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Tracking of proton flow during transition from anaerobiosis to steady state in rat liver mitochondria

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(1) The hydrophobic pH indicator Bromthymol blue and the hydrophilic pH indicator Phenol red have been used to follow the redox-pump-linked proton flows during transition from anaerobiosis to static head. The domains monitored by the pH indicators, whether external or internal, and the localization of the dye, whether free or membrane bound, have been identified by recording the absorbance changes following addition of nigericin or valinomycin to anaerobic or aerobic mitochondria and the effects of permeant and impermeant buffers. (2) After addition of the H^+/K^+ exchanger, nigericin, to anaerobic mitochondria, Phenol red and Bromthymol blue record an alkalinization and an acidification, respectively, indicating that while the hydrophilic pH indicator faces an external domain, the hydrophobic pH indicator faces, at least partly, an internal domain. The latter effect is sensitive to phosphate and to phosphate carrier inhibitors. On the other hand, addition of nigericin to aerobic mitochondria leads to an increased Bromthymol blue absorbance, which reflects an alkalinization, indicating that the pH indicator faces an external domain. The reorientation of the dye from the internal to the external domain is a function of the uncoupler concentration and thus of the membrane potential (cf. Mitchell et al. (1968) Eur. J. Biochem. 4, 9–19). (3) The amount of oxygen required for the transition from anaerobiosis to static head has been determined by following in parallel the extent of oxidation of cytochrome *aa*$_3$ and the rise of $\Delta\tilde{\mu}_{H^+}$. With succinate as substrate, 50% levels of cytochrome oxidation are obtained at 0.125 ngatom oxygen/mg and 50% of Safranine response at about 0.2 ngatom oxygen/mg. These amounts of oxygen correspond to an H^+ displacement of about 0.8–1.2 ngatom/mg on the basis of the H^+/O stoichiometry. It is concluded that mitochondria are in presteady state below, and in static head above, displacement of 2–3 ngatom H^+/mg. This figure is very close to the original calculation of Mitchell (Mitchell, P. (1966) Biol. Rev. 41, 445–502). (4) Transition, by oxygen pulses, of EGTA-supplemented mitochondria from anaerobiosis to either presteady state or static head state results in a response of the hydrophilic pH indicator, Phenol red, which is negligible in amount and/or kinetically unrelated to the $\Delta\tilde{\mu}_{H^+}$ rise. The fact that H^+ extrusion in the bulk aqueous phase is negligible also in presteady state excludes proton cycling as an explanation. Addition of oxygen pulses to Sr^{2+}-supplemented anaerobic mitochondria results in an H^+ extrusion whose amount and rate is proportional to the Sr^{2+} concentration. The increase in rate and amount of the H^+ extrusion is accompanied by a proportional depression of $\Delta\tilde{\mu}_{H^+}$. (5) Transition, by oxygen pulses, of EGTA- and Mg^{2+}-supplemented mitochondria from anaerobiosis to static head state results in a decreased absorbance of Bromthymol blue. The decrease in absorbance is insensitive to impermeant buffers but partially sensitive to phosphate and the effect of phosphate is abolished by *N*-ethylmaleimide. The decrease in absorbance is proportional to the amount

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Abbreviations: J_e, rate of electron transfer; J_H^p, rate of proton pumping; J_H^l, proton flux through leaks; L_H^l, proton membrane conductance; n_e, H^+/e^- stoichiometry; $\Delta\tilde{\mu}_{H^+}$, transmembrane proton electrochemical potential gradient; $\Delta\psi$ transmembrane electrical potential gradient; EGTA, ethylenebis[oxyethylenenitrilo]tetraacetic acid; NEM, *N*-ethylmaleimide; Mops, 3-(*N*-morpholino)propanesulfonic acid; $TPMP^+$ triphenylmethylphosphonium ion; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; P_i, inorganic phosphate; ATP, adenosine 5′-triphosphate; ATPase, adenosine triphosphatase.

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of oxygen in the range between zero and 0.6 ngatom O/mg and fully sensitive to the addition of protonophores. (6) It appears that hydrophilic and hydrophobic pH indicators may be used to record pH changes occurring at the two sides of the hydrophobic barrier and at the membrane level. It is further proposed that the redox pump-linked proton extrusion takes place at the membrane level.

Introduction

In energy-transducing systems such as mitochondria, bacteria and chloroplasts, the energy transduction is generally accepted to depend on the operation of the redox and ATPase proton pumps, that is the transfer of electrons or hydrolysis of ATP[1] is obligatorily coupled to vectorial transfer of H^+ ions [1]. This view may be formalized as a thermodynamically reversible system of redox-driven, light-driven and ATP-driven H^+ pumps embedded with similar orientation in the same membrane and coupled together by the proton electrochemical gradient $\Delta\tilde{\mu}_{H^+}$ [2,3].

Mitchell [1] has calculated that the osmotically resistant membrane of rat liver mitochondria has an electrical capacity of 1 $\mu F/cm^2$. Hence to charge the coupling membrane at a potential of 200 mV, 12 500 electronic charges should be displaced per μm^2 or about 2 pequiv./cm^2. Assuming that the coupling membrane can be identified with the cristae membrane and that the area of this cristae membrane is about 40 m^2/g protein, it follows that the charge displacement required for a membrane potential of some 200 mV during oxidative phosphorylation would correspond to about 0.8 μequiv./g protein. Mitchell's calculations have important implications both with respect to the electrical properties of the membrane and to the mechanism of charge displacement leading to formation of the transmembrane potential. However, only the latter part of the calculation has been the object of experimental testing, namely that concerning the extent of H^+ ejection.

Several reports have appeared [4,5] claiming a negligible H^+ ejection in liver mitochondria when care is taken to remove Ca^{2+} with chelating agents [6,7] or ion-exchange resins [8]. In *Paracoccus denitrificans* Scholes and Mitchell [9] reported a large H^+ ejection only in the presence of the permeant anion SCN^-. The lack of significant H^+ ejection in the absence of permeant ion movement has been attributed to the development of a delocalized membrane potential which inhibits subsequent H^+ ejection and causes back-flow of H^+ into the inner space. However, Gould and Cramer [10,11] have shown that in a series of repeated oxygen pulses given to *Escherichia coli*, the H^+/O ratio in the absence of SCN^- is independent of the size of the pulse. In *P. denitrificans*, Hitchens and Kell [12,13] have further shown, by use of a fast-responding oxygen electrode, that a significant kinetic discrepancy exists between oxygen reduction and H^+ translocation. The discrepancy disappears upon addition of SCN^-, or by replacing intact cells with osmotically active protoplasts of *P. denitrificans*.

By using the lipophilic pH indicator neutral red, Junge and collaborators [14–16] observed absorption changes which were interpreted as due partly to alkalinization of the outer medium and quenched by bovine serum albumin, and partly to acidification as occurring in the thylakoid lumen. Recently, Taylor and Jackson [17,18], using the photosynthetic bacterium, *Rhodopseudomonas capsulata*, have observed H^+ extrusion upon initiation of photo-induced electron transfer. The H^+ release showed a complex relationship with the duration of illumination, but a small fast component of the H^+ extrusion was reported to be closely correlated with $\Delta\tilde{\mu}_{H^+}$ as determined by the carotenoid band shift followed at 503 nm.

We have reported that addition of oxygen pulses to anaerobic rat liver mitochondria results in negligible H^+ extrusion in the absence of permeant ions [19,20]. The question, however, arises as to whether the extrusion of these negligible amounts of H^+ ions might be sufficient to achieve a static head state where the net H^+ efflux becomes equal to zero. The answer to this question can be provided by determining the extent to which the oxygen pulses bring the system in static head state. The assessment of the thermodynamic state of the mitochondria during oxygen pulses furthermore allows us to test Mitchell's calculation as to the relationship between charge displacement and electrical properties of the coupling membrane.

Antonini et al. [21] reported that Bromthymol blue binds to hemoglobin and that the binding alters the oxygen equilibrium. Chance and Mela [22–25] reported that while uptake of Ca^{2+} is accompanied by H^+ release in the medium, the opposite result is detected by the pH indicator Bromthymol blue, this indicating that the dye records matrix rather than aqueous pH. Chance and Mela [22–25] then suggested that Bromthymol blue can be used to indicate the pH inside of the cristae membrane of intact respiring mitochondria because it is tightly bound at a site on the inner side of the coupling membrane. Similar results were also obtained with submitochondrial particles, bacterial chromatophores and chloroplasts grana. Mitchell et al. [26], however, showed that: (a) Bromthymol blue measures simultaneously both the external and internal pH of mitochondria; and (b) that the amount of dye measuring the internal pH is not constant in the various metabolic states. In 1968, Azzone et al. [27] reported changes of Bromthymol blue

absorbance in mitochondria which were independent of external pH changes and linked to the functional state of the mitochondria. No correlation was, however, presented between the changes of absorbance of the dye and its membrane localization.

In the present study, we have analyzed the transition from anaerobiosis to static head by following the redox state of cytochrome aa_3 and the response of the $\Delta\psi$ probe, Safranine, after addition of increasing pulses of oxygen. This approach indicates that the charge displacement required to charge the coupling membrane to a membrane potential of, say, 200 mV is about 3 ngatom H^+/mg a figure which is only slightly higher than that originally calculated by Mitchell. Furthermore, we have utilized hydrophilic and hydrophobic pH indicators to track the proton flow during transition from anaerobiosis to static head. While Phenol red is almost exclusively dissolved in water, Bromthymol blue is both dissolved in water and bound to the membrane, BTB_w and BTB_m, and monitors the pH of both external and internal domains BTB_e and BTB_i. We will then show that addition of O_2 pulses to anaerobic mitochondria results in a negligible response of the hydrophilic pH indicator Phenol red and in significant decrease of absorbance of the hydrophobic pH indicator Bromthymol blue. This latter dye response may be interpreted as an increased acidification of an external domain which possesses the properties of a membrane phase.

Materials and Methods

Rat liver mitochondria were prepared in 0.25 M sucrose, 10 mM Tris (pH 7.4), 0.1 mM EGTA, according to standard procedures [28], and all experiments were performed within 4 h of preparation. In some preparations, the last washing was carried out in an EGTA-free medium and the final resuspension was made in bovine serum albumin supplemented medium. Mitochondrial protein was assayed with the biuret method using serum albumin as a standard. The composition of standard incubation medium was: 0.28 M sucrose, 5 mM succinate, 0.5 mM Mops/Tris, 0.5 mM EGTA, 20 μM NEM, 5 μM rotenone, 1 μg/mg oligomycin and catalase, at 25 °C. The pH of the incubation medium was adjusted to value 7.4 by adding HCl or NaOH. In some measurements, EGTA was omitted. In the experiments with Bromthymol blue, in the presence of variable permeant and/or impermeant buffers, the sucrose concentration was varied to obtain an osmolarity of the media corresponding to 300 mosM.

Spectrophotometric studies were performed in temperature-controlled, stirred cuvette using an AMINCO DW2a dual wavelength spectrophotometer. Safranine was used as indicator of membrane potential [29] and Phenol red or Bromthymol blue as pH indicator [19]. The change in absorbance of Safranine and of Phenol red were followed at 520 minus 554 nm, and at 576 minus 620, respectively. Response of Phenol red was calibrated by using a standard solution of HCl (0.01 and 0.001 M). Reduction of cytochrome aa_3 was monitored at 605 minus 625 nm. The absorbance change of Bromthymol blue (30 μM) was followed spectrophotometrically at 618 minus 700 nm. Bromthymol blue has a sulfonic side chain and acquires a negative charge on the chromophoric group upon ionization. The ionization of the chromophoric group has a marked effect on the binding of the dye to mitochondria or submitochondrial particles in that the protonated dye is strongly bound, while the nonprotonated dye is very weakly bound. The hydrophobic interaction of the dye with the membrane is accompanied by a large pK shift of the dye. In practice, while the pK of the free dye is 7.1, the pK of the dye in the presence of mitochondrial membranes is between 8.1 and 8.3 [30]. The molar extinction coefficient of Bromthymol blue is also markedly dependent on the dielectric constant of the medium decreasing from 14700 in water to 800 in 50% ethanol where the dielectric constant is 52. The spectral analysis indicated that the change of absorbance following the addition of oxygen to anaerobic mitochondria was due to an acid-base transition.

For studies on anaerobic–aerobic transitions, the medium was maintained at the desired temperature under bubbling nitrogen prior to use. The cuvettes were equipped with fitted Plexiglass stoppers with a shaft sufficient to reduce the reaction volume to 3 ml and a 1–2 mm diameter opening to accept a syringe needle. After addition of the reaction medium (final volume 3 ml) and mitochondria (3 mg/ml), the stoppered cuvette was incubated with stirring at the desired temperature until complete anaerobiosis, as determined by continuously following the oxygen consumption in parallel samples. 10 min was generally sufficient to obtain anaerobiosis and stabilization of the dye uptake. Aerobic transitions were initiated by the injection of calibrated hydrogen peroxide solutions to the cuvette. Hydrogen peroxide solutions were calibrated by measurements of oxygen released in presence of catalase. Oxygen concentration was measured polarographically with a Clark electrode (Yellow Spring) equipped with a Teflon membrane in a closed thermostatted and stirred vessel using air-saturated medium as standard. The transmembrane electrical potential, $\Delta\psi$, was evaluated from distribution of the lipophilic ion triphenylmethylphosphonium ($TPMP^+$) essentially as described in Luvisetto et al. [31] (cf. also Ref. 32).

Safranine was a product of BDH Chemicals, purified according to standard methods [33]. Phenol red and Bromthymol blue were purchased from Merck. All other reagents were of maximal purity commercial grade.

Results

The domains where the pH changes take place may be defined as external or internal according to whether the response of the pH indicator is in the same or in the opposite direction with respect to that of a pH glass electrode. The two forms of Bromthymol blue facing the internal and the external domains, at the two sides of the hydrophobic barrier, may be denoted as BTB_i and BTB_e. Furthermore, the hydrophobic nature of Bromthymol blue introduces an additional operational distinction according to whether the dye is sensitive to buffers dissolved in water, BTB_{we} and BTB_{wi}, or insensitive to buffers and presumably membrane bound, BTB_{me} and BTB_{mi}.

The localization of Phenol red and Bromthymol blue

When nigericin is added to respiratory inhibited mitochondria, which have a K^+ content of 70–100 nmol/mg^{-1} and are incubated in a K^+-free medium, the antibiotic catalyzes a large efflux of K^+ from the matrix and an equivalent H^+ uptake [32]. Fig. 1C shows that addition of nigericin resulted in a large increase of absorbance of Phenol red (alkalinization) which was sensitive to the concentration of impermeant buffers. In contrast, addition of nigericin resulted in a large decrease in Bromthymol blue response (acidification) when the mitochondria were incubated in the presence, and in an increase of Bromthymol blue response (alkalinization) when incubated in the absence, of 10 mM $MgCl_2$ (Fig. 1A). Since, under the conditions of Fig. 1, the responses of the glass pH electrode and of Phenol red are that of an alkalinization [32], the experiment suggests that in the presence, but not in the absence of Mg^{2+}, the response of Bromthymol blue reflects the pH of an internal domain. This means that (a) the dye behaves as a hydrophobic weak acid diffusing across the membrane and attaining presumably a ΔpH determined distribution and (b) due to an asymmetric distribution the dye may become an indicator of the internal pH of the vesicular structure. Fig. 1A also shows that the extent of decrease of Bromthymol blue response increases with the increase of the impermeant buffer concentration. An increased concentration of impermeant buffers does not alter the number of protons transferred in the matrix in exchange with K^+, but only reduces the extent of alkalinization of the bulk aqueous phase. Hence, the larger decrease of Bromthymol blue absorbance in the presence of impermeant buffers reflects the abolition of the pH changes recorded by the fraction of Bromthymol blue dissolved in the external bulk phase. Fig. 1B also shows the effect

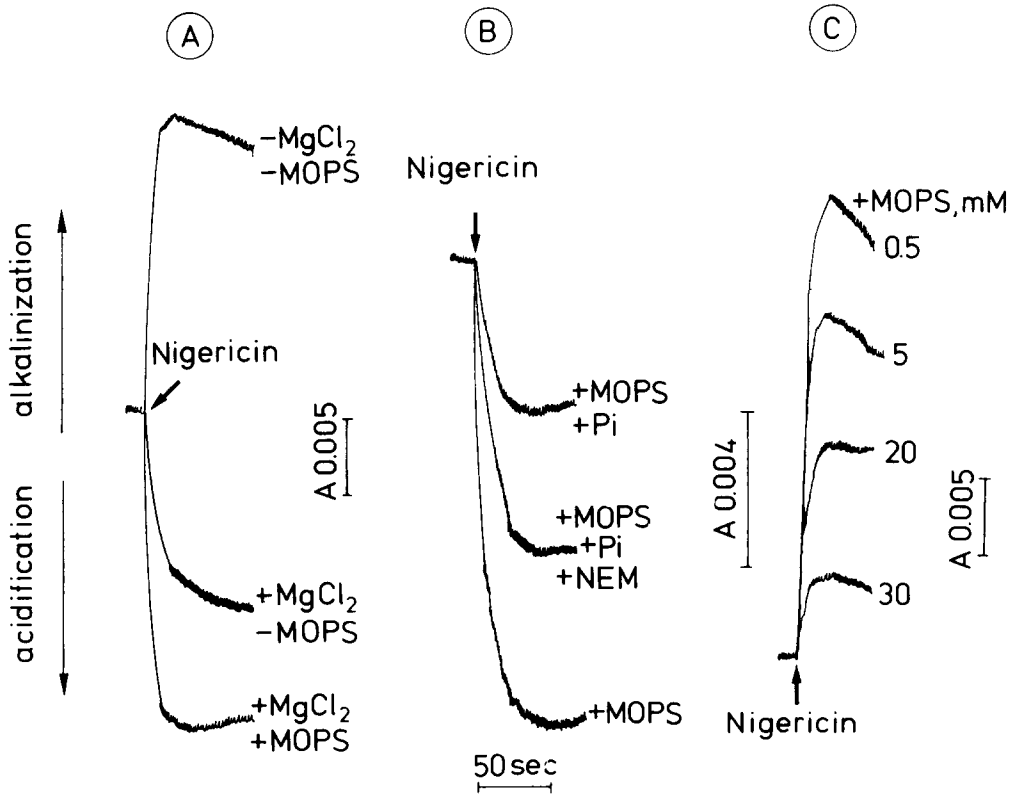

Fig. 1. Responses of Bromthymol blue and Phenol red after addition of nigericin to respiratory inhibited mitochondria. Left (A): response of Bromthymol blue in the presence and absence of $MgCl_2$ (10 mM), and in presence of impermeant buffer Mops (30 mM). Middle (B): response of Bromthymol blue in the presence of $MgCl_2$ (10 mM) and Mops (30 mM), in the presence of *N*-ethylmaleimide (100 μM) and/or P_i (20 mM). Right (C): effect of impermeant buffer on the the Phenol red response. 3 mg/ml RLM were incubated in the presence of Bromthymol blue (30 μM) or Phenol red (60 μM) for 10 min.; 50 ng/mg antimycin was then added; followed, after 10 s, by 27 pmol/mg nigericin.

of P_i on the Bromthymol blue response. The extent of absorbance change decreased with the addition of phosphate and the effect was abolished in the presence of the phosphate carrier inhibitor *N*-ethylmaleimide. The inhibitory effect of phosphate on the nigericin-induced dye response was proportional to the phosphate concentration (not shown). The experiment thus indicates that dye response is decreased in the presence of weak acids in which protons are co-transported into the matrix.

The question arises as to whether the dye responses recorded in Fig. 1 do not change when an electrical field develops across the inner membrane. The experiment of Fig. 2A shows the dye response after addition of nigericin to respiring mitochondria. It is seen that addition of nigericin to aerobic mitochondria resulted in an alkalinization, in contrast with the acidification observed in Fig. 1B. The experiment thus shows that a reorientation of the dye under the electrical field had taken place and that the dye presumably monitors a more external domain in respiring with respect to the nonrespiring mitochondria, i.e., BTB_i decreases and BTB_e increases. The term reorientation is used here solely to indicate that what is of interest here is the functional polarity of the dye, cf. Mitchell et al. [26].

Fig. 2A also shows the effect of the impermeant buffer on the nigericin-induced response. First, the extent of dye response was practically unchanged when the buffering power of the medium was increased. Second, while in a highly buffered medium the alkaline response of the dye was monotonic, in the Mops-free medium the dye indicated first an alkalinization and then an acidification. The pattern of the dye response in the Mops-free medium is very similar to that recorded by the pH glass electrode [32] and partially reflects the pH changes in the bulk aqueous phase following addition of nigericin. In fact, the acidification of the matrix due to the H^+/K^+ exchange is followed by the redox pump-linked proton extrusion which leads to acidification of the outer phase. In conclusion, the experiment indicates that in the highly buffered medium the dye response is mainly provided by a fraction of membrane-bound indicator, BTB_{me}, whose response is independent of the buffer power of the medium. Finally, Fig. 2A shows that in respiring mitochondria the nigericin-induced alkaline dye response was insensitive to the addition of phosphate. The insensitivity to phosphate of the nigericin-induced increase of dye response, in aerobic mitochondria, contrasts with the sensitivity to phosphate of the decrease of dye response in anaerobic mitochondria

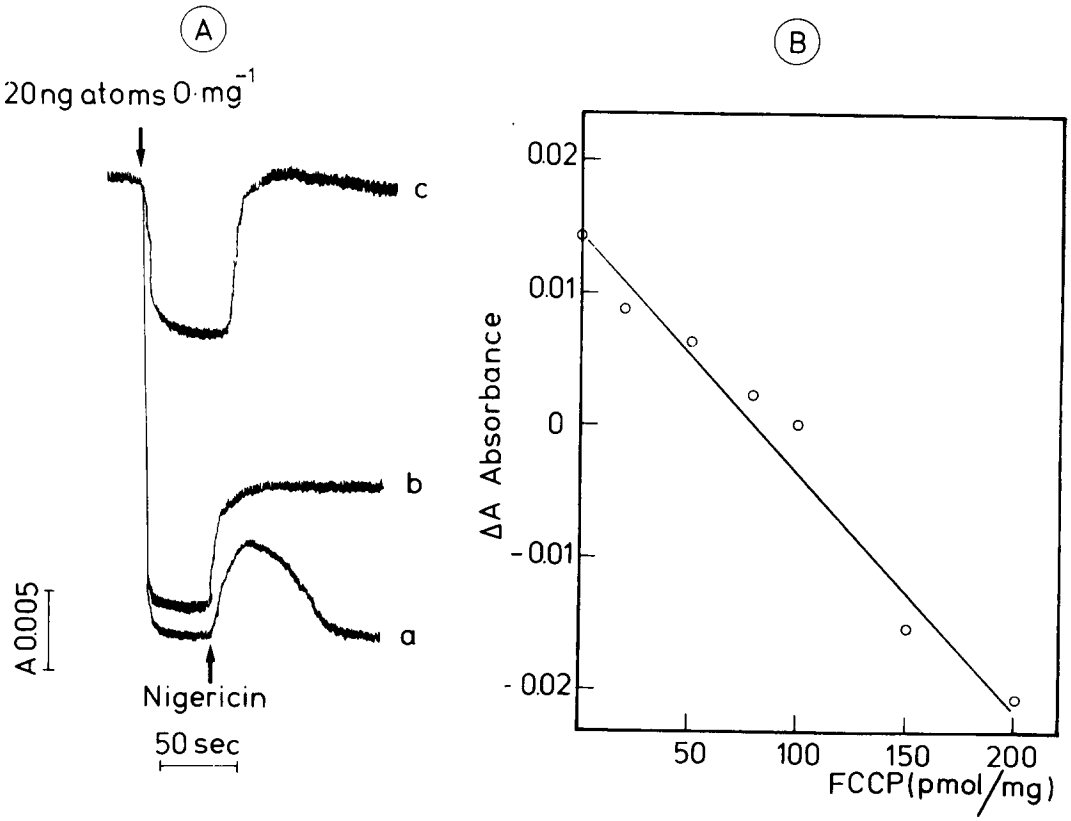

Fig. 2. Nigericin-induced Bromthymol blue response in respiring mitochondria. Effect of P_i and FCCP. Left (A): Curve (a), unbuffered medium; curve (b), presence of 30 mM Mops; curve (c) presence of 30 mM Mops and 20 mM P_i. Experimental conditions as in Fig. 1 except that antimycin was omitted and reaction was initiated by the addition of excess of oxygen. Right (B): Absorbance change of Bromthymol blue after nigericin pulse in respiring mitochondria as a function of increasing concentration of FCCP. Respiring mitochondria were first treated with increasing concentrations of FCCP and then pulsed with nigericin.

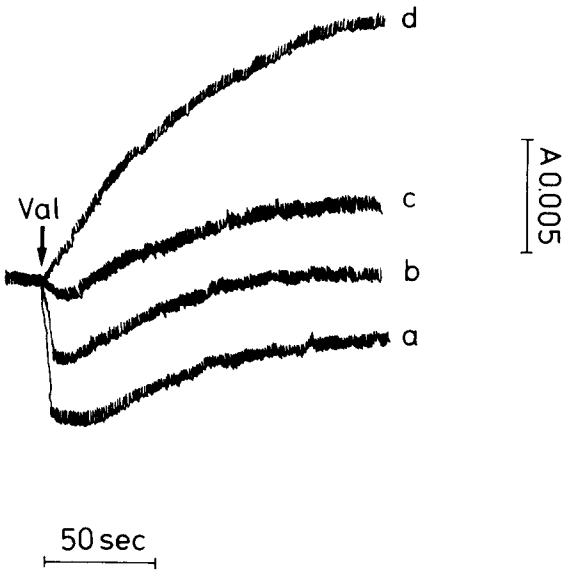

Fig. 3. Effect of P_i on valinomycin-induced Bromthymol blue response. Curve (a), 1 mM P_i; curve (b), 10 mM P_i; curve (c), 20 mM P_i; curve (d) without P_i; curves (a), (b) and (c) with 10 mM Mg^{2+}. Experimental conditions as in Fig. 1. Mitochondria were supplemented with antimycin and then treated with valinomycin (150 ng/mg). 30 mM Mops.

and further supports a more external orientation of the dye under aerobic conditions. The transition of the nigericin-induced dye response from increase (alkalinization) to decrease (acidification) was a function of the increase of concentration of a classical protonophore like FCCP, Fig. 2B. Since the major effect of FCCP is that of causing a collapse of the transmembrane potential, the experiment supports the view that the reorientation of the dye from being an indicator facing the internal domain to being an indicator facing the external domain is a function of the electrical field [26].

Addition of valinomycin to respiratory inhibited mitochondria incubated in a K^+-free medium, initiates a K^+ diffusion down the K^+ electrochemical gradient and generates a transmembrane potential which can be used to assess the electrophoretic response of Bromthymol blue without redox pump catalyzed proton movement. Fig. 3 shows that addition of valinomycin to anaerobic mitochondria caused a biphasic response of Bromthymol blue, first a rapid decrease and then a slow increase of absorbance. The biphasic response presumably reflects two phenomena, both driven by the K^+ diffusion potential, first the influx of protons in the

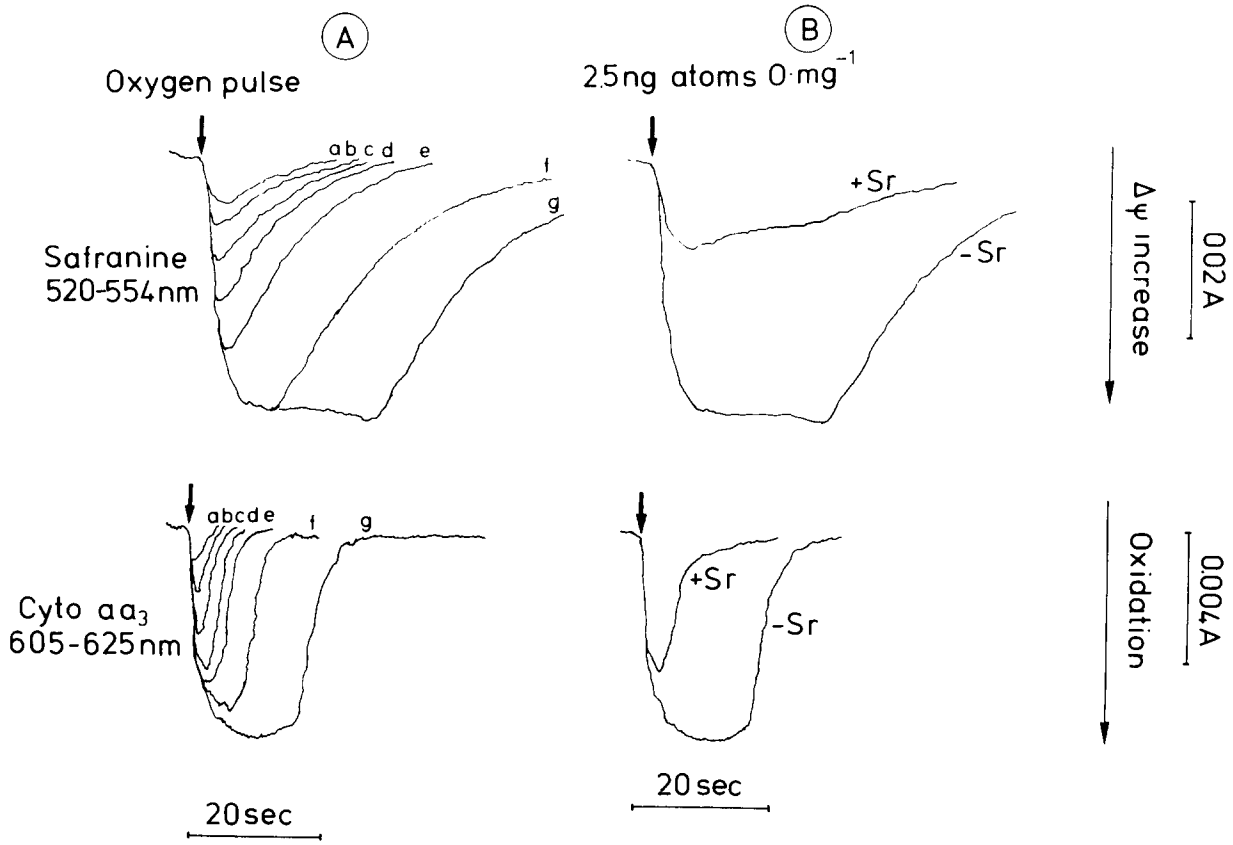

Fig. 4. Kinetics of Safranine (upper) and cytochrome aa_3 (lower) responses as a function of the oxygen concentration in the absence (A) or in the presence of $SrCl_2$ (B). Mitochondria (3 mg/ml) were incubated for 10 min under anaerobic conditions in the presence of 20 μM Safranine. In A, the reaction was initiated by the addition of different aliquots of calibrated H_2O_2 solutions corresponding to: 0.025 (a), 0.050 (b), 0.125 (c), 0.25 (d), 0.5 (e), 1.25 (f), 2.50 (g) ngatom O/mg. In parallel samples, the reduction of cytochrome aa_3 was recorded in the absence of Safranine. In (B), experimental conditions were essentially as in (A), except for the presence of $SrCl_2$ (100 μM), where indicated; 2.5 ngatom O/mg. In B, EGTA was omitted.

matrix via the proton leaks and second the extrusion of the dye. In fact, the translocation of Bromthymol blue from the membrane to the aqueous phase results in an increase of absorbance, since the absorbance of the dye increases as a function of the increase of the dielectric constant of the medium. This interpretation is further supported by the effect of the phosphate buffer which had no effect on the slow increase of dye response, while it abolished almost completely the rapid phase of decreased dye response. Fig. 3 also shows that when valinomycin was added to mitochondria incubated in Mg^{2+}-free medium the phase of increase of absorbance was slightly larger and faster, presumably as a consequence of larger and more rapid extrusion of the dye from the membrane.

Turnover of redox pumps and $\Delta\tilde{\mu}_{H^+}$ in presteady state and steady state

Addition of small but increasing amounts of O_2 to anaerobic mitochondria results in a presteady state situation characterized, in principle, by the outflow of protons and by the progressive rise of $\Delta\tilde{\mu}_{H^+}$. Mitochondria supplemented with O_2 amounts exceeding that required for the presteady state attain a stationary state, denoted as static head, and are characterized by a net flow of H^+ ions equal to zero and by a maximal level of transmembrane potential. Mitochondria in static head respire at a rate which is determined by the extent of passive proton permeability and of slipping within the redox pump. We have analyzed the evolution of the presteady state by comparing the turnover of the redox pump with the rise of $\Delta\psi$.

Fig. 4A shows the effect of gradually increasing oxygen pulses to anaerobic mitochondria on the Safranine response and the redox state of cytochrome aa_3. The increase of the oxygen pulse was accompanied by a proportional increase, in extent and length of time, of both Safranine response and cycle of cytochromes oxidation. A comparison between Safranine response and cytochrome oxidation indicates that the cycle of the dye lasted for a time about 30% longer than that of the cytochromes. Furthermore, also the rate of restoration of the anaerobic state was considerably faster when monitored on the cytochromes. This difference may be due to two factors. First, the Safranine response involves a multistep process [29] and is limited by the rate of diffusion of the dye across the membrane, which is not the case for the cytochromes. Second, the Safranine response is linear with the potential only in a limited range and, especially in the high potential range, the increase of $\Delta\psi$ is not accompanied by a proportional increase of Safranine response [29]. This implies that when the membrane potential declines, after termination of the oxygen, the initial phase of potential decline is not readily detected by the dye. Fig. 4B shows the effect of the presence of a permeant cation, such as Sr^{2+} on the responses of Safranine and of the cytochromes. It is seen that in the presence of Sr^{2+} both the responses of Safranine and of the cytochrome were reduced in extent. Furthermore, the length of time of the responses was markedly reduced as a consequence of the faster rate of oxygen consumption.

Fig. 5 shows a plot of the Safranine and cytochrome responses at increasing oxygen pulses as obtained in the absence and in the presence of 100 μM Sr^{2+}. 50% response was obtained at about 0.125 ngatom O/mg in the case of cytochrome aa_3 and at about 0.2 ngatom O/mg in the case of safranine. By multiplying this

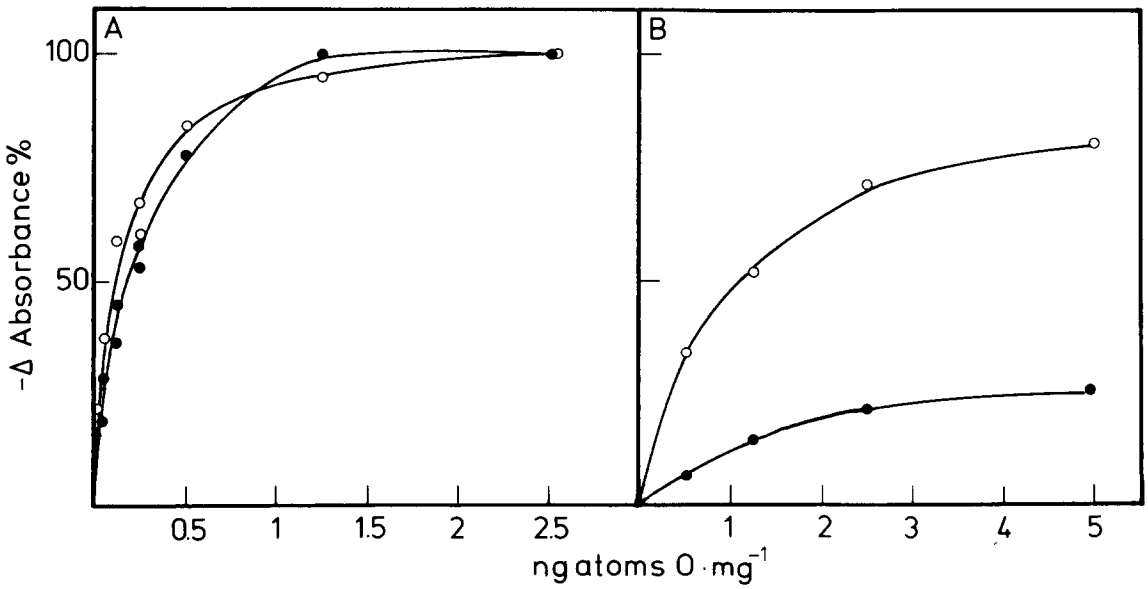

Fig. 5. Relationship between cytochrome aa_3 and Safranine responses in absence (panel a) and presence (panel b), of permeant cations as a function of the oxygen concentration. Reduction of cytochrome aa_3 (o) and response of Safranine (●). In panel b, the medium was supplemented with 100 μM $SrCl_2$ and EGTA was omitted. Experimental procedure was as in Fig. 4.

figure for the most recent estimate of the stoichiometry of the redox proton pumps during succinate oxidation ($H^+/O = 7$, cf. Ref. 34) we come to a charge displacement, for 50% cytochrome oxidation, of 0.8 ngatom H^+/mg and for 50% Safranine response of 1.4 ngatom H^+/mg. Fig. 5 also shows that the presence of 100 μM Sr^{2+} resulted in a large increase in the dimension of the oxygen pulses required to achieve a corresponding extent of responses of the cytochromes and of Safranine. This is what would be expected for a permeant species whose effect is that of increasing the electrical capacitance of the membrane.

Cytochrome aa_3 is an internal pigment whose response is determined by the rate at which oxygen reaches the respiratory chain and whose kinetics reflect the rate of oxidation, in thermodynamic equilibrium with $\Delta\tilde{\mu}_{H^+}$, of the electron carriers during the oxygen pulse. The cytochrome kinetics thus indicates that, whatever the behavior of the individual mitochondrion, there is an overall electron carrier oxidation, increasing in extent and length of time, proportionally to the increase of the oxygen pulse between 0 and 0.5 ngatom O/mg. This redox behavior of the average mitochondrion is closely paralleled by the Safranine response. The fact that the duration of the cycle of cytochrome oxidation increases with the amount of oxygen and that the attainment of the static head state requires about 4 s, indicates the adequacy of the experimental approach to detect the transition. In conclusion the experiments of Figs. 4–5 provide an answer to the question of the distinction between presteady state and steady state during oxygen pulses. Only when the oxygen pulses are larger than 0.5 ngatom O/mg do mitochondria enter into a steady state condition, also denoted as static head, where the membrane potential is maximal and the net charge displacement is equal to zero.

Phenol red and Bromthymol blue responses during oxygen pulses

Fig. 6A and B show the responses of the $\Delta\psi$ dye Safranine and of the pH indicator Phenol red during oxygen pulses carried out either in the absence of permeant cations (and supplemented with 0.5 mM EGTA) or in the presence of increasing concentrations of Sr^{2+}. It is seen in Fig. 6A that the Safranine response was maximal when the mitochondria were depleted of permeant cations by the addition of EGTA and then reduced proportionally to the increase of the Sr^{2+} con-

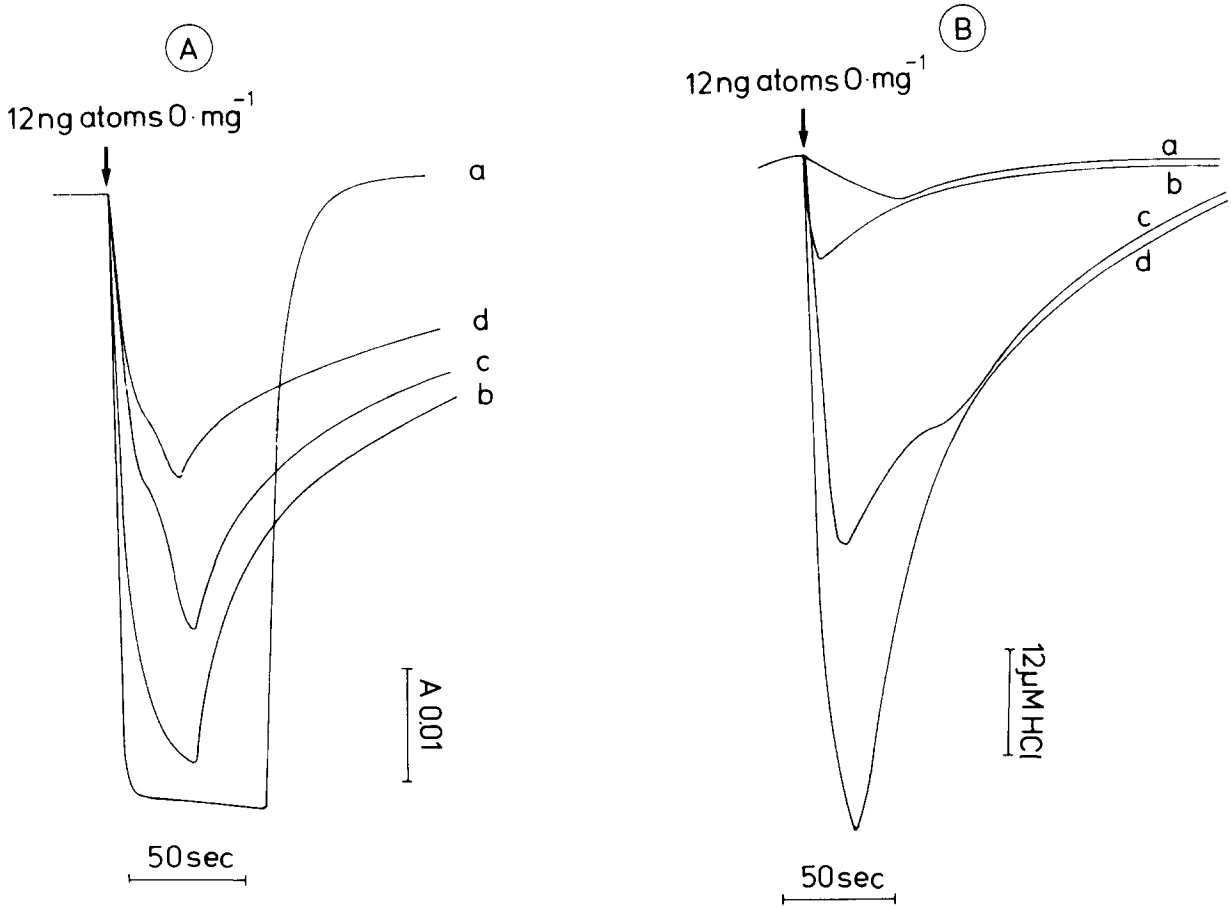

Fig. 6. Kinetics of Safranine (left) and Phenol red (right) responses after oxygen pulses in the presence of 0.5 mM EGTA (a) and in the absence of EGTA and presence of increasing concentrations of $SrCl_2$: 0 μM Sr (b), 50 μM Sr (c), 100 μM Sr (d). Mitochondria (3 mg/ml) were incubated for 10 min under anaerobic conditions with 20 μM Safranine, or 60 μM Phenol red, in parallel sample. Reaction was initiated with 12 ngatom O/mg.

centration. In accordance with Fig. 5, the time required for the consumption of O_2 was considerably reduced in the presence of Sr^{2+}.

Fig. 6B shows the response of Phenol red during oxygen pulses carried out under conditions similar to those of Fig. 6A. Mitochondria were pulsed with O_2 either in the presence and in the absence of EGTA or in the presence of 50 and 100 μM Sr^{2+}. It is seen that practically no appreciable or kinetically significant ejection of H^+ occurred in the presence of EGTA while a fast ejection did occur when EGTA was omitted from the medium. The abolition of the fast H^+ ejection by EGTA suggests that the effect is dependent on the movement of membrane-associated Ca^{2+} (cf also Refs. 35, 4, 5). In the presence of 50 and 100 μM Sr^{2+}, there was a marked increase in the amount of ejected H^+. The experiments of Fig. 6B thus indicate the existence of a Phenol red response which is dependent of the presence of permeant cations (or of membrane-associated Ca^{2+}, cf Refs. 5 and 35). The cation-dependent Phenol red response increases proportionally to the free cation concentration in the medium and is very fast. It has been argued [17,18] that, under the conditions used by Gould and Cramer [10,11] and by Hitchens and Kell [12,13], the amount of proton ejection accompanying the rise of potential would have been too small to be detected by the used methodology. Under the conditions of Fig. 6, the Phenol red assay indicates a maximal H^+ release of 0.16 ng/mg during the time required to attain the static head state. This is about one-tenth the amount of H^+ release expected on the basis of oxygen consumption.

The possibility that the absence of H^+ movement during the initiation of electron transport is an artifact of the conditions employed is always a concern. The appearance of H^+ ions may be masked, for instance, by the movement of H^+-buffering species, such as an influx of weak acid or an efflux of weak base. The most likely problem would be the influx of inorganic phosphate. Indeed, addition of *N*-ethylmaleimide, an inhibitor of the phosphate carrier [36], has been frequently used to increase the amount of protons ejected after oxygen pulses. We have analyzed the effect of increasing concentrations of *N*-ethylmaleimide on the H^+ ejection occurring in the presence of Sr^{2+} (results not shown). The increase of the *N*-ethylmaleimide concentration from 0 to 50 μM resulted in only a negligible change in the amount of ejected H^+ parallel to the

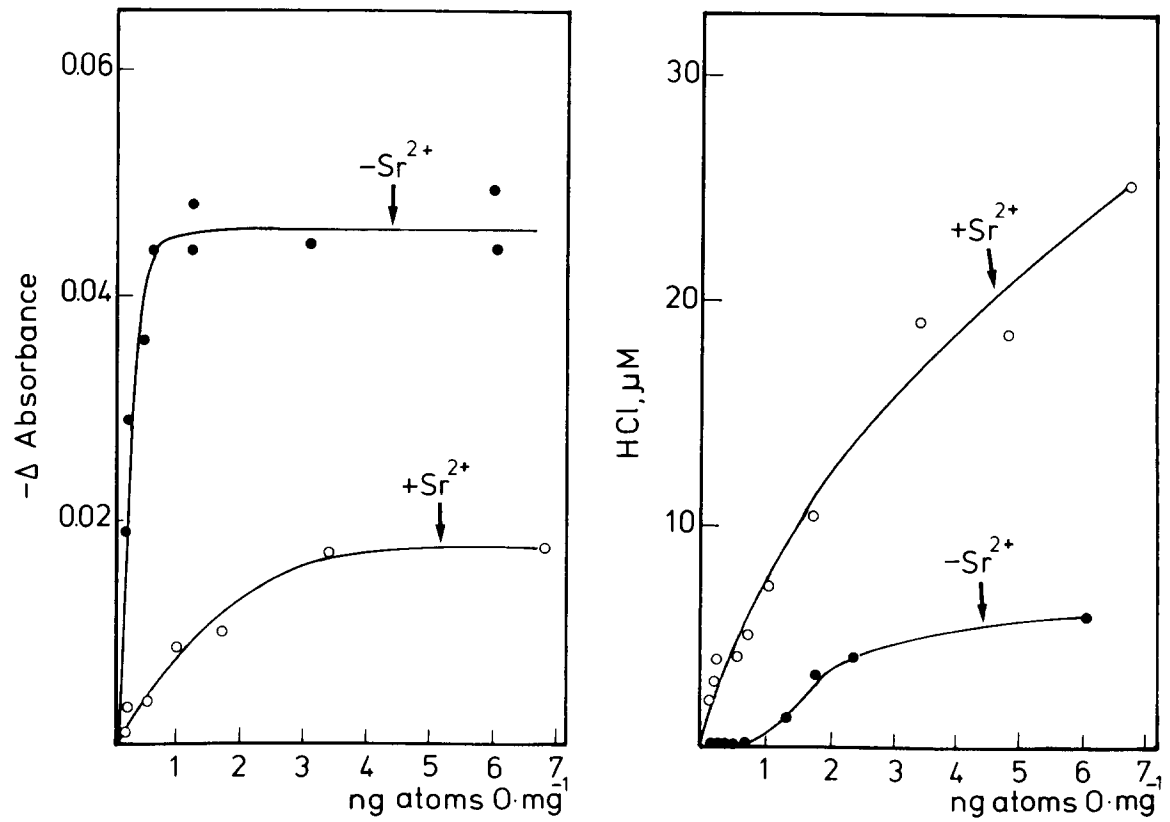

Fig. 7. Rise of membrane potential (left panel) and Phenol red response (right panel) as a function of the oxygen concentration in the absence or in the presence of 100 μM $SrCl_2$. The membrane potential was measured as variation of 20 μM Safranine response and the proton release from the response of 60 μM Phenol red. The figures for the proton release refer to the total extent of Phenol red response during the whole oxygen consumption and not to that part of the response which is kinetically related to the membrane potential rise. Measurements with Safranine and Phenol red were performed in parallel samples.

uptake of Sr^{2+}, while causing a considerable modification of the kinetics of the H^+ reuptake after termination of the O_2 consumption. In fact, while in the presence and absence of *N*-ethylmaleimide the release of H^+ from the mitochondria was slight and very slow, the larger was the *N*-ethylmaleimide concentration in the medium and the more rapid and the more complete was the H^+ influx into the mitochondria after termination of oxygen consumption. Such behavior is in accord with the properties of *N*-ethylmaleimide as an inhibitor of the phosphate carrier and reflects the state of the accumulated Sr^{2+} in the matrix, i.e., either precipitated as phosphate salt or bound to the membrane.

Fig. 7 shows the relationship between rise of $\Delta\psi$ and H^+ ejection at increasing oxygen pulses in the absence or in the presence of 100 μM Sr^{2+}. It is seen that, in the absence of Sr^{2+}, the Safranine response increased in the range between 0 and 0.4 ngatom O/mg at which oxygen concentration it reached the static head level. In the same range of oxygen concentrations, no H^+ ejection was observed. Only at the higher oxygen concentrations there was a small H^+ release showing, however, the characteristics of a slow process, i.e., the acidification recorded at the high oxygen concentrations took place during lengths of time orders of magnitude larger than required for the Safranine response and was kinetically unrelated to the $\Delta\psi$ changes. Fig. 7 analyzes also the effect of the proton ejection on the level of $\Delta\psi$ as determined with mitochondria incubated in the presence of a constant amount of $SrCl_2$ (100 μM). It may be seen that the rate of H^+ ejection was inversely related to the magnitude of $\Delta\psi$. Thus, this experiment shows the quantitative relationships between the rate of ejection of charge-compensated protons and the suppression of $\Delta\psi$.

Fig. 8 shows that the oxygen pulse was accompanied by a decrease of absorbance of Bromthymol blue. The oxygen induced responses of the dye were insensitive to the addition of impermeant buffers, indicating that the decrease of absorbance of Bromthymol blue cannot be attributed to an acidification in the bulk aqueous phase. Furthermore, Fig. 8 also shows that the oxygen-induced response was highly sensitive to phosphate.

Fig. 9 shows the response of Bromthymol blue following addition of increasing amounts of oxygen as required to support the transition from anaerobiosis to static head. The response of the dye increased proportionally to the oxygen concentration and reached a maximum at the oxygen concentration capable of supporting the attainment of static head, indicating that the decreasc in dye absorbance is also dependent on the thermodynamic state of the system. Fig. 9 also shows the effect of FCCP on the oxygen-induced acidification. The response of Bromthymol blue was sensitive to

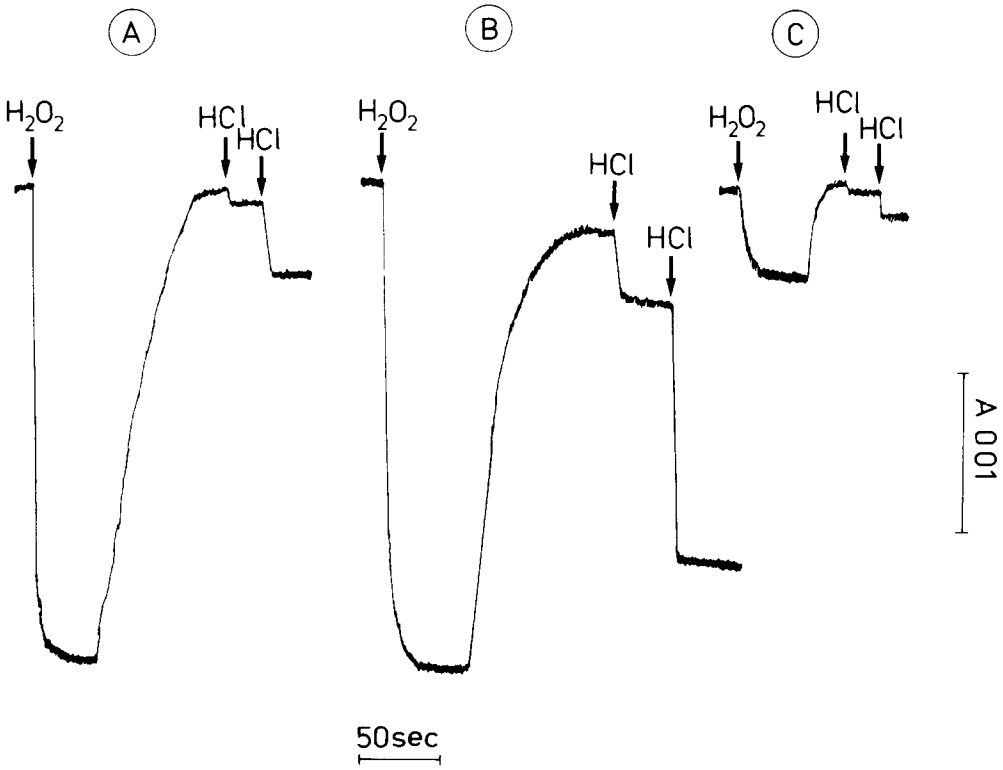

Fig 8. The response of Bromthymol blue after addition of oxygen pulses to anaerobic mitochondria. 3 mg/ml RLM were incubated in the presence of 30 μM Bromthymol blue and of 10 mM $MgCl_2$ for 10 min. Reaction was initiated by adding 12 ngatom O/mg; 1 and 5 nmol HCl/mg were added where indicated. Curve (A): presence of 30 mM Mops; Curve (B) unbuffered medium; Curve (C) presence of 30 mM Mops and 20 mM P_i.

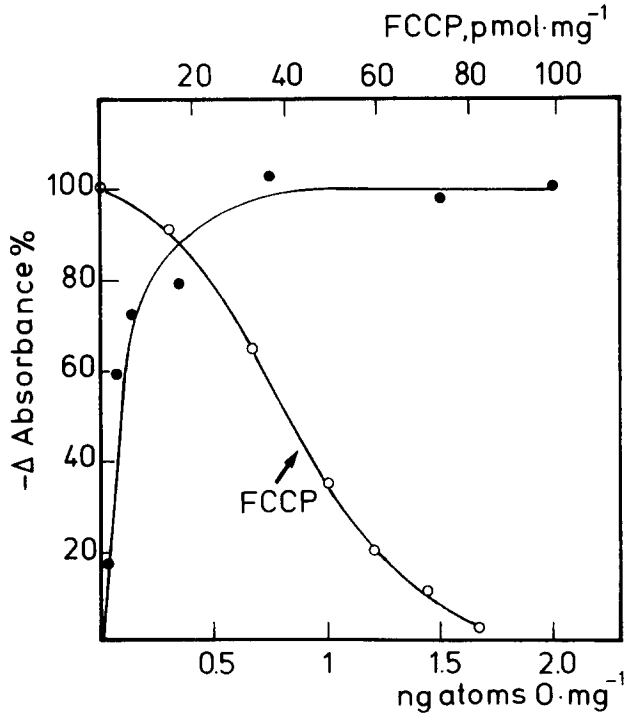

Fig. 9. Relationship between extent of Bromthymol blue response as a function of increasing concentrations of oxygen or of FCCP. Experimental conditions as in Fig. 8, 10 mM $MgCl_2$ and 30 mM Mops. The effect of FCCP on the oxygen-induced Bromthymol blue response was obtained in the presence of 2.5 ngatom O/mg.

uncouplers in the same range of uncoupler concentrations which cause stimulation of the respiration or depression of $\Delta\psi$. About 50% uncoupling was observed at about 40 pmol/mg, indicating that the phenomenon recorded by Bromthymol blue is a function of the level of energized state of the mitochondria.

The effect of phosphate on the oxygen-induced Bromthymol blue response was proportional to its concentration and the inhibition by phosphate was abolished by *N*-ethylmaleimide. The inhibition by phosphate was also removed by mersalyl at mersalyl concentrations, approx. 15 nmol/mg protein (results not shown), capable of inducing full inhibition of the phosphate carrier. As expected, the inhibitory effect of mersalyl was evident when mersalyl was added before, but not after, phosphate, while the inhibitory effect of *N*-ethylmaleimide was evident whether added before or after phosphate. The extent of the oxygen-induced acidification was also sensitive to the addition of acetate, although acetate concentrations almost one order of magnitude higher than those of phosphate were required.

The effect of phosphate on the oxygen-induced responses of Bromthymol blue was compared with that of a typical electrical probe such as TPMP as measured with the TPMP electrode (results not shown). Addition of 20 mM phosphate buffer caused a marked inhibition of the dye response, while it resulted in an enhancement of the electrical probe response. The experiment indicates that the response of Bromthymol blue principally differs from that of a $\Delta\psi$ probe.

Discussion

In analyzing the pH indicator responses three aspects deserve consideration: (a) that proton pumping across a hydrophobic barrier gives rise to opposite pH changes in the two domains separated by the barrier; (b) that the charged dyes may undergo a translocation or a reorientation under the strong electrical field developed during respiration (cf. Mitchell et al. [26]); and (c) that the protein- and membrane-bound dyes may monitor the pH of non-aqueous environments; all conditions favoring the binding of the dyes will favor the detection of membrane domains and vice versa. The domains monitored by the indicators have been identified on the basis of two criteria: (a) effects following addition of two ionophores, such as nigericin or valinomycin, producing well-established changes in proton electrochemical gradient; and (b) modifications induced by permeant and impermeant buffers on the dye responses.

While Phenol red is a very hydrophilic dye and records essentially the pH of the bulk aqueous phase, the response of the hydrophobic pH indicator Bromthymol blue is much more complex in that the dye monitors the pH of both the internal and the external domains: (a) a fraction of the dye BTB_e monitors the external domains, providing a response which is similar to that of a pH glass electrode or a hydrophilic pH indicator; and (b) another fraction of the dye BTB_i monitors the internal domains responding in a manner which is opposite to that of a glass electrode or a hydrophilic pH indicator. By combining the use of Mg^{2+} with that of high concentrations of impermeant buffers, Bromthymol blue may be used as an indicator of the pH changes in the internal domains or of the ΔpH across the membrane. Support to both these views is provided by the sensitivity to phosphate of the nigericin-induced dye response in anaerobic mitochondria and by the sensitivity to the phosphate carrier inhibitor *N*-ethylmaleimide of the phosphate effect.

Since Bromthymol blue is negatively charged it may undergo an electrophoretic movement under the electrical field. This translocation may be considered responsible for the oxygen-induced decrease of absorbance. Such a mechanism may receive indirect support by the observation that the nigericin pulse indicates a more external localization of the dye in aerobic with respect to anaerobic mitochondria. However, an extrusion of the dye from the membrane to the water phase, should be accompanied by an increase and not by a decrease of absorbance because it is a shift from a phase of lower to a phase of higher dielectric constant: compare with the dye response following the valinomycin-induced K^+

diffusion potential which is, apart from a rapid initial phase, that of a slow increase of absorbance. A membrane potential driven extrusion of the dye during the oxygen pulse is also in contrast with other evidence. Firstly, as shown in Fig. 3, the valinomycin-induced increase of absorbance is a very slow process. Secondly, the valinomycin-induced slow increase of dye absorbance is slightly diminished by the addition of phosphate while the response of other potential probes, such as TPMP, is enhanced by phosphate, because the latter augments the membrane potential. We conclude that there is no major dye extrusion from the membrane to the water phase which can explain the oxygen-induced dye response.

The nigericin-induced response of Bromthymol blue indicates a dye facing the external domains more in respiring than in non-respiring mitochondria due to a reorientation of the membrane-bound dye under the electrical field. That Bromthymol blue predominantly records pH changes occurring at the level of the membrane, BTB_m, and not of the bulk aqueous phase, is supported by the properties of the dye response during the nigericin pulse in aerobic mitochondria: although both Bromthymol blue and the glass electrode record an alkalinization, the former, BTB_{me}, is insensitive, while the latter is sensitive to the buffer concentration in the medium.

Proton flows during oxygen pulses

The recent studies of Taylor and Jackson [17,18] utilizing the photosynthetic bacterium, *R. capsulata*, in which $\Delta\tilde{\mu}_{H^+}$ generation was followed by carotenoid band shifts and H^+ extrusion by pH-indicating dyes showed a rapid phase of H^+ extrusion which kinetically seemed to parallel the rise of $\Delta\tilde{\mu}_{H^+}$. On the other hand, numerous reports agree on the observation that, in the absence of counter-ion movement, the amount of H^+ ions appearing in the bulk aqueous phase during the generation of $\Delta\tilde{\mu}_{H^+}$ in mitochondria or bacteria is very small or negligible [5,9]. There is, however, little agreement as to the explanation of the proton scarcity.

Net H^+ extrusion should occur only during the transition from anaerobiosis to aerobic static head. This transition may be described as a presteady state where the rate of H^+ pumping (J_H^p) exceeds the leak rate (J_H^l). In presteady state the electron transfer rate (J_e) would be high (state 3) and $\Delta\tilde{\mu}_{H^+}$ less than maximal. Once static head is attained, $\Delta\tilde{\mu}_{H^+}$ will have reached a maximum and J_e will have become minimal (state 4). Then, $J_H^p = J_H^l$ and no further net H^+ extrusion will occur. Then, after attainment of static head, the H^+ extrusion occurs only as a consequence of reactions which lead to the consumption of $\Delta\tilde{\mu}_{H^+}$.

The explanation proposed by Mitchell [5] for the lack of H^+ ejection implies that mitochondria or bacteria have already achieved static head, where the net H^+ efflux is equal to zero, even at the lowest oxygen pulse. In the experiments by Gould and Cramer with *E. coli* [10,11], and by Hitchens and Kell with *P. denitrificans* [12,13], the only H^+ extrusion consistently detected increased proportionally to the increase of the oxygen pulses, an observation which would be inconsistent with the interpretation proposed by Mitchell. In the experiments reported here the Phenol red response at increasing oxygen pulses is both kinetically and quantitatively unrelated to the generation of $\Delta\tilde{\mu}_{H^+}$.

The arguments that (a) the initial electrogenic H^+ extrusion required to generate $\Delta\tilde{\mu}_{H^+}$ would be too small to be detected by the present assay system and (b) that mitochondria are in static head state even at the lowest oxygen concentration, are ruled out by two considerations. First, the concept of the obligatory coupling between electron transfer and vectorial H^+ translocation implies that the extent of oxygen consumed to achieve the static head can be converted into the amount of H^+ ions translocated by multiplying the amount of O_2 for the stoichiometry of the H^+ pumps. From the experiments shown in Figs. 4–5, the number of H^+ ions translocated is between 2.4 and 3.2 ngion/mg. This number of H^+ ions is more than 30-times larger than the sensitivity of the Phenol red response. Second, the cytochrome aa_3 and Safranine responses provide the direct criteria for the determination of the establishment of the static head state. With succinate as substrate, increases in the oxygen pulse up to 0.5 ngatom/mg are accompanied by an increase in the extent of cytochrome and Safranine responses. Only above 0.5 ngatom O/mg further increase in oxygen causes no further increase in cytochrome and Safranine responses. The pattern of the cytochrome and Safranine responses thus indicates that with oxygen pulses of up to 0.5 ngatom O/mg, the system is in a presteady state condition, i.e., the rate of H^+ extrusion by the redox H^+ pump exceeds by definition that of H^+ back-flow. In conclusion, only above 0.5 ngatom O/mg, i.e., when the system has achieved static head, a mechanism of H^+ cycling can explain the absence of net H^+ ion extrusion.

The value for the H^+ ions translocated during achievement of static head $\Delta\tilde{\mu}_{H^+}$ can be compared with the value of 1 ngatom H^+/mg calculated by Mitchell from estimates of inner membrane capacitance and surface area [1]. The relative agreement between the two values supports the basic correctness of Mitchell's calculation. This may appear surprising if one considers the number of assumptions involved particularly in the calculation of the membrane surface area. It will be a matter of future experimentation to establish the extent to which this figure may change, especially under condition of cristae deconvolution or membrane stretching.

The polarity of the redox pumps predicts that during respiration the external domains become acid with a decreased absorbance of the dye, BTB_e, monitoring the

external domains. The question arises as to whether the external domain monitored by Bromthymol blue during the oxygen-induced pulses corresponds to the outer water phase. The answer is negative on the basis of two facts: (a) this domain is not accessible to either glass electrode or hydrophilic pH indicators, such as Phenol red, and (b) the Bromthymol blue response is insensitive to the concentration of impermeant buffers in the outer medium. The insensitivity to impermeant buffers of the oxygen pulse-induced acidification recalls the insensitivity of the nigericin pulse-induced alkalinization in aerobic mitochondria. In both cases the dye response is functionally related to the pH changes of the external medium but is insensitive to impermeant buffers. This dye may be operationally defined as BTB_{me}.

That the oxygen-induced acidification is sensitive to the addition of phosphate and that the phosphate effect is abolished by *N*-ethylmaleimide, indicates operation of the phosphate carrier. Thus, the phosphate sensitivity of the oxygen-induced decrease in dye absorbance indicates that this response is dependent on the ΔpH between the internal and external membrane layers.

A simple scheme accounts for the effects observed. Bromthymol blue is seen as a hydrophobic pH indicator, which undergoes: (a) a ΔpH-dependent distribution across the membrane with a major fraction of the indicator facing, in anaerobic mitochondria, the internal domain and (b) an electrical field dependent orientation, with the chromophoric group facing the internal in anaerobic, and the external domain in aerobic mitochondria. The distribution of the pH dyes, BTB_e and BTB_i, is therefore determined by ΔpH similarly to that of other permeant weak acids, with the only difference that part of the dye is membrane bound. Given this condition, the formation of ΔpH, as it occurs during an oxygen pulse, leads to a series of events including: first, a redistribution of the dye, proportionally to the dimension of the ΔpH, between external and internal domains ; second, an electrical field dependent on reorientation of the dye towards the external domain: BTB_{mi} into BTB_{me}. This scheme explains why collapse of ΔpH by phosphate leads to depression of the oxygen-induced decrease of dye response. The acidification recorded by Bromthymol blue during the oxygen pulse is seen as a consequence of the redox pump catalyzed uptake of protons from the matrix. This uptake leads on one side to the formation of a ΔpH and on the other to the ejection of protons into a membrane layer close to the outer surface. These protons are not accessible to a dye localized in the outer water phase but are so to a dye localized in the membrane phase facing the outer medium.

In concluding it may be useful, as Taylor and Jackson [17] have done in *R. capsulata*, to separate also in mitochondria the H^+ translocation into two categories i.e., the primary electrogenic H^+ displacement, and the charge-compensated H^+ movements. The primary electrogenic H^+ displacement (3 ngion H^+/mg), which occurs in presteady state, is responsible for the rise of $\Delta\tilde{\mu}_{H^+}$ and does not involve counter ion movement. It is also not detectable by hydrophilic pH indicators in rat liver mitochondria. The charge-compensated H^+ movements occur only in the presence of counter-ion movements and are a consequence of the permeant ion-induced increase of the electrical capacitance of the inner membrane. The charge-compensated H^+ movements, when present, are, in amount and rate, to be easily measured by pH electrodes or hydrophilic pH indicators. Proton movements observed during respiration-driven ion transport processes in mitochondria are usually of this latter type as indicated by the depression of $\Delta\tilde{\mu}_{H^+}$ almost invariably observed. While the charge-compensated H^+ movements occur mostly after establishment of the stationary state condition, the electrogenic H^+ displacement occurs in presteady state (or after perturbation of the stationary state).

The reason as to why electrogenic H^+ translocation is observed in one (*R. capsulata* or thylakoids) but not in the other (mitochondria) system remains obscure. One alternative is that of an excess of fixed buffering groups in the space between the outer and inner mitochondrial membranes. This would diminish the response of the small amount of dye present in the intermembrane space and would allow the protons to re-enter the matrix at the end of the pulse without ever seeing the bulk aqueous phase. The other, more likely, alternative is that of a difference in state of the membrane structure of the two systems and particularly in the state of the ionic clouds close to the membrane surface and then in the proton mobility. Kamp et al. [37] have recently suggested that the invisibility of H^+ ions may be due to electrostatic effects consequent to the presence of a protein layer adjacent to the membrane which restricts equilibration of charges. As noted by Hong and Junge [16], in studies with freeze-thawed and intact chloroplasts, in the latter material the internal space (where the protons are released) lacks the properties of an aqueous bulk phase.

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