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Quantitation of the Energetic State in Mitochondria and Submitochondrial Vesicles with 8-Anilino-1-Naphthalene Sulfonic Acid

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Abstract

Some of the apparently anomalous findings made with the fluorescent probe 8-anilino-1-naphthalene sulfonic acid (ANS) have been reinvestigated using rat liver mitochondria. The results have been found compatible with current views on energy conservation.

The direction of fluorescence and proton flux changes under different conditions have been delineated. The relation of these results to consideration of membrane polarity and organization is discussed.

The reliability of ANS fluorescence changes in determining the level of energization of mitochondria and submitochondrial preparations is discussed.

Changes in the fluorescence-intensity of ANS† (ΔF_E) and pH (ΔH^+_E) have been used for the quantitation of energy transformed by the flow of electrons through the mitochondrial respiratory chain. Mitchell and his associates (cf. Greville[1]) demonstrated that the H^+/O ratio calculated from the transitory acidification elicited by mixing a limited amount of oxygen with anaerobic mitochondria is 6 and 4 for β-hydroxybutyrate and succinate, respectively. ΔF_E of ANS was shown by Ernster and Nordenbrand[2] to bear a quantitative relationship to the respiratory control and the oxidation-reduction level of cytochrome *b* in SMP for succinate respiration. The assumed difference in polarity of inner membranes of intact mitochondria as compared to SMP was matched by a parallel difference in the direction of ΔH^+_E and ΔF_E.[3] However, some other reports exist in the literature which show discrepancies both in direction and magnitude ΔH^+_E[4] and ΔF_E.[5] Hence, the aim of the present study was to reinvestigate certain factors which affect the use of ΔH^+_E and ΔF_E for quantitation of changes in energization of mitochondria and SMP.

Methods

Rat liver mitochondria were isolated in a medium containing 0·33 M sucrose, 1 mM EDTA and 1 mM Tris-chloride (pH 7·5) as described by House and Packer.[5] Respiration was determined polarographically, ΔH^+_E by measuring pH changes with a combination

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† Abbreviations used: ANS, 8-anilino-1-naphthalene sulfonic acid; ΔF_E and ΔH^+_E, O_2 dependent change in fluorescence and H^+ in mitochondria and SMP; SMP, submitochondrial preparation.

glass electrode, and ΔF_E as described by Packer, Donovan and Wrigglesworth.[6] Low angle fluorescence of ANS was measured from the front face of a 1 cm^2 quartz cuvette between 455 and 475 nm after excitation by incident light between 290 and 404 nm. The ethidium bromide fluorescence was also measured at 590 nm with excitation at 360 nm.[7] The reaction vessel consisted of a cuvette adjusted with a perforated plastic cover, i.e., a semi-closed system in which two of the three functions studied (fluorescence, proton transfer and respiration) could be assayed simultaneously. Protein was determined according to Lowry *et al.*[8]

Results and Discussion

Correlation Between ΔF_E and ΔH^+_E in Mitochondria

O_2 pulses given to mitochondria were generated under similar conditions to those employed by Mitchell and Moyle[9] except that tightly coupled fresh mitochondria were

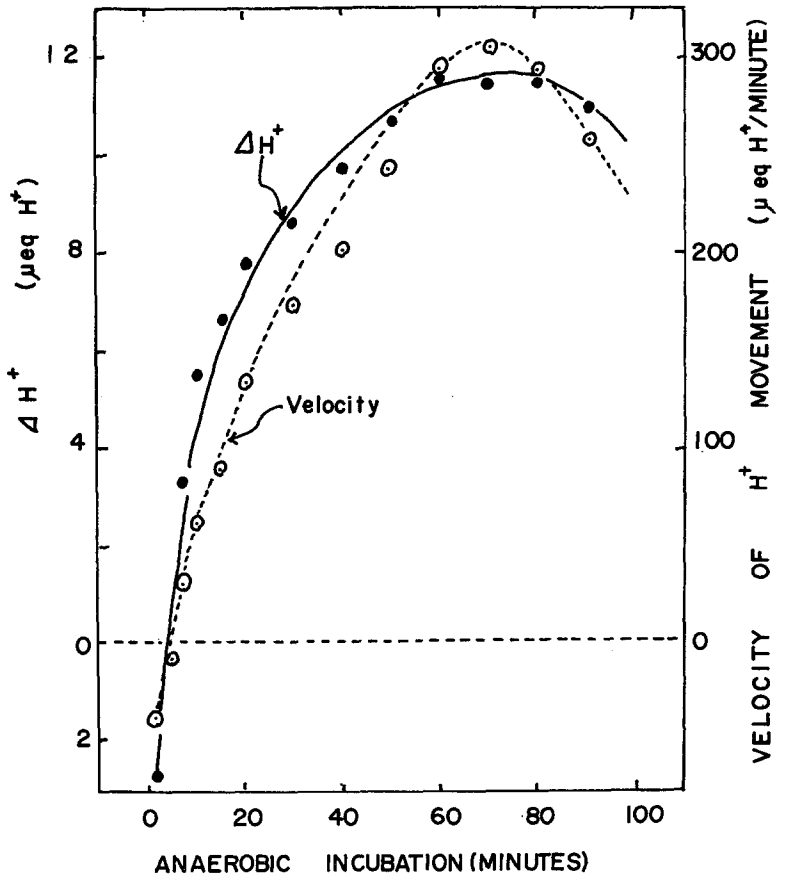

Figure 1. Effect of anaerobic preincubation on the respiration-dependent proton flux in mitochondria. Mitochondria (1·0 mg protein/ml) were preincubated at 25° in a choline chloride (150 mM) and Na succinate (3 mM) medium (pH 7·1) previously flushed with N_2 before the addition of mitochondria. After various anaerobic preincubation times an oxygen pulse of 6 mμatoms was applied and the ΔH^+_E recorded.

used instead of aged mitochondria. Mitochondria were added to a medium containing 0·15 M sucrose and 3 mM succinate which had been previously depleted of oxygen by bubbling nitrogen. After anaerobiosis, an oxygen pulse was given and the ensuing

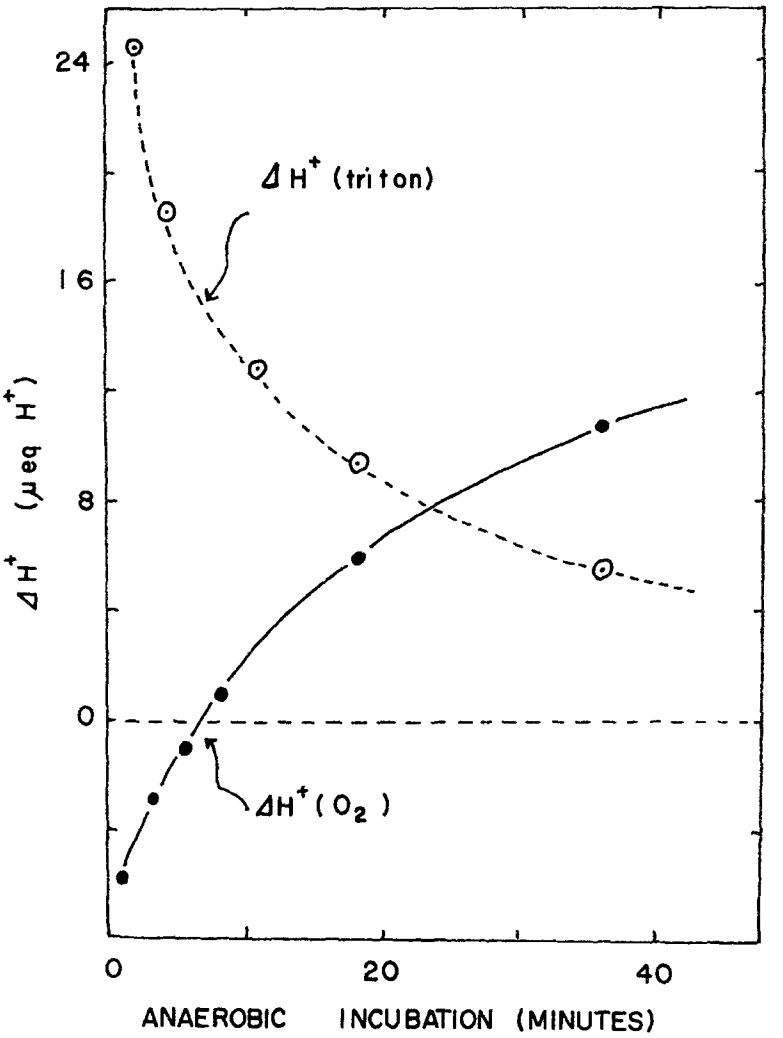

Figure 2. Relation between H^+ uptake elicited by Triton addition and H^+ production induced by an oxygen pulse in mitochondria. Conditions as in Fig. 1 with the exception that medium was 0·15 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 3 mM Na succinate and mitochondria (3·5 mg protein/ml). Oxygen pulse was 6 mμatoms/ml.

transient pH change recorded. The time interval between the onset of anaerobiosis and the oxygen pulse was varied as shown in Fig. 1. Both the extent of proton ejection and the initial velocity of the pH change vary with anaerobic preincubation time. In mitochondria without anaerobic preincubation the response to the oxygen pulse is a small H^+ uptake rather than H^+ ejection. When the anaerobic preincubation time is somewhat

lengthened, $\Delta H^+_E = 0$ and when the incubation time is further prolonged, progressive increases occur in the velocity of proton ejection and maximum ΔH^+_E until about 70 min of anaerobic preincubation has elapsed.

Packer and Utsumi[4] reported that when Triton is added at various time intervals (instead of an oxygen pulse) proton uptake occurs. Figure 2 demonstrates the magnitude of Triton-induced proton uptake as compared with oxygen-induced proton ejection, both shown as a function of anaerobic preincubation time. The two curves appear

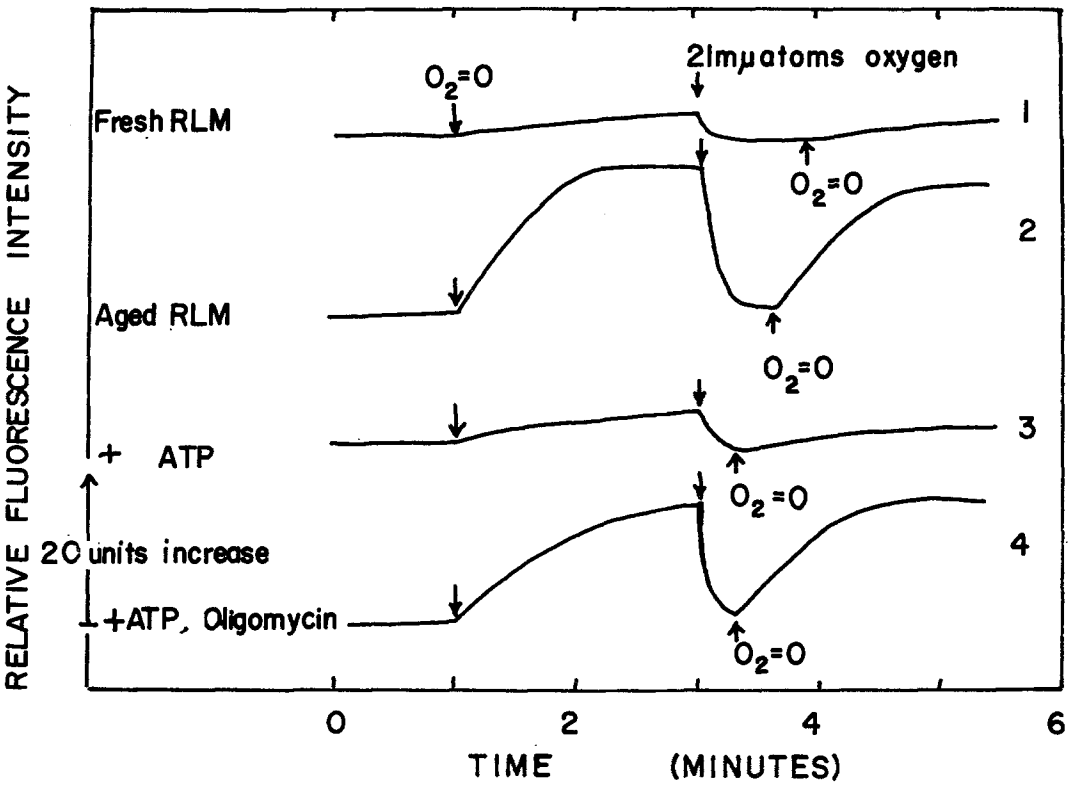

Figure 3. ΔF_E in fresh and "aged" mitochondria. Mitochondria (1·0 mg protein/ml) were "aged" for 30 min under anaerobic preincubation at 25°. Where indicated, 150 mM ATP and 0·4 μg oligomycin/ml were present.

complementary, namely, the greater the oxygen-induced proton ejection, the smaller the Triton-induced proton uptake.

These results can be explained by assuming that immediately after the onset of the anaerobic state the mitochondria remain energized by endogenous high-energy components. The small proton uptake induced by an oxygen pulse at this stage probably represents consumption of protons due to synthesis of ATP from endogenous ADP and inorganic phosphate as this activity is suppressed by oligomycin. The pre-existing proton gradient cannot be increased further by applying an oxygen pulse, but it can be abolished by Triton. With longer incubation time, the pre-existing proton gradient is lower and can be restored to its original level by applying an oxygen pulse.

ANS Fluorescence

Changes in the energy level of mitochondria were also followed using ANS fluorescence as the indicator (Fig. 3). The onset of anaerobiosis is not accompanied by fluorescence changes in fresh mitochondria. After a short time only a minute increase in fluorescence accompanies an oxygen pulse. However, in aged mitochondria, the onset of anaerobiosis is accompanied by large increases in fluorescence and after an oxygen pulse large de-

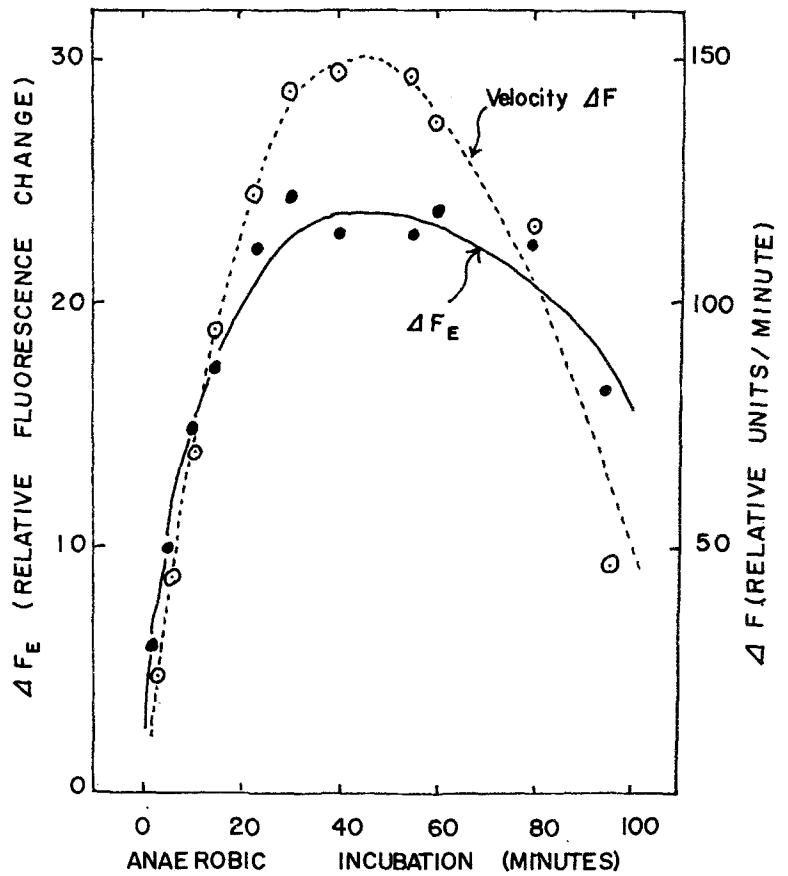

Figure 4. Effect of anaerobic preincubation upon ΔF_E of ANS in mitochondria. Conditions as in Fig. 1.

creases in fluorescence occur (curve 2, Fig. 3). It can also be seen (curves 3 and 4, Fig. 3) that ATP partially restores aged mitochondria to the coupled state, an effect which is oligomycin-sensitive. The extent and initial velocity of ANS fluorescence changes are plotted as a function of the time interval of anaerobic preincubation in Fig. 4. There is a striking similarity between these curves and those presented in Fig. 1 for proton flux.

Thus, the magnitude of the proton gradient as well as the ANS fluorescence apparently serve equally well for the characterization of the state of energization of mitochondria. The pH and fluorescence changes linked to the aerobic–anaerobic transition seem to serve as a semiquantitative measure for momentary energy level of the mitochondria.

However, a question can be raised whether the fluorescence changes directly measure changes in proton gradient. To answer this question a comparison was made of the

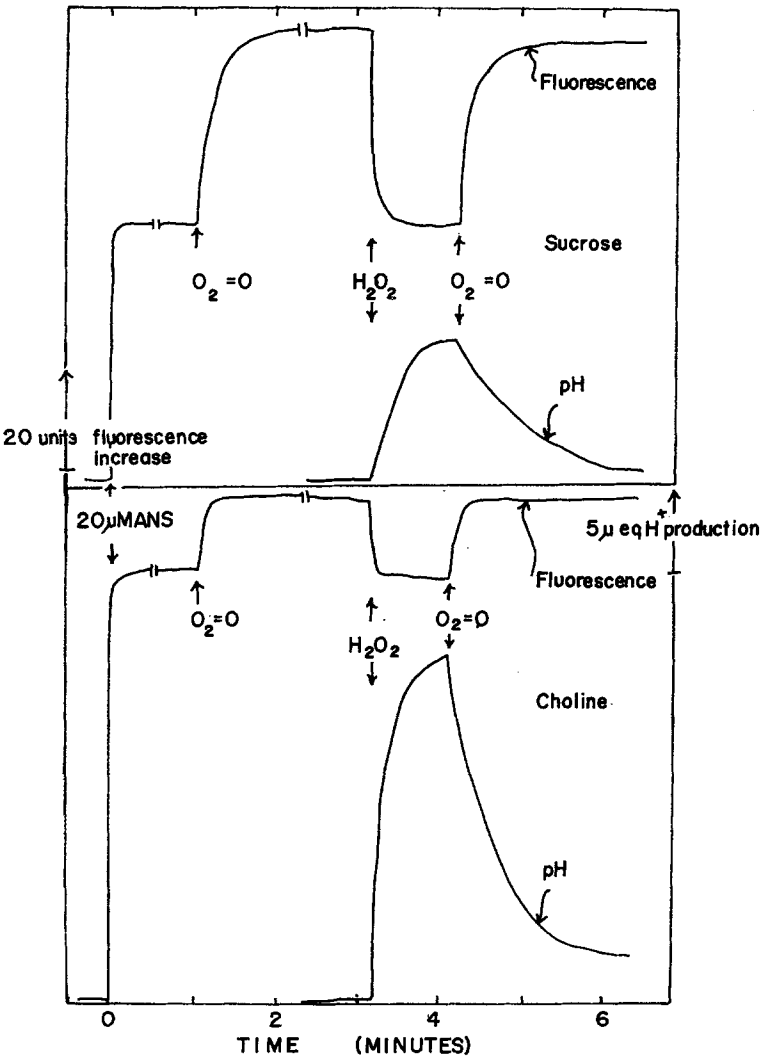

Figure 5. Comparison of ΔF_E and ΔH^+_E in mitochondria in sucrose and choline chloride media. Mitochondria (2 mg protein/ml) were aged for 30 min during anaerobic preincubation at 25° and were incubated in the medium of 0·15 M sucrose or choline chloride which contained 3 mM sodium succinate, 50 units of catalase, 20 μM ANS and 1 mM Tris-HCl. Oxygen pulse was made by addition of H_2O_2.

responses of mitochondria in low electrolyte (sucrose) and high electrolyte (choline chloride) media. Figure 5 shows that ΔH^+_E increases while ΔF_E decreases in the choline medium.

The responses of SMP were also investigated since they are depleted of endogenous energy sources and should therefore respond more abruptly to variations in the external

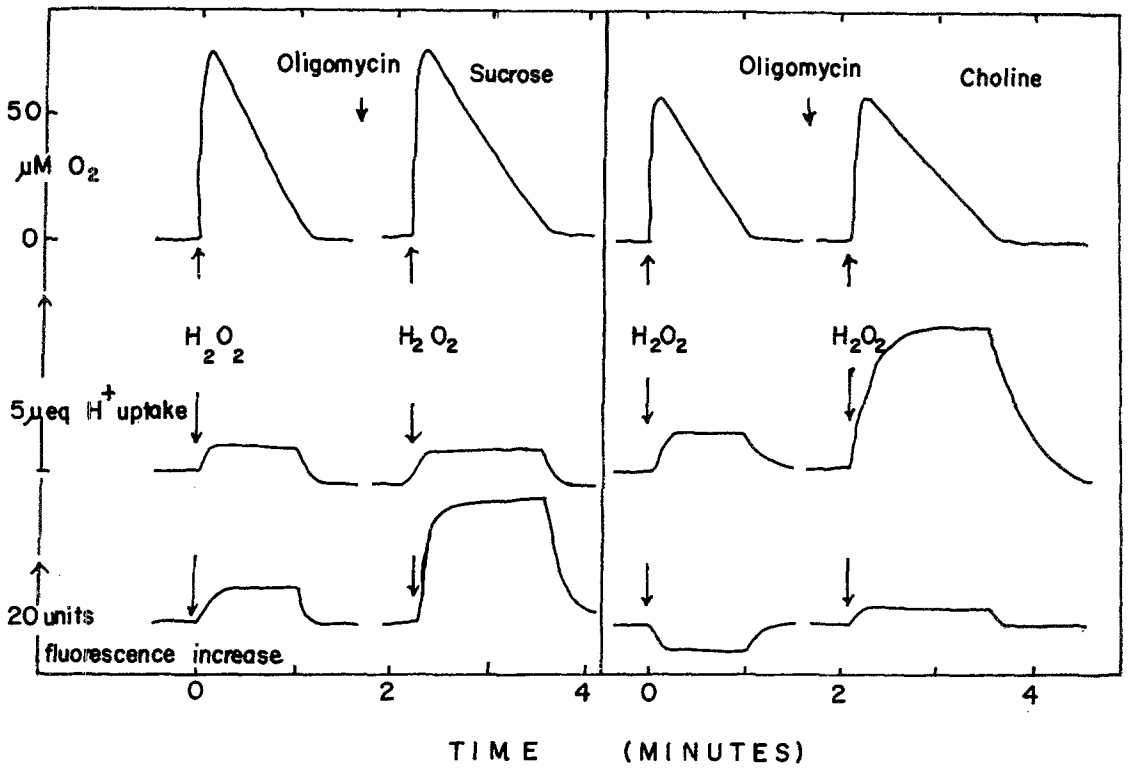

Figure 6. Interrelation between ΔF_E and ΔH^+_E in SMP in sucrose and choline chloride media. Conditions as in Fig. 5 except, instead of mitochondria, SMP (1 mg protein/ml) were used. Where indicated 0·2 μg/ml oligomycin was present.

TABLE I. pH dependence of respiration, ANS fluorescence and proton flux of submitochondrial preparation in sucrose and choline chloride media. Conditions were as in Fig. 6 except that no buffer was added

	Medium					
	Sucrose			Choline chloride		
pH:	6	7	8	6	7	8
Respiration (mμatom/mg/min)						
−Oligomycin	45	115	300	—	137	—
+Oligomycin	40	100	165	—	105	—
ANS (relative fluorescence change)						
−Oligomycin	−2·0	7·5	8·0	10	−7·0	−3·0
+Oligomycin	2·0	30·0	30·0	0	2·0	12·0
ΔpH (mμeq H^+/mg protein)						
−Oligomycin	−0·02	0·11	0·07	0	0·13	0·15
+Oligomycin	0·26	0·19	0·09	0·32	0·55	0·32

conditions. The experiment is given in Fig. 6. The influence of the ion environment is similar in SMP and in mitochondria. In sucrose ΔH^+_E is small even in the presence of oligomycin. In choline addition of oligomycin increases ΔH^+_E more than threefold.

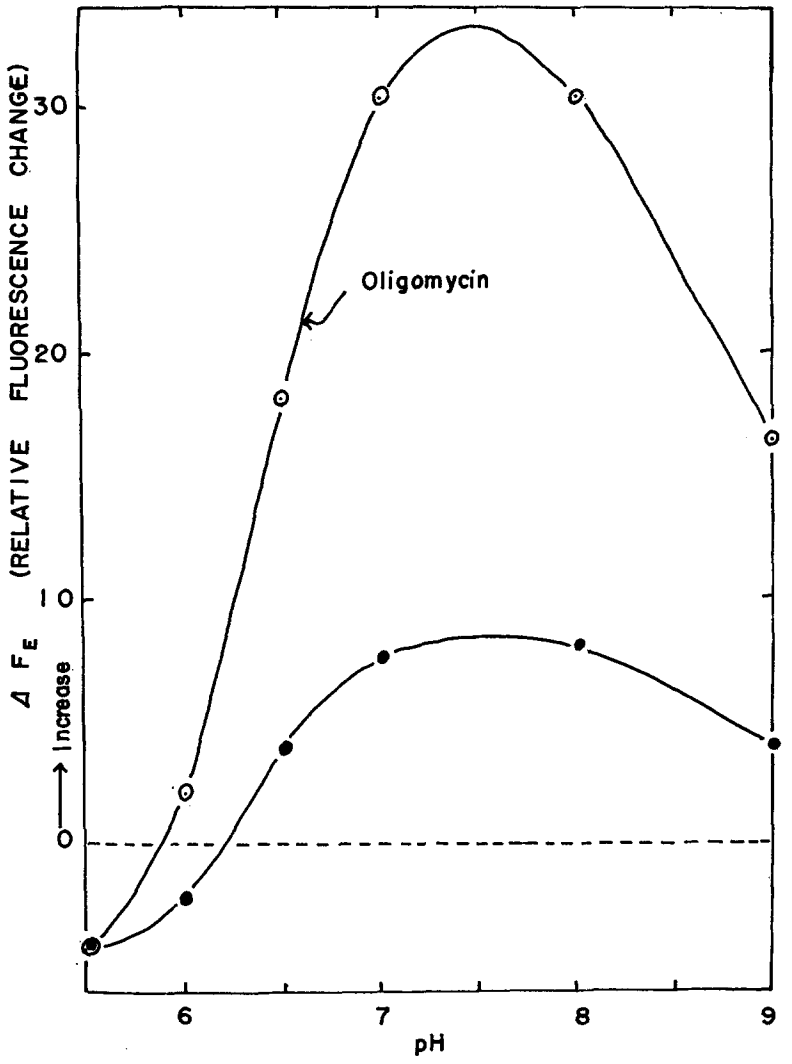

Figure 7. pH dependence of ΔF_E in SMP. Conditions as in Fig. 6 except that 10 mM Tris-Cl was also added. The assay mixture was adjusted to the required pH by NaOH or HCl after addition of SMP. No significant changes in pH were noted during experiments.

Fluorescence increases when oxygen is injected into the anaerobic system with sucrose–succinate medium but decreases with an oxygen pulse in the choline chloride–succinate medium. Oligomycin increases ΔF_E in sucrose and makes it positive in choline. It is known that at low concentrations oligomycin restores energy-linked functions in beef heart[10] and rat liver SMP[11] which may explain this effect. To further investigate the

interrelation between ΔF_E and ΔH^+_E in SMP, aerobic–anaerobic energy transitions were performed at various pH values (Table I). pH affects the aforementioned two parameters in the opposite manner, namely, low pH favors large proton fluxes and smaller fluorescence changes. At the pH values examined the fluorescence is enhanced in a sucrose medium and proton fluxes are enhanced in the choline chloride medium. Furthermore, under unfavourable conditions (low pH and choline chloride medium and

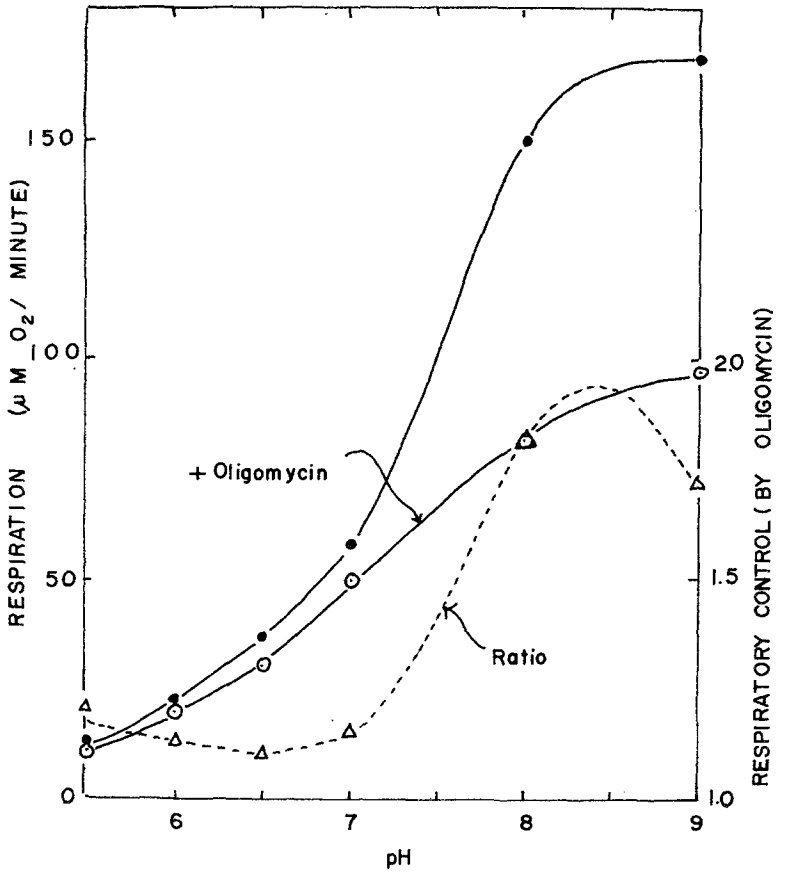

Figure 8. Influence of oligomycin upon the pH dependence of succinate oxidation. Conditions as in Fig. 6.

a lack of oligomycin) there is a tendency for a reversal of the direction of ΔF_E. Figures 7 and 8 reveal that the extent of fluorescence changes qualitatively appear related to the respiratory rate and oligomycin sensitivity of the respiration of SMP (cf. ref. 5). Small ΔF_E or reversal of the sign of this change corresponds to slow respiratory rate and/or oligomycin insensitivity.

Thus, both in mitochondria and SMP changing from a sucrose medium to a choline chloride medium increases ΔH^+_E but decreases ΔF_E. According to the chemiosmotic hypothesis

$$\text{PMF} = \Delta\Psi - \zeta\Delta\text{pH}$$

since PMF is constant, an increase in the ΔpH leads to a decrease in $\Delta\Psi$, indicating that ΔF_E could measure changes in membrane potential. However, alternatively one could

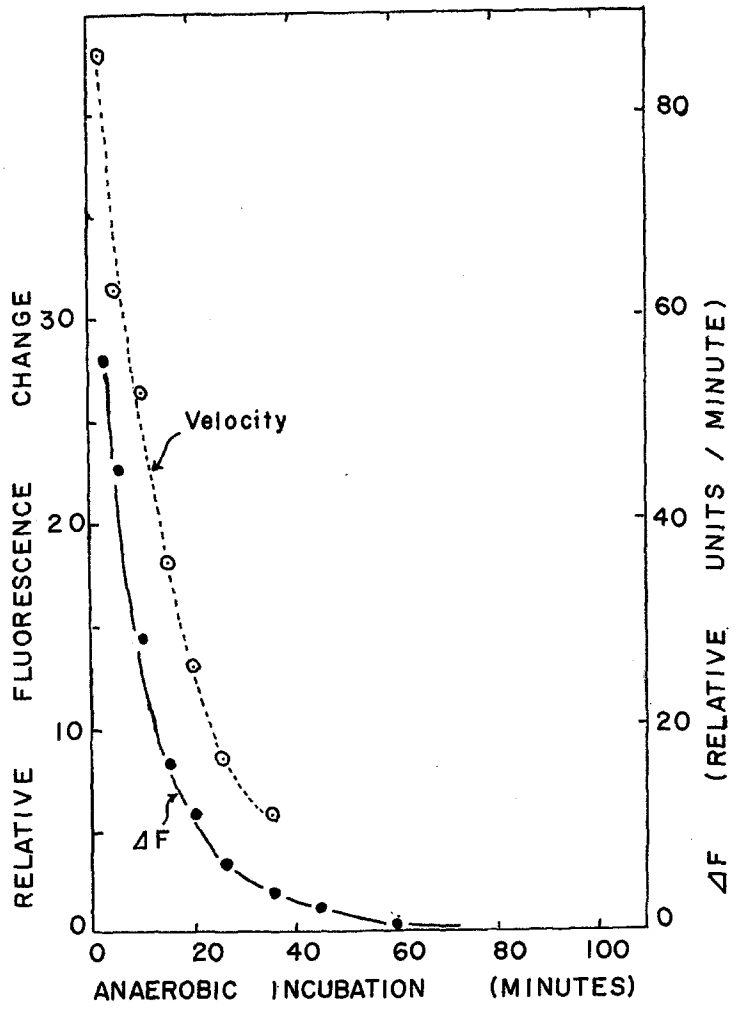

Figure 9. Effect of anaerobic preincubation on ethidium bromide fluorescence in mitochondria. Incubation conditions as in Fig. 1 with the exception that 10 mM ethidium bromide and 50 units catalase were added. Oxygen pulse was made by addition of H_2O_2.

also visualize, in accord with the chemical theory of energy conservation, that ΔF_E indicates change in the level of high-energy intermediates (states)

$$\Delta F_E \sim \Delta[\sim].$$

Choline chloride, by stimulating an energy-dependent proton pump, dissipates some of the $\sim$, and as a corollary also lowers ΔF_E. To choose between these two alternatives

studies using fluorescence probes like ethidium bromide which preferentially respond to changes in charge distribution or probes, such as light-scattering, which reflect changes in conformation are required.

Ethidium Bromide Fluorescence

Ethidium bromide fluorescence has been reported[7] to be a sensitive indicator of changes in charge distribution. Figure 9 summarizes oxygen pulse experiments made as a function of anaerobic incubation time in mitochondrial suspensions. The fluorescence intensity was decreased by an oxygen pulse. Contrary to the findings made with ANS fluorescence, proton flux, and light-scattering responses, the response of this probe becomes inhibited as the anaerobic incubation time is increased. This suggests that charge distribution changes accompanying oxygen pulse experiments diminish under conditions that enhance proton permeability and ANS fluorescence changes. Similar experiments have been performed using ethidium bromide in SMP but no change in fluorescence intensity in response to oxygen pulses was observed either in sucrose or in choline chloride medium in the pH range 6–8. Although it has been claimed that ANS fluorescence changes reflect changes in charge distribution accompanying energization of the membrane, the experiments with ethidium bromide do not appear to substantiate this suggestion. Ethidium bromide responses were found to be absent in SMP and to diminish in mitochondria under conditions which favor an increase in ANS fluorescence and proton fluxes.

Asymmetry vs. Population of SMP

Azzi[3] has related the difference in direction of the energy-dependent fluorescence changes in mitochondria and in SMP to the opposite structural polarity of the membrane. If this assumption is applied to our data on SMP it would mean that pH changes or the composition of the medium can change the symmetry of the membranes. Alternatively, the SMP population could be a mixture of vesicles, one with a membrane polarity opposite to that of intact mitochondria and one with a membrane polarity similar to that of mitochondria. If we assume that only the former type of population can participate in energy transformation and can interact with oligomycin it will be apparent that the mitochondrial type of fluorescence response will be observed only under conditions which are unfavourable for coupling. The population distribution of SMP types is now under intensive investigation by techniques of freeze-cleavage and -etching electron microscopy.

Acknowledgement

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