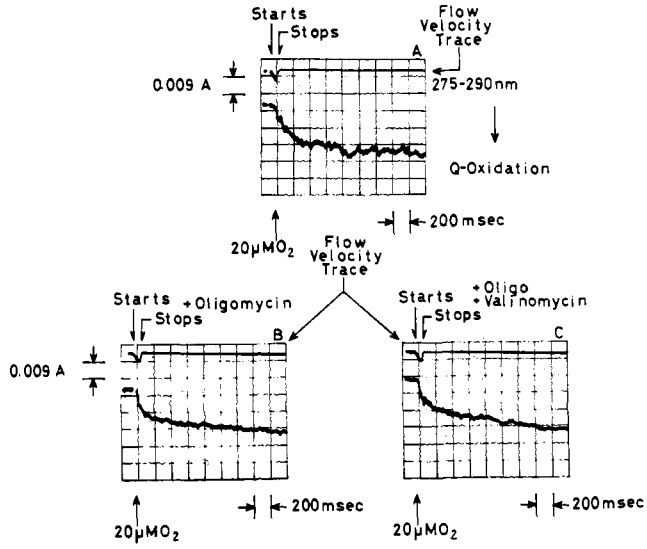

FIGURE 5: Kinetics of ubiquinone oxidation induced by oxygen pulses of anaerobic EDTA particles. The experimental conditions and procedure were those described in the footnote to Table II. Additions were: 2 μg/ml of oligomycin and 0.5 μg/ml of valinomycin.

Further discrimination between the two redox phases of flavoproteins and *b* cytochromes was given by their differential sensitivity to antimycin A. Figure 4 shows, in fact, that antimycin at concentrations which suppressed the oxidation of cytochrome *b* and the slow phase of flavoprotein oxidation did not abolish the fast phase of flavoprotein oxidation and the oxygen-induced reduction of cytochrome *b* (*cf.* Erecinska *et al.*, 1972). That the process was not an artifact was shown by its suppression when oxygen was added in the presence of Na_2S. Furthermore the absorbance traces of this figure clearly show that cytochrome *b* interference in the flavoprotein measurement is negligible.

In Figure 5 the effect of oligomycin and valinomycin on the oxidation of ubiquinone is presented. Oligomycin depressed the rate of Q oxidation; valinomycin relieved to a large extent this inhibition. The effect of oligomycin and valinomycin on the rate of Q oxidation was quantitatively comparable to the effect they had on the oxidation of the fast-reacting cytochrome *b*.

Separate controls showed that the stimulation by valinomycin of the rate of oxidation of flavoprotein, ubiquinone, and *b* cytochromes (see Table IV) did specifically require K^+. No appreciable effect was observed when the cationic species present was Tris or Na^+.

Effect of Salts on Proton Translocation and Kinetics of Cytochrome b. It has been found that salts of anions, to which the mitochondrial membrane is permeable, stimulate the respiration-driven proton uptake in submitochondrial particles (Papa *et al.*, 1970a,b). Figure 6 shows the effect of KSCN on the respiration-driven proton uptake and the $t_{1/2}$ of cytochrome *b* oxidation during oxygen pulses of succinate-supplemented submitochondrial particles. Increasing concentrations of KSCN caused a progressive stimulation of the respiration-driven proton uptake. Maximum stimulation was reached at 5 mM KSCN. In the same concentration range the stimulation of proton uptake was paralleled by an increase of the rate of cytochrome *b* oxidation as indicated by the progressive decrease of the $t_{1/2}$ of this process. The addition of nigericin and/or valinomycin, in the presence of 7 mM KSCN, had practically no further effect on the rate of cytochrome *b* oxidation.

pH Dependence of the Kinetics of b Cytochromes. In Table IV data are presented on the effect of the pH of the particle suspension on the kinetics of cytochrome *b* oxidation in the anaerobic–aerobic transition. It can be seen that the inhibition by oligomycin of the absorbance decrease at 566–575 nm dramatically decreased as the pH was increased from 7.0 to 8.5. The consequence was that in the presence of oligo-

TABLE IV: Influence of the pH on the Kinetics of Cytochrome *b* Oxidation in the Anaerobic–Aerobic Transition of EDTA Particles.[a]

	Cytochrome *b* Oxidation, $t_{1/2}$ (msec)					
	560–540 nm			566–575 nm		
	pH					
Additions	7.0	7.75	8.5	7.0	7.75	8.5
	147	162	200	200	140	170
Oligomycin	316	225	270	1080	580	236
Oligomycin, valinomycin	295	307	236	1100	500	221
Oligomycin, valinomycin, KCl	175	105	141	210	178	165
Oligomycin, valinomycin, KCl, nigericin	165	150	250	210	187	165

[a] The reaction mixture (final volume 20 ml) contained: 250 mM sucrose, 10 mM Tris-succinate, 5 mM Tris-malonate, 20 mM Tris-HCl, 1.5 mg/ml of particle protein. Additions were: 2 μg/ml of oligomycin, 0.5 μg/ml of valinomycin, 20 mM KCl, 0.5 μg/ml of nigericin. The experimental conditions and procedure were exactly those described in the footnote to Table II. For other details, see Methods.

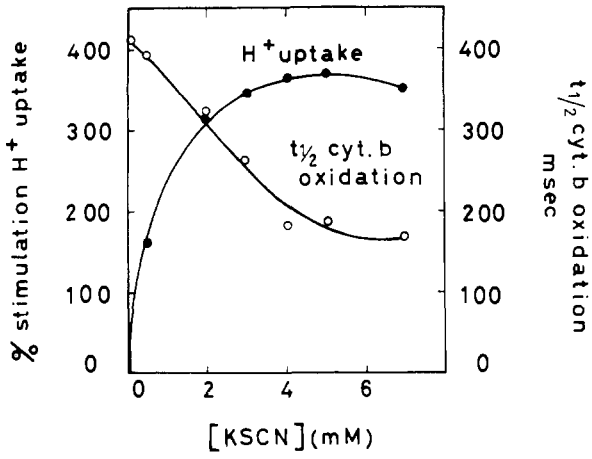

FIGURE 6: Effect of KSCN concentration on the extent of proton uptake and $t_{1/2}$ of cytochrome *b* oxidation induced by oxygen pulses of anaerobic EDTA particles. The experimental conditions and procedure were, for the measurement of proton uptake, those described in the footnote to Table I, for the measurement of the $t_{1/2}$ of cytochrome *b* oxidation, those of the footnote to Table II. In both cases 2 μg/ml of oligomycin was present in the reaction mixture.

mycin the rate of cytochrome *b* oxidation at 566–575 nm was at pH 8.5 fourfold higher than that at pH 7.0. The absorbance decrease at 560–540 nm was less effectively inhibited by oligomycin than that at 566–575 nm. Furthermore at 560–540 nm the $t_{1/2}$ of the oxygen-induced absorbance decrease was, in the presence of oligomycin, scarcely affected by changing the pH. The differential pH dependence of the absorbance decrease at 560–540 and 566–575 nm, observed in the presence of oligomycin, was largely abolished by the addition of valinomycin plus K^+.

Discussion

Oligomycin restores in EDTA submitochondrial particles the capacity for respiratory control, oxidative phosphorylation, and energy-linked transhydrogenase (Lee and Ernster, 1965, 1966). More recently it has been found that oligomycin enhances the respiration-driven proton uptake by EDTA particles (Papa *et al.*, 1970a; Papa, 1970, 1971; Montal *et al.*, 1970). This last effect of oligomycin is exerted at concentrations equal to those restoring the other energy-transducing reactions and appears to be due to inhibition of the passive proton back flow[2] (Table I; Papa *et al.*, 1970a; Papa, 1971). It should be stressed that the amount of oligomycin required for maximal inhibition of passive diffusion of the protons taken up by respiring particles (Papa *et al.*, 1970a; Papa, 1971) was much smaller than that required for maximal inhibition of the ATPase activity of EDTA particles (Lee and Ernster, 1965, 1966). Oligomycin depresses by two- to fourfold the rate of oxidation of flavoproteins, ubiquinone, and *b* cytochromes in the anaerobic–aerobic transition of succinate-supplemented EDTA particles. The conclusion that can be drawn from these facts is that in EDTA particles the energy made available by respiration is dissipated by a cyclic flow of protons across the mitochondrial membrane. Oligomycin, by inhibiting the decay of the proton gradient established by respiration, prevents dissipation of the respiratory energy, this being, now, principally conserved as the thermodynamic potential difference across the membrane of protons ($\Delta\tilde{\mu}_H$) and alkali metal cations, which secondarily exchange with protons (Cockrell and Racker, 1969; Montal *et al.*, 1970; Papa, 1970, 1971).

The respiration-driven proton uptake by EDTA particles, supplemented with oligomycin and succinate as respiratory substrate, is three- to fourfold stimulated by salts of lipophilic anions (Figure 6; Papa *et al.*, 1970a,b) or by valinomycin plus K^+ (Table I; Papa *et al.*, 1970a; Papa, 1971). Stimulation of electron-flow-driven proton translocation by valinomycin plus K^+ has also been observed in intact mitochondria (Mitchell and Moyle, 1967; Chappell and Haarhoff, 1967), chromatophores (Von Stendigk and Baltscheffsky, 1966; Jackson *et al.*, 1968), and chloroplasts (Von Stedingk, 1970; Karlish and Avron, 1971). Permeant anions are accumulated by submitochondrial particles along with protons (Grinius *et al.*, 1970; Papa, 1970, 1971; Papa *et al.*, 1972). In the presence of valinomycin respiration induces K^+ extrusion from the particles (Cockrell and Racker, 1969; Montal *et al.*, 1970). The steady-state respiratory rate of oligomycin-supplemented EDTA particles is not affected by valinomycin plus K^+ or by salts of permeant anions (see also Papa *et al.*, 1970a; Montal *et al.*, 1970). Valinomycin *plus* K^+ and salts of permeant anions give, however, a marked stimulation of the rate of oxidation of flavoproteins, ubiquinone, and *b* cytochromes in the anaerobic–aerobic transition. These data provide valuable circumstantial evidence for the electrogenicity of the respiration-driven proton pump. In this case the $\Delta\tilde{\mu}_H$ consists of a chemical (ΔpH) and an electrical ($\Delta\psi$) component. Both exert a back pressure on the proton pump and electron flow. Dissipation of the $\Delta\psi$, through distribution of positively or negatively charged ions in the electric field, results in a stimulation of proton uptake and electron flow. This stimulation persists until the ensuing extra ΔpH replaces the dissipated $\Delta\psi$, then the rate of electron flow subsides to the control value. Nigericin which mediates a 1:1 H^+/K^+ exchange diffusion and thus converts the ΔpH into $\Delta\psi$ has no significant effect on electron flow either at the steady state or in the anaerobic–aerobic transition. When both the ΔpH and $\Delta\psi$ are dissipated by the combined addition of valinomycin and nigericin the steady-state respiratory rate is greatly stimulated. The three- to fourfold stimulation of the rate of proton uptake and the equally great stimulation of the oxidation of respiratory carriers brought about by valinomycin plus K^+, as well as the lack of any effect of the addition of nigericin in the presence of valinomycin on the kinetics of respiratory carriers, indicate (*cf.* Mitchell, 1966, 1968) that the major part of the $\Delta\tilde{\mu}_H$ established by the proton pump is represented by a $\Delta\psi$.

Mitchell (1966, 1968) has proposed that electron flow is linked to proton translocation across the mitochondrial membrane since the respiratory chain is bent back on itself in a series of loops, each one composed of a hydrogen-carrying and an electron-carrying arm. According to this view the redox poise of the electron and hydrogen carriers may be differently affected by the $\Delta\psi$ and the ΔpH components of the $\Delta\tilde{\mu}_H$ established by the proton pump, oxidoreduction of electron carriers in the normal direction being opposed by $\Delta\psi$, oxidoreduction of hydrogen carriers being opposed by ΔpH (Mitchell, 1968). The experimental finding is, however, that conversion of ΔpH into $\Delta\psi$, brought about by nigericin plus K^+, has no effect on the $t_{1/2}$ of oxidation of flavoproteins and *b* cytochromes in EDTA particles. Collapse of the $\Delta\psi$ by valinomycin plus K^+ stimulated, to about the same extent,

[2] It has been found that oligomycin inhibits proton diffusion also in phospholipids micelles (M. Montal, 1971, personal communication; A. Azzi, 1971, personal communication).

the rate of oxidation of flavoproteins, ubiquinone, and of one of the *b* cytochromes. It has also been reported (Montal *et al.*, 1970) that at the steady state the conversion of ΔpH into $\Delta\psi$ or *vice versa* of $\Delta\psi$ into ΔpH gives no significant change of the redox level of cytochromes in EDTA particles respiring with succinate in the presence of oligomycin.

Mathematical analysis of the kinetics of the absorbance decrease of *b* cytochromes, measured at 560–540 and 566–575 nm during the anaerobic–aerobic transition of succinate-supplemented particles (Figure 2), showed this to correspond, at both the wavelength couples, to the oxidation of two chemically distinct ferrocytochrome species. In the absence of oligomycin the $t_{1/2}$ of oxidation was 70–110 msec for one species, 390–470 msec for the other one. The fast-oxidized species showed an E_{560}/E_{566} ratio > 1; for the slow species this ratio was <1. When energy dissipation was prevented by oligomycin the kinetics constant of the oxidation of the slow-reacting *b* cytochrome was much more reduced than that of the fast-reacting *b* cytochrome. Furthermore oligomycin increased the reduction level of the slow-reacting *b* cytochrome both in the anaerobic and the aerobic state. The fast- and the slow-reacting *b* cytochromes described here can be respectively identified with cytochrome b_K and cytochrome b_T of pigeon heart and rat liver mitochondria (Wilson and Dutton, 1970; Dutton *et al.*, 1970; Chance *et al.*, 1970; Sato *et al.*, 1971). Energization of these mitochondria with ATP increases the reduction level of b_T (Sato *et al.*, 1971) and depresses its rate of oxidation in the anaerobic–aerobic transition (Chance *et al.*, 1970).

The fast-reacting cytochrome *b* with an oxidation $t_{1/2}$ of 70–110 msec in the absence and 140–170 msec in the presence of oligomycin finds its place on the oxygen side of flavoproteins ($t_{1/2}$, 240 msec in the absence and 500 msec in the presence of oligomycin) and ubiquinone ($t_{1/2}$, 190 msec in the absence and 415 msec in the presence of oligomycin). The slow-reacting *b* cytochrome, with an oxidation $t_{1/2}$ of 390–470 msec in the absence and of 1700 msec in the presence of oligomycin, can hardly be placed on the main path of the respiratory chain; it should be on a side path. Oxygenation of anaerobic succinate-supplemented EDTA particles caused a transient, antimycin-insensitive, reduction of *b* cytochromes (Erecinska *et al.*, 1972). Our data indicate that it is the slow-reacting cytochrome b_{566} that is reduced and identify the flavoproteins as a reductant of cytochrome b_{566}.

The inhibition of the respiration-linked proton turnover by oligomycin is accompanied by a much more profound inhibition of the oxidation of the slow cytochrome *b* than of the fast cytochrome *b*, flavoproteins, and ubiquinone. Activation of the proton pump by valinomycin plus K^+ stimulates preferentially the oxidation of the slow cytochrome *b*. If one compares the kinetic parameters of the respiratory carriers in the anaerobic–aerobic transition of submitochondrial particles with those of the proton pump, it appears that of the various components of the respiratory chain the slow cytochrome *b* has the oxidation $t_{1/2}$ closest to the $t_{1/2}$ of the respiration-driven proton uptake (Papa, 1971). These observations would suggest a specific relationship between oxidoreduction of this species of cytochrome *b* and the proton pump. Further correlation between the slow-reacting *b* cytochrome and proton translocation in the mitochondrial membrane is indicated by the specific pH dependence of the inhibitory effect of oligomycin on its oxidation. These data as well as the known pH dependence of the redox potential of cytochrome *b* (Straub and Colpa-Boonstra, 1962; Urban and Klingenberg, 1969) make further investigation of a possible role of *b* cytochromes in the proton pump of mitochondria particularly interesting.

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Effect of Local Anesthetics on Phospholipases from Mitochondria and Lysosomes. A Probe into the Role of the Calcium Ion in Phospholipid Hydrolysis†

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ABSTRACT: Local anesthetics which contain a tertiary amine have marked effects on solubilized and membrane bound phospholipase A_2 of rat liver mitochondria. The compounds with greatest influence, dibucaine and butacaine, had opposite effects; at 10–50 μM dibucaine stimulated whereas butacaine inhibited. At higher concentrations (200–300 μM) dibucaine inhibited whereas butacaine stimulated which indicates that local anesthetics might have more than one mechanism of action. Studies on the mechanism of inhibition by dibucaine led to the following conclusions. (1) The substrate phosphatidylethanolamine does not bind dibucaine tightly enough to account for the inhibition. (2) The inhibition is uncompetitive, with respect to substrate. (3) The inhibition is greatest during the initial part of the reaction which suggests that the availability of the active site of the enzyme to the 2-acyl ester of phosphatidylethanolamine is reduced. This does not imply, however, that the physical interaction of the enzyme with the liposome is decreased when dibucaine is present. (4) Under the appropriate conditions, Ca^{2+} protects against inhibition by dibucaine, but Ca^{2+} concentrations higher than 1 mM are also inhibitory which suggests a common binding site for the two compounds on the enzyme. Lysosomal phospholipases A_1 and A_2 were also inhibited by either dibucaine or Ca^{2+} which further suggests the mechanism of inhibition by dibucaine is similar to that found with the mitochondrial enzyme. Dibucaine causes a severalfold increase in the hydrolysis of phosphatidylethanolamine of mitochondria in the absence of added Ca^{2+}. On the other hand, when the membrane-bound enzyme was fully stimulated by Ca^{2+}, dibucaine inhibited. These results are interpreted as being the result of two effects of dibucaine; one, dibucaine displaces Ca^{2+} from the membrane which makes it more available to the phospholipase, and two, when sufficient Ca^{2+} is available for maximal activity, dibucaine inhibits.

Nearly a decade ago Lehninger and his coworkers (1964) established that in mitochondria there is relationship between lipolytic activity and structure-linked functions such as respiratory control. More recently the phospholipase A_2 of mitochondria (EC 3.1.1.4) responsible for this lipolytic activity was described and its involvement in mitochondrial swelling was more clearly defined (Waite *et al.*, 1969b). This phospholipase is located primarily in the outer membrane (Nachbaur and Vignais, 1968; Waite, 1969) although some activity has been found in the inner membrane (Waite, 1969). Extraction of the mitochondria with ammoniacal acetone solubilized the enzyme which has now been partially purified and characterized (Waite and Sisson, 1971).

Recent work by Seppala *et al.* (1971) and Scherphof and Scarpa (1972) showed that local anesthetics such as dibucaine and butacaine inhibit both the phospholipase A_2 activity of the venom from *Crotalus adamanteus* and of pancreas, and the hydrolysis of mitochondrial phospholipid catalyzed by the mitochondrial phospholipase A_2. It was observed also that dibucaine competes with Ca^{2+} for binding sites on membranes (Scarpa and Azzi, 1968; Kwant and Seeman, 1969) and to phospholipids (Blaustein, 1967). The question arose: does dibucaine inhibit the phospholipase A_2 directly or is the effect primarily on the mitochondrial membrane and only secondarily

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