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On the Mechanism of Action of Oligomycin and Acidic Uncouplers on Proton Translocation and Energy Transfer in "Sonic" Submitochondrial Particles

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Abstract

A study is presented of the effect of acidic uncouplers and oligomycin on energy-linked and passive proton translocation, oxidative phosphorylation, and energy-linked nicotinamide-adenine-nucleotide transhydrogenase in EDTA submitochondrial particles from beef-heart. A flow potentiometric technique has been applied to resolve the kinetics of the initial rapid phase of the redox proton pump. Rapid kinetics analysis shows that carbonyl-cyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP) does not exert any direct effect on redox-linked active proton transport. The uncoupling action of FCCP on oxidative phosphorylation and energy-linked transhydrogenase is shown to be quantitatively accounted for by its promoting effect of passive proton-diffusion across the mitochondrial membrane. Oligomycin depresses passive proton diffusion in EDTA sonic particles and this effect accounts for the coupling action exerted by the antibiotic on oxidative phosphorylation and energy-linked transhydrogenase. In fact, rapid kinetic analysis demonstrates that oligomycin does not directly affect the redox-linked proton pump. The present results show that there does not exist any labile intermediate in the redox-linked proton pump which is sensitive to acidic uncouplers.

Introduction

Previous investigations have provided evidence showing that respiration-driven vectorial proton translocation in mitochondria and submito-

chondrial particles, obtained by sonication, consists of a single electrogenic flux [1–5] (also see [6, 7]; cf. [8, 9]). In this paper a kinetic analysis of the action of an acidic uncoupler, carbonyl-cyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP), and of oligomycin on proton translocation in "sonic" submitochondrial particles is presented. The effects exerted by FCCP and oligomycin on the parameters of proton translocation are compared to those exerted on oxidative phosphorylation and energy-linked nicotinamide-adenine-nucleotide transhydrogenase.

Acidic uncouplers increase and oligomycin depresses passive proton diffusion across the membrane of "sonic vesicles." On the other hand, both substances do not exert any direct effect on uphill redox-linked proton transport. These findings show that the redox-linked proton pump does not involve labile, uncoupler-sensitive intermediates. The effects of FCCP and oligomycin on proton diffusion account for the uncoupling and coupling action that these substances exert on other energy-transducing reactions in sonic particles. Part of the results presented in this paper have been previous communicated in a preliminary form [1, 5, 10].

Materials and Methods

Valinomycin was a generous gift of Dr. W.O. Godtfredsen of Leo Pharmaceutical Products, Ballerup, Denmark. FCCP was a gift of Dr. C.P. Lee. Oligomycin was purchased from Sigma Chemical Company.

Preparation of submitochondrial particles

Mg-ATP submitochondrial particles were prepared from beef-heart mitochondria according to Löw and Vallin [11], EDTA submitochondrial particles according to Lee and Ernster [12] (see also [5]). Sonication of heavy beef-heart mitochondria was carried out using an Ultrasonic Branson Sonifier, Model W 185, for 60 sec at 70 W.

Measurements of respiration and H^+ translocation

Submitochondrial particles (1–4 mg protein/ml) were incubated in a basic reaction mixture containing 250 mM sucrose and 0.2 mg/ml purified catalase (E.C.1.11.1.6). Respiration was activated by repetitive pulses of 0.4–3% H_2O_2 (5 μl/ml). The pH of the suspension and O_2 concentration were monitored potentiometrically by electrodes immersed in the reaction mixture. Respiration was measured with a Clark oxygen electrode (Yellow Springs Instrument Co. Inc., Mod. 5331). The pH was monitored with a fast responding glass electrode (Ingold KG,

Frankfurt/Main, Germany) or a Beckman combination electrode (no. 39030, Beckman Instruments International, Geneva, Switzerland). The incubation mixture was thermostated to ±0.01° C. For other details and experimental conditions see [5] and Figs. 1–7.

Oxidative phosphorylation

Submitochondrial particles (1 mg protein/ml) were incubated at 25° C in the reaction mixture described in the legend to Fig. 3. Oxygen uptake was measured manometrically. Phosphate esterification was measured by determining glucose-6-phosphate enzymically with glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) [12], correction being made for the adenylate-kinase activity (E.C.2.7.4.3) of the particles. This activity was measured by determining the disappearance of added ADP enzymically with NADH, phospho-enol-pyruvate, pyruvate kinase (E.C.2.7.1.40), and lactate dehydrogenase (E.C.1.11.27).

Nicotinamide-adenine-nucleotide transhydrogenase

Submitochondrial particles (1–1.2 mg protein/ml) were incubated at 25° C in the reaction mixture described in the legend to Fig. 3. The energy-linked transhydrogenase activity was measured by determining the disappearance of $NADP^+$; this was estimated enzymically with glucose-6-phosphate dehydrogenase [14]. Correction was made for the nonenergy-linked transhydrogenase by subtracting from the total $NADP^+$ disappearance that occurred in the presence of 2 mM KCN and 0.2 µg/ml antimycin.

Mathematical analysis

The potentiometric traces of the anaerobic proton diffusion from submitochondrial particles were converted into proton equivalent with the aid of a Hewlett-Packard 9810A Calculator and then treated by a douple exponential equation (see [2]).

Flow analysis of fast redox-linked H^+ translocation

The kinetics of proton translocation was analysed with a Roughton-type continuous-flow pH meter (mixing ratio 1:60) with a resolution time of 10 msec [15, 16]. The electrodes used were a 50-100 MΩ glass electrode (Ingold KG, Frankfurt/Main, Germany) and a calomel electrode connected to the incubation mixture in the measuring cell of the apparatus through a saturated KCl bridge. The electrodes were connected to a Vibron Precision Electrometer (Model 62A) set up at a sensitivity at the

output of 24 V/pH unit. The input capacitance of the electrometer was 1 pF. The electrometer output was displayed on a Honeywell strip chart Recorder, model Electronik 194. The sensitivity of the recorder was adjusted to give a 5 cm deflection/V. This circuit allowed the pH to be measured with a precision of 0.001 pH unit. Potential changes were quantitated as proton equivalents by double titration with standard solutions of KOH and HCl. These titrations were performed by following the pH changes which occurred within 30 msec after the addition to aerobic or KCN-inhibited aerobic mitochondria of KOH or HCl at a final concentration of 35 μM; the short time interval used avoids titration of the interior of the particles. The syringes of the continuous-flow pH meter, the mixing chamber and the measurement compartment were surrounded by a water bath thermostated at $\pm 0.01^{\circ}$ C.

Results and Discussion

Effect of FCCP on proton translocation and energy transduction

Figure 1 shows that FCCP added to anaerobic, succinate-supplemented Mg-ATP sonic particles depresses the extent of proton uptake which ensues upon activation of respiration with oxygen pulses, speeds up the anaerobic passive proton back-flow, but apparently has no effect on the initial rate of the aerobic proton uptake. These effects of FCCP on proton translocation are accompanied by acceleration of oxygen consumption.

The effect of FCCP on the initial rate of fast aerobic proton translocation was further analysed by means of the continuous-flow pH meter described in *Materials and Methods.* Previous work carried out by means of flow techniques has revealed that rapid aerobic oxidation of reduced respiratory carriers is associated with synchronous proton release from intact mitochondria [16, 17, 19] and synchronous proton uptake by "sonic" submitochondrial particles [5, 16, 18, 19]. The experiment, presented in Fig. 2, shows that FCCP, at a fully uncoupling concentration of 0.5 μM, has no effect on the initial rate of proton uptake induced by rapid aerobic oxidation of anaerobically reduced respiratory carriers in sonic particles. Only 300 msec after oxygenation does FCCP start to depress proton uptake; this being clearly due to enhanced proton back-flow induced by the proton-conducting acidic uncoupler. In order to measure maximum capacity of aerobic proton uptake, the EDTA particles used in the present experiment were supplemented with valinomycin plus K^+ and oligomycin (see [20-22] and below). Similar results were obtained in EDTA particles in the absence of these substances and in Mg-ATP particles.

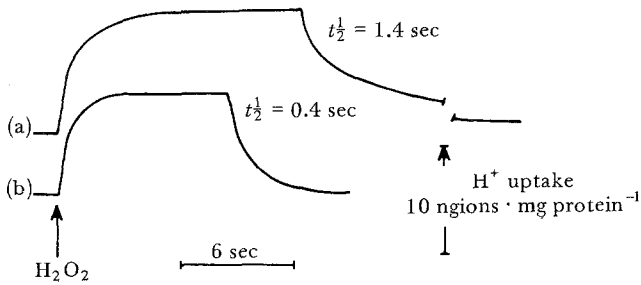

Figure 1. Respiration-induced H^+ translocation in Mg-ATP particles showing effect of FCCP. Submitochondrial particles (1.2 mg protein/ml) were incubated in a reaction mixture containing 250 mM sucrose, 10 mM potassium succinate, and 0.4 mg/ml purified catalase. Final volume, 1.5 ml. Final pH, 7.5. Temperature, 25° C. (a) Control; (b) plus 0.5 μM FCCP.

Figure 3 compares the effect of FCCP on oxidative phosphorylation, energy-linked transhydrogenase and aerobic proton uptake in Mg-ATP particles. On increasing the FCCP concentration from 0.05–0.5 μM, oxidative phosphorylation and energy-linked transhydrogenase were progressively, completely uncoupled. The respiration-linked proton

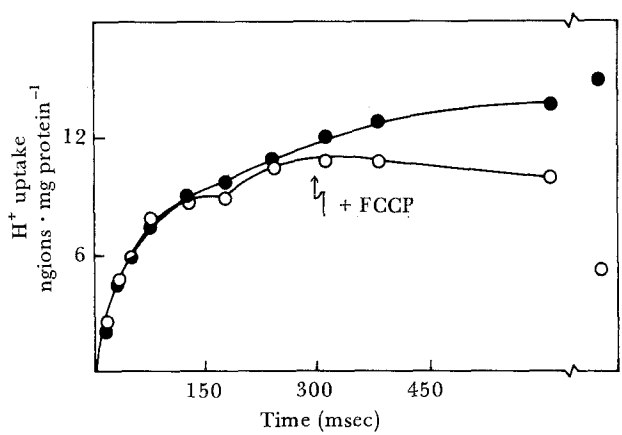

Figure 2. Kinetic analysis of aerobic proton uptake in EDTA submitochondrial particles. The kinetics of proton uptake was analysed with a Roughton-type continuous-flow pH meter. Mixing ratio, 1:60 (v:v). The main syringe containing 250 mM sucrose, 5 mM potassium succinate, 3.5 mM potassium malonate, 30 mM KCl, 2 μg/mg protein oligomycin, 0.5 μg/mg protein valinomycin, and EDTA particles 2 mg/ml. Final pH, 6.8. Temperature, 20° C. The smaller syringe contained an oxygen-saturated mixture consisting of 250 mM sucrose and 30 mM KCl. Where added, the concentration of FCCP was 0.5 μM.

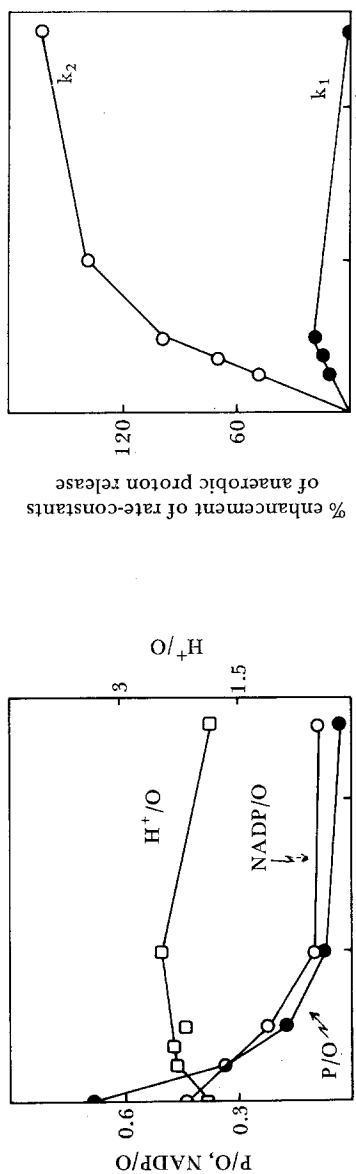

Figure 3. Effect of FCCP onoxidative phosphorylation, energy-linked nicotinamide-adenine-nucleotide transhydrogenase and respiration-induced proton translocation in Mg-ATP particles. Experimental conditions were as follows: (a) Oxidative phosphorylation; 200 mM sucrose, 20 mM glucose, 10 mM potassium succinate, 5 mM $MgCl_2$, 5 mM H_3PO_4, 1 mM EDTA, 0.1 mM ADP, 7 units hexokinase, and Mg-ATP particles 0.98 mg protein/ml. Final volume, 1 ml. Final pH, 7.5. Temperature, 30°C. (b) Energy-linked transhydrogenase; 200 mM sucrose, 1 μg/mg protein rotenone, 10 mM potassium succinate, 5 mM semicarbazide, 2 mM NADH, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM NADP, 15 units alcohol dehydrogenase, and 1 mg protein/ml Mg-ATP particles. Final volume, 1 ml. Final pH, 7.5. Temperature, 30°C. (c) Proton translocation; 250 mM sucrose, 0.2 μg/ml catalase, 15 mM sodium succinate, 15 mM NaSCN and submitochondrial particles 1.1 mg protein/ml. Final volume, 1.5 ml. Final pH, 7.5. Temperature, 30°C.

uptake, on the other hand, was not inhibited, as shown by the fact that the H^+/O ratio was unchanged by FCCP. Similar results on the insensitivity of the H^+/O ratio to uncouplers have been reported by Mitchell and Moyle [20] and Hinkle and Horstman [22].

It has previously been shown that the kinetics of the anaerobic decay of the respiration-linked proton gradient in sonic particles is biphasic [2]. The process appears to consist of two parallel first-order reactions. A fast reaction which has the characteristics of a proton/monovalent-cation exchange and a slow reaction which appears to consist of a single, passive electrogenic flux [2].

The experiment of Fig. 3 shows that the uncoupling action of FCCP correlates with its stimulatory effect on the slow proton diffusion reaction across the mitochondrial membrane. The fast proton diffusion reaction is practically unaffected by FCCP [2].

Effect of oligomycin on proton translocation and energy transduction

Lee and Ernster reported in 1965 [23] that in EDTA sonic particles, relatively low concentrations of oligomycin restore oxidative phosphorylation and energy-linked nicotinamide-nucleotide transhydrogenase and also cause inhibition of respiration, which is released by uncouplers. This effect of oligomycin, which was observed also in other types of highly resolved submitochondrial particles [22], was explained by assuming that in these vesicles, oligomycin prevents the hydrolysis of a high-energy chemical intermediate [23]. Subsequent work in our laboratory [5, 21] has shown that oligomycin depresses the proton permeability of EDTA particles, which is relatively high, and enhances the respiration-driven proton uptake (see also [20, 22]). Hence we concluded that oligomycin prevents uncoupling of the particles by inhibiting energy-wasting cyclic flow of protons across the membrane (see also [20, 22]). This latter explanation is favoured by the close correspondence of the oligomycin titre for inhibition of respiration and restoration of energy-transfer reactions on one hand and the oligomycin titre for inhibition of passive proton diffusion on the other (see [23, 3, 21, 22]). The alternative possibility that oligomycin restores energy coupling by inhibiting the hydrolysis of high-energy chemical intermediates appears to be ruled out by the following experiments.

Figure 4 shows that oligomycin enhances the extent of the respiration-linked proton uptake by EDTA particles also in the presence of a fully uncoupling concentration of FCCP. The concentrations of oligomycin causing half maximal stimulation of the proton uptake were practically the same in the presence and in absence of FCCP.

Figure 5 illustrates the effect of an excess of oligomycin on fast proton uptake induced in EDTA particles by aerobic oxidation of endogenous respiratory carriers. Oligomycin, added to succinate-reduced

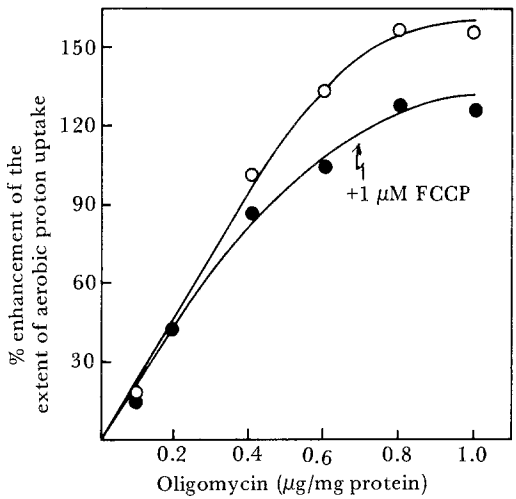

Figure 4. Effect of oligomycin on the respiration-linked proton uptake by EDTA particles in the absence and presence of FCCP. Submitochondrial particles (1.1 mg protein/ml) were incubated in a reaction mixture containing 250 mM sucrose, 15 mM potassium succinate, 30 mM KCl, 0.4 mg/ml catalase, and 0.1 μg/ml valinomycin. Final volume, 1.5 ml. pH, 7. Temperature, 30° C.

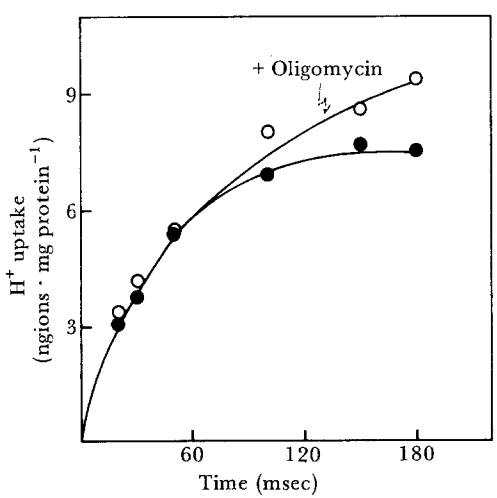

Figure 5. Effect of oligomycin on the kinetics of the respiration-linked proton uptake in EDTA submitochondrial particles. The main syringe contained 250 mM sucrose, 10 mM potassium succinate, 30 mM KCl, 0.5 μg/mg protein valinomycin, and EDTA particles 2 mg/ml. Final pH, 7.5. Temperature, 20° C. The smaller syringe contained an oxygen saturated mixture as described in the legend to Fig. 3. Where added, the concentration of oligomycin was 2 μg/mg protein.

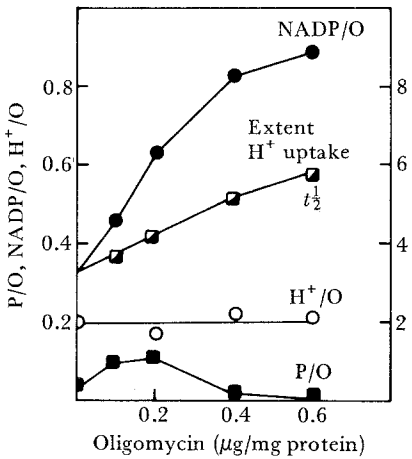

Figure 6. Titration of the effect of oligomycin on oxidative phosphorylation, energy-linked nicotinamide-adenine-nucleotide transhydrogenase and respiration-induced proton translocation in EDTA submitochondrial particles. The reaction mixtures for oxidative phosphorylation and energy-linked transhydrogenase were those described in the legend to Fig. 4. Final volume, 1 ml. Final pH, 7.5. Temperature, 25° C. The reaction mixture for proton translocation contained 250 mM sucrose, 0.4 mg/ml catalase, 15 mM potassium succinate, and submitochondrial particles (1.5 mg protein/ml). Final volume, 1.5 ml. Final pH, 7.5. Temperature, 25° C. □—□ represents extent H^+ uptake ngions · mg protein^{-1}; ■—■, $t_{\frac{1}{2}}$ H^+ release (sec).

particles in the anaerobic state, had no effect on the initial rate of aerobic proton uptake. Only at an interval of 100 msec after oxygenation, did oligomycin cause a certain enhancement of net proton uptake, an effect which is clearly explained by the inhibitory action of oligomycin on passive proton back-flow.

In Fig. 6 a titration of the effect of oligomycin on the respiration-linked proton translocation, energy-linked transhydrogenase, and oxidative phosphorylation in EDTA particles is presented. It can be seen that the progressive depression of proton diffusion caused by oligomycin, as shown by the enhancement of the $t_{\frac{1}{2}}$ of the anaerobic proton release, was accompanied by parallel enhancement of the extent of the aerobic proton uptake and of the NADP/O ratio. The P/O ratio was increased by oligomycin up to a concentration of about 0.2 μg/mg protein and then inhibited; this secondary inhibition being due to the direct inhibitory effect of the antibiotic on the phosphorylation reaction. In contrast with these effects on the NADP/O and P/O ratio, oligomycin had no significant effect on the H^+/O ratio. It should be noted that the H^+/O ratios of Fig. 6 were obtained from measurements

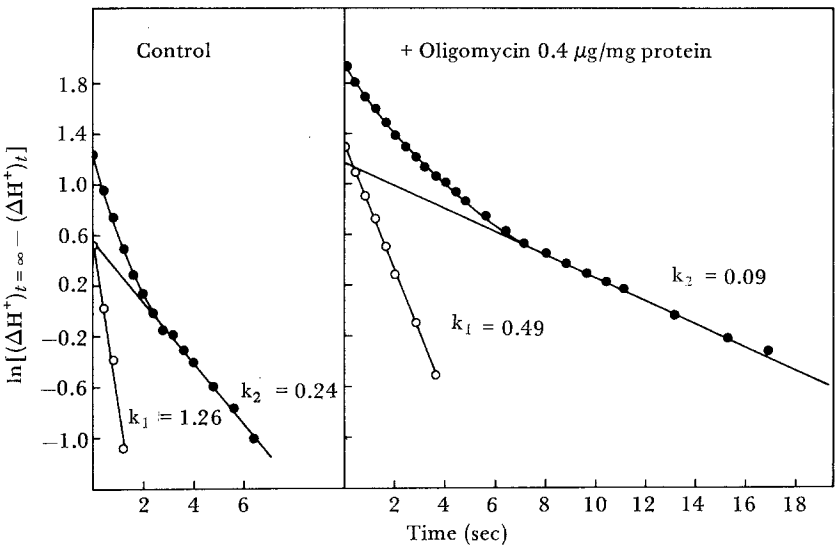

Figure 7. Double exponential analysis of the kinetics of the anaerobic proton release from EDTA submitochondrial particles showing effect of oligomycin. EDTA particles (2.6 mg protein) were incubated in 1.5 ml of a reaction mixture containing 250 mM sucrose, 0.4 mg/ml catalase, 15 mM potassium succinate. Final pH, 7.8. Temperature, 25° C.

of the proton turnover and respiratory rate, at the aerobic steady-state in the absence of agents which destroy the membrane potential. Thus these ratios are much lower than those measured in the presence of permeant ions with the same procedure (see Fig. 3 and [24]) or with the oxygen-pulse technique and correction for proton back-flow [20, 22].

The results presented in Figs 5 and 6 clearly rule out the possibility that the stimulation of aerobic proton uptake by oligomycin is due to prevention of hydrolysis of high-energy chemical intermediates. If this were the case, oligomycin should increase the initial rate of uphill aerobic proton uptake and the H^+/O ratio at the steady-state.

As mentioned above, aerobic proton back-flow is accounted for by at least two parallel reactions [2]; the experiment of Fig. 7 shows that oligomycin, at least under the prevailing conditions used, apparently inhibits both the fast and the slow reactions of passive proton diffusion.

Conclusions

The data presented allow the following conclusions to be drawn:

(1). The uncoupling action of acidic uncouplers on energy transfer reactions in the mitochondrial membrane can be quantitatively

accounted for by their proton-conducting property (cf. [6]). By enhancing the rate of passive diffusion of protons across the mitochondrial membrane FCCP induces an energy-dissipating cyclic flow of protons and this is directly responsible for the inhibition of oxidative phosphorylation and energy-linked nicotinamide-adenine-nucleotide transhydrogenase. There is no other more direct action of FCCP on the primary energy-conserving act of the respiratory chain as shown by the lack of any effect of this substance on the initial rate of redox-linked active proton transport.

(2). The coupling action exerted by oligomycin in EDTA submitochondrial particles is a direct consequence of its inhibitory action on passive proton diffusion across the mitochondrial membrane. Oligomycin inhibits energy-dissipating cyclic flow of protons across the membrane, so that more energy is made available for oxidative phosphorylation and energy-linked nicotinamide-adenine-nucleotide transhydrogenase.

There is no other direct effect of oligomycin on the primary energy-conserving act of the respiratory chain, as shown by the fact that this antibiotic has no effect on the initial rate of the redox-linked active proton transport; neither does it affect the steady-state H^+/O ratio.

(3). It is concluded that there does not exist any labile intermediate in the redox-linked proton pump which is sensitive to acidic uncouplers.

(4). The high selectivity of oligomycin for the ATPase complex as well as the observation [22] that the inhibitory action exerted by oligomycin on passive proton diffusion in submitochondrial particles deficient in coupling factor 1 (soluble ATPase) is mimicked by the combined addition of coupling factor 1 and the oligomycin-sensitivity-conferring factor suggest that the inhibition of proton diffusion by oligomycin is a result of its interaction with a component of the ATPase-complex directly involved in the ATP-driven proton pump. The possibility that the oligomycin-sensitivity-conferring factor is directly involved in proton transport has in fact already been entertained by other authors [25, 26]. The passive proton diffusion in sonic submitochondrial particles (as well as intact mitochondria [20]) consists of at least two parallel processes [2]. As reported above, there exist data indicating that a fast phase of the proton back-flow in sonic particles [2], as well as in intact mitochondria [20], consists of a proton/monovalent cation exchange diffusion. In EDTA particles, both of the proton-diffusion reactions, into which the proton back-flow can be resolved by mathematical analysis [2], appear to be inhibited by oligomycin. Further studies are needed to clarify the mechanism of action of oligomycin on proton-diffusion reactions, as well as their relationship to the ATPase and monovalent cation transport in the mitochondrial membrane.

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