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Control of electron transfer in the cytochrome system of mitochondria by pH, transmembrane pH gradient and electrical potential

The cytochromes b–c segment

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1. A study is presented of the effects of pH, transmembrane pH gradient and electrical potential on oxidoreductions of *b* and *c* cytochromes in ox heart mitochondria and 'inside-out' submitochondrial particles. 2. Kinetic analysis shows that, in mitochondria at neutral pH, there is a restraint on the aerobic oxidation of cytochrome b_{566} with respect to cytochrome b_{562}. Valinomycin plus K^+ accelerates cytochrome b_{566} oxidation and retards net oxidation of cytochrome b_{562}. At alkaline pH the rate of cytochrome b_{566} oxidation approaches that of cytochrome b_{562} and the effects of valinomycin on *b* cytochromes are impaired. 3. At slightly acidic pH, oxygenation of antimycin-supplemented mitochondria causes rapid reduction of cytochrome b_{566} and small delayed reduction of cytochrome b_{562}. Valinomycin or a pH increase in the medium promote reduction of cytochrome b_{562} and decrease net reduction of cytochrome b_{566}. 4. Addition of valinomycin to mitochondria and submitochondrial particles in the respiring steady state causes, at pH values around neutrality, preferential oxidation of cytochrome b_{566} with respect to cytochrome b_{562}. The differential effect of valinomycin on oxidation of cytochromes b_{566} and b_{562} is enhanced by substitution of ^1H_2O of the medium with ^2H_2O and tends to disappear as the pH of the medium is raised to alkaline values. 5. Nigericin addition in the aerobic steady state causes, both in mitochondria and submitochondrial particles, preferential oxidation of cytochrome b_{562} with respect to cytochrome b_{566}. This is accompanied by *c* cytochrome oxidation in mitochondria but *c* cytochrome reduction in submitochondrial particles. 6. In mitochondria as well as in submitochondrial particles, the aerobic transmembrane potential ($\Delta\psi$) does not change by raising the pH of the external medium from neutrality to alkalinity. The transmembrane pH gradient (ΔpH), on the other hand, decreases slightly. 7. The results presented provide evidence that the $\Delta\psi$ component of the aerobic $\Delta\bar{\mu}_{H^+}$ (the sum of the proton chemical and electrical activities) exerts a pH-dependent constraint on forward electron flow from cytochrome b_{566} to cytochrome b_{562}. This effect is explained as a consequence of anisotropic location of cytochromes b_{566} and b_{562} in the membrane and the pH-dependence of the redox function of these cytochromes. Transmembrane ΔpH, on the other hand, exerts control on electron flow from cytochrome b_{562} to *c* cytochromes.

The cytochrome *b–c* segment of the respiratory chain consists of five redox centres, four of which, two *b* cytochromes, one non-haem iron–sulphur protein and cytochrome c_1, are arranged in a complex, complex *b–*c_1 or III (Rieske, 1976; Erecinska *et al.*, 1976; Engel *et al.*, 1979). The mechanism by which the two reducing equivalents donated by ubiquinol are accepted by the redox centres of the *b–*c_1 complex and transferred to cytochrome *c* (Baum *et al.*, 1967; Wikström, 1973; Rieske, 1976) and transmembrane $\Delta\bar{\mu}_{H^+}$ is generated (Lawford & Garland, 1973; Leung & Hinkle, 1975; Mitchell, 1976; Papa *et al.*, 1975*a*, 1977) is still unsettled.

The available evidence shows the existence in mitochondria of two different cytochrome *b* species, cytochrome b_{566} (b_T or b_L, low potential) and cytochrome b_{562} (b_K or b_R, high potential), identified

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by differences in spectral properties, midpoint redox potentials and kinetics (Chance *et al.*, 1970; Dutton & Wilson, 1974; Rieske, 1976; Wikström, 1973; Wainio, 1977). A dimeric *b* cytochrome has been isolated from ox heart mitochondria with two redox centres of different midpoint potentials (von Jagow *et al.*, 1978; cf. Weiss, 1976).

Cytochromes *b* exhibit peculiar energy-linked reduction, which is explained either as the expression of a specific increase of the E_m (midpoint potential) of cytochrome b_{566} (Chance *et al.*, 1970; Dutton & Wilson, 1974; Wilson & Erecinska, 1975), or as the result of reversed electron flow (Wikström & Lambowitz, 1974), or, more specifically, of equilibria shifts associated with the transmembrane potential (Mitchell, 1972, 1976).

Another property of *b* cytochromes that has not so far received sufficient attention is represented by the pH-dependence of their midpoint redox potential observed in intact mitochondria (Straub & Colpa-Boonstra, 1962; Urban & Klingenberg, 1969; Wilson *et al.*, 1972), isolated b–c_1 complex and purified *b* cytochromes (von Jagow *et al.*, 1978). This pH-dependence, which applies to both *b* cytochromes of complex III (Dutton & Wilson, 1974; von Jagow *et al.*, 1978), indicates the existence of allosteric linkage (Wyman, 1968) between the redox state of the haem iron and protolytic equilibria in the apoprotein (Papa, 1976; Papa *et al.*, 1973*a*, 1979).

The present paper characterizes the effects of pH and the electrical ($\Delta\psi$) and chemical (ΔpH) components of the respiration-linked transmembrane $\Delta\tilde{\mu}_{H^+}$ on oxidoreductions of *b* and *c* cytochromes. It is shown that $\Delta\psi$ exerts a specific pH-dependent 'crossover effect' on forward electron flow from cytochrome b_{566} to cytochrome b_{562}. Transmembrane ΔpH controls, on the other hand, electron flow from cytochrome b_{562} to *c* cytochromes.

Materials and methods

Chemicals

Valinomycin was obtained from Sigma Chemical Co., nigericin from Eli Lilly Laboratories, catalase from Boehringer-Mannheim, ^2H_2O from Bio-Rad Laboratories, [^{14}C]sucrose, ^{86}RbCl and ^3H_2O from The Radiochemical Centre, and [^{14}C]5,5-dimethyloxazolidine-2,4-dione and [^{3}H]methyltriphenylphosphonium bromide from New England Nuclear Corp.

Preparation of mitochondria and submitochondrial particles

Heavy-fraction ox heart mitochondria were prepared as described by Löw & Vallin (1963). Submitochondrial particles were prepared by exposing mitochondria to ultrasonic energy in the presence of EDTA (Lee & Ernster, 1968).

Determination of the protonmotive force

$\Delta\psi$ and ΔpH in respiring mitochondria were determined by the distribution of ^{86}Rb or [^{3}H]methyltriphenylphosphonium bromide and [^{14}C]5,5-dimethyloxazolidine-2,4-dione respectively, across the inner membrane (Nicholls, 1974; Schuldiner & Kaback, 1975).

Mitochondria were incubated in the reaction mixture (see the legend to Table 1) and bubbled with oxygen. Samples of the suspension were then centrifuged in the presence of catalase (0.2 mg/ml) and 2 mM-H_2O_2, at 20000 *g*. Portions of the $HClO_4$ extract of the pellet and of the supernatant were counted for radioactivity.

$\Delta\psi$ in respiring submitochondrial particles was determined by the transmembrane distribution of SCN^- (Papa *et al.*, 1973*b*; Kell *et al.*, 1978). Particles were incubated in the reaction mixture (see the legend to Table 1) in the presence of catalase (0.2 mg/ml) at 25°C (±0.1°C) under a stream of argon. The uptake of SCN^- was monitored by an SCN^--sensitive electrode (Sens-Ion model 201; Amel, Milan, Italy). Once the particle suspension was made anaerobic by succinate oxidation, respiration was activated by addition of 6 μl of 2.5% H_2O_2/ml. The internal volume of the particles was taken as 1.4 μl/mg of protein (Papa *et al.*, 1973*c*).

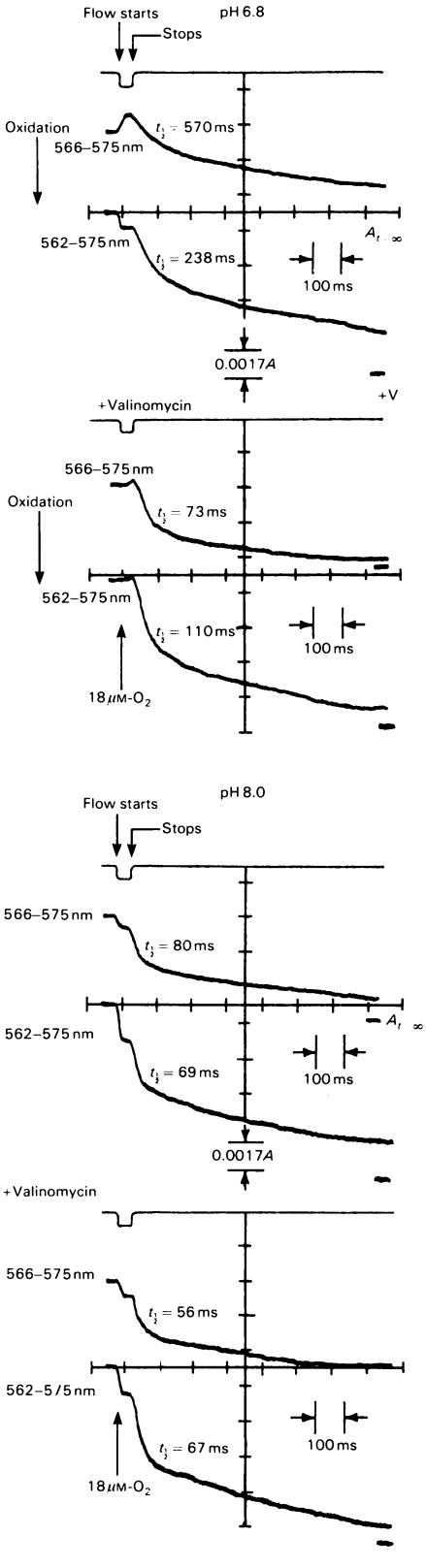
Measurement of oxidoreduction of cytochromes

Oxidoreduction of cytochromes was monitored with a Johnson Foundation dual-wavelength spectrophotometer. The slit-width of monochromators was 0.1–1 mm and the two beams were directed on to the observation cell by a mirror oscillating at 400 Hz.

Kinetic analysis. A stopped-flow apparatus (Chance *et al.*, 1967) with mixing ratio of 1:70 was used. The main compartment of the apparatus was filled with the suspension of mitochondria. The oxygen in the suspension was allowed to be consumed by succinate oxidation. Oxygen pulses were delivered from the side syringe as oxygenated reaction mixture.

The reaction time at the point of observation in the flow spectrophotometer (continuous-flow phase) ranged, from experiment to experiment, between 6 and 9 ms, depending on the speed of the discharge (see Papa *et al.*, 1975*a*).

Steady-state measurements. The suspension of mitochondria or submitochondrial particles, supplemented with succinate, was allowed to become anaerobic in the observation cell. Respiration was activated by addition of 1–5 μl of 3% H_2O_2/ml. The total amount of reducible cytochromes was obtained by addition of solid sodium dithionite. Both the static cuvette and the flow cell were thermostatically controlled at 20 or 25 ± 0.01°C.



Results

Kinetics of aerobic oxidation of b cytochromes

Fig. 1 shows the effect of pH and valinomycin on the kinetics of aerobic oxidation of cytochromes b_{566} and b_{562}, monitored at the wavelength couples 566–575 nm and 562–575 nm, in ox heart mitochondria whose respiratory carriers were reduced by succinate in anaerobiosis. At pH 6.8 the oxygen-induced absorbance decrease at 566–575 nm was preceded by a small absorbance increase (completed in the flow period, 8 ms) and was slower than that monitored at 562–575 nm. In the latter case no initial absorbance increase was observed. Valinomycin (plus K^+) accelerated the absorbance decrease at both wavelength couples. This acceleration was much more marked at 566–575 nm than at 562–575 nm.

At pH 8 the oxygen pulse induced immediate absorbance decreases at both 566–575 and 562–575 nm, which were faster than at pH 6.8 and practically synchronous. Valinomycin specifically accelerated the absorbance decrease at 566–575 nm, but the effect was smaller than at pH 6.8.

The comparison of the kinetic patterns of the absorbance changes at 566–575 with those at 562–575 nm shows that cytochromes b_{566} and b_{562} respond differently to pH and valinomycin. A closer quantitative evaluation of the difference in the response of cytochromes b_{566} and b_{562} can be provided by calculating, from the observed absorbance changes, the respective absorbance changes of the two *b* cytochromes. These were obtained (see the legend to Fig. 2) on the basis of the fractional contributions of cytochromes b_{566} and b_{562} at 562–575 and 566–575 nm reported for ox heart mitochondria (Berden *et al.*, 1972) and submitochondrial particles (von Jagow, 1975). It can be

Fig. 1. *Effect of pH and valinomycin on the kinetics of aerobic oxidation of b cytochromes in ox heart mitochondria*

The fast kinetics of *b*-cytochrome oxidation was analysed as described in the Materials and methods section. The main syringe of the flow apparatus contained 200 mM-sucrose, 10 mM-potassium succinate, 10 mM-potassium malonate, 30 mM-KCl, oligomycin (2 μg/mg of protein), rotenone (1 μg/mg of protein) and ox heart mitochondria (2.5 mg of protein/ml). The temperature was 25°C. The smaller syringe contained an oxygen-saturated mixture consisting of 200 mM-sucrose and 30 mM-KCl. Where indicated, valinomycin (0.1 μg/mg of protein) was present in the mitochondrial suspension. The small horizontal absorbance traces ($A_{t=\infty}$) show the absorbance at the end of aerobic oxidation of *b* cytochromes. For other details, see the Materials and methods section.

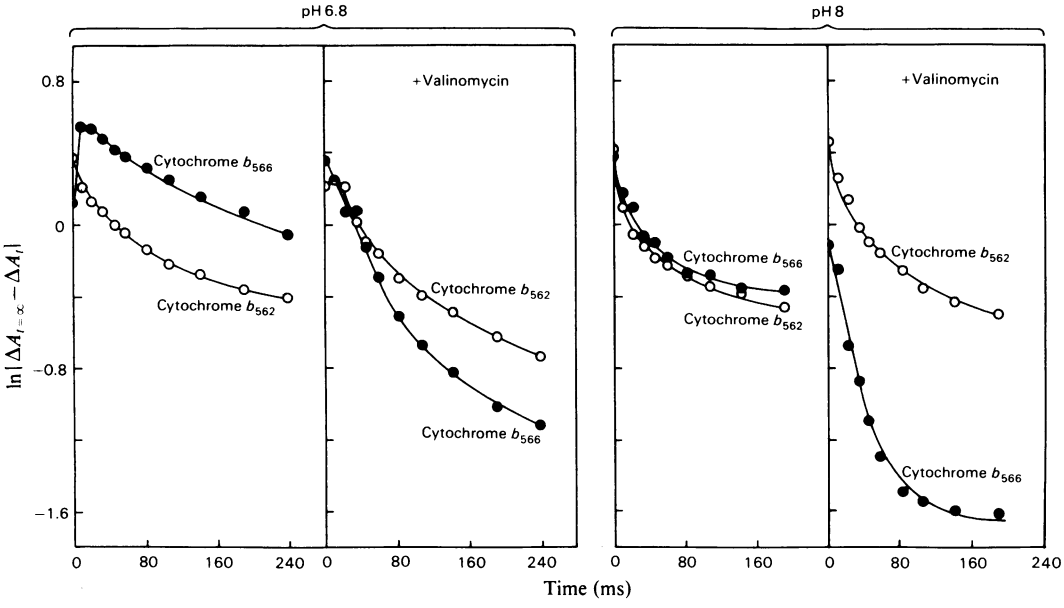


Fig. 2. *First-order plots of absorbance changes of cytochrome b_{566} and b_{562} at 566–575 nm caused by oxygenation of anaerobic ox heart mitochondria.*

The respective absorbance changes of cytochromes b_{566} and b_{562}, presented in arbitrary units, were obtained from the deflections (in cm) of the absorbance traces at 566–575 nm of Fig. 1, by applying eqn. (1):

$$\frac{\Delta A_{566-575} - y_1}{\Delta A, T_{566-575} \cdot 0.6} = \frac{\Delta A_{562-575} - (y_1 \cdot R)}{\Delta A, T_{562-575} \cdot 0.2} \quad (1)$$

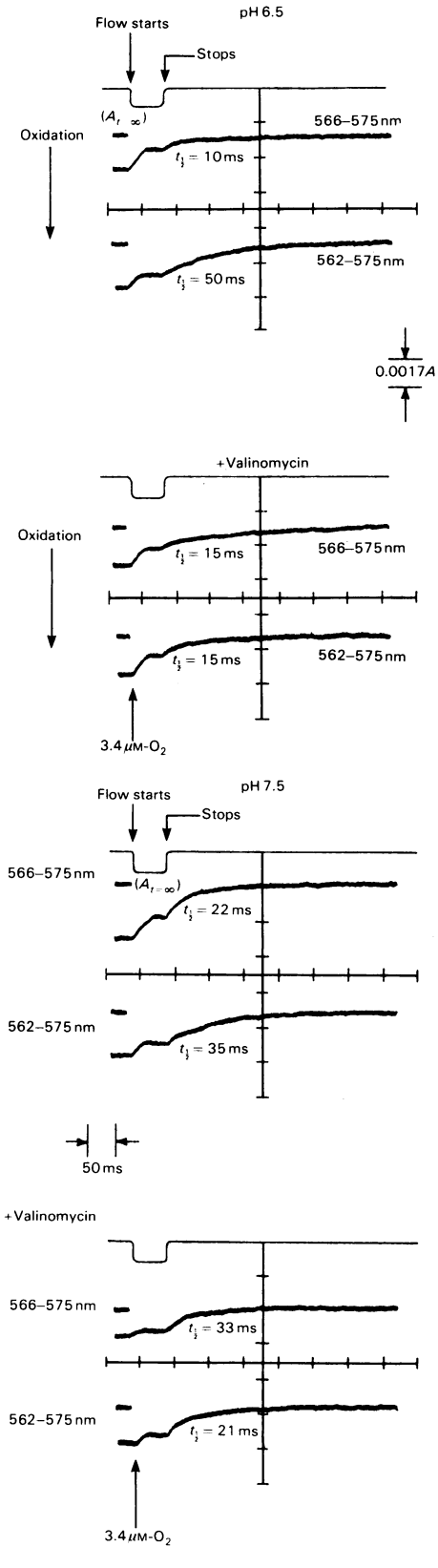
This was also used (Figs. 6–9) to calculate the respective redox transitions of the two *b* cytochromes, expressed as a percentage of the total dithionite-reducible amounts. The first quantity in eqn. (1) refers to measurements at 566–575 nm and the second to measurements at 562–575 nm; $\Delta A, T_{566-575}$ and $\Delta A, T_{562-575}$ represent the total absorbance changes, y_1 the absorbance change of cytochrome b_{562} at 566–575 nm and R the ratio of the absorption coefficients of cytochrome b_{562} at 562–575 and 566–575 nm. The factors 0.2 and 0.6 represent the relative contributions of cytochrome b_{566} to the total ΔA (dithionite-reduced minus fully oxidized) at 562–575 and 566–575 nm respectively (Berden *et al.*, 1972; von Jagow, 1975). $A_{t=\infty}$ represents the total extent of absorbance decrease (in cm), caused by aerobic oxidation of *b* cytochromes. The values used for the first-order plot can be converted into absolute absorbance changes by using the calibration factor shown in Fig. 1.

noted that these represent the lower estimate, as compared with other measurements in different mitochondria (Dutton *et al.*, 1972; Wikström, 1973; Wilson & Erecinska, 1975), for the contribution of cytochrome b_{566} at 566–575 and 562–575 nm.

The first-order plots of the respective responses of cytochromes b_{566} and b_{562} to oxygenation (Fig. 2) show, at pH 6.8, a first phase (up to ~20 ms) characterized by rapid oxidation of cytochrome b_{562} and reduction of cytochrome b_{566}. In the second phase, cytochrome b_{566} started to be oxidized, and this was accompanied by decline in the net rate of cytochrome b_{562} oxidation. Valinomycin accelerated cytochrome b_{566} oxidation. In its presence the initial reduction of cytochrome b_{566} was suppressed and a lag in the oxidation of cytochrome b_{562} appeared. At pH 8 cytochrome b_{566} started to be immediately oxidized at a rate close to that for cytochrome b_{562} oxidation. Valinomycin enhanced the rate of cytochrome b_{566} oxidation (this effect was smaller than that at pH 6.8) and decreased the initial rate of cytochrome b_{562} oxidation.

Oxygen-induced reduction of b cytochromes

Oxygenation of anaerobic mitochondria, supplied with succinate and antimycin, results in rapid reduction of *b* cytochromes (Erecinska *et al.*, 1972; Wikström, 1973). In Fig. 3 the effects of pH and valinomycin on this reduction of *b* cytochromes is presented. Comparison of the kinetics of the absorbance increase at 566–575 and 562–575 nm indicates that the reduction of cytochrome b_{566} was, especially at pH 6.5, faster than that of cytochrome b_{562}. At pH 7.5 the rate of cytochrome b_{566} reduction was lower than at pH 6.5; the reverse was observed in the case of cytochrome b_{562}. Valinomycin ac-



celerated the reduction of cytochrome b_{562}, but slowed down that of cytochrome b_{566}. These effects of valinomycin were more pronounced at pH 6.5 than at pH 7.5.

Steady-state redox transitions of cytochromes

The $\Delta\bar{\mu}_{H^+}$ set up across the membrane by respiration exerts at the steady state (State 4; Chance & Williams, 1956) back pressure on oxidation of cytochromes. The influences of $\Delta\psi$ and ΔpH were respectively analysed by testing the effect on the redox levels of cytochromes of $\Delta\psi$ collapse by valinomycin and ΔpH collapse by nigericin.

Fig. 4(*a*) shows that, at pH 6.9, the addition of valinomycin to mitochondria respiring with succinate caused oxidation of *b* cytochromes and reduction of *c* cytochromes. Nigericin, added when the cytochromes had attained a new redox level, caused oxidation of both *b* and *c* cytochromes.

In submitochondrial particles (Fig. 4*b*), addition of oligomycin, which enhances respiration-linked proton uptake (Papa *et al.*, 1970), resulted in reduction of *b* cytochromes. Oligomycin induced also transient reduction of *c* cytochromes. Valinomycin caused rapid oxidation of *b* cytochromes and reduction of *c* cytochromes. These initial transitions were followed by partial re-reduction of *b* cytochromes and reoxidation of *c* cytochromes, which can be ascribed to replacement of $\Delta\psi$ by generation of extra ΔpH (Papa *et al.*, 1972). Further addition of nigericin caused oxidation of *b* cytochromes and reduction of *c* cytochromes.

In Fig. 5(*a*), spectral analysis is presented of the absorbance changes of *b* and *c* cytochromes observed when nigericin, instead of valinomycin, was added to respiring mitochondria and submitochondrial particles. In mitochondria, nigericin caused oxidation of both *b* and *c* cytochromes. From the position of the peak, oxidation of cytochrome b_{562} can clearly be identified. In submitochondrial particles, nigericin caused oxidation of cytochrome b_{562} but reduction of *c* cytochromes.

Fig. 3. *Effect of pH and valinomycin on the kinetics of oxygen-induced reduction of b cytochromes in antimycin-inhibited anaerobic ox heart mitochondria*

The main syringe contained 200mM-sucrose, 30mM-KCl, 5mM-potassium succinate, oligomycin (2μg/mg of protein), antimycin (0.5μg/mg of protein) and ox heart mitochondria (2mg of protein/ml). The smaller syringe contained a mixture of 200mM-sucrose and 30mM-KCl. Where indicated, valinomycin (0.1μg/mg of protein) was present in the mitochondrial suspension. The temperature was 25°C. The small horizontal absorbance traces ($A_{t=\infty}$) show the absorbances at the end of aerobic reduction of *b* cytochromes.

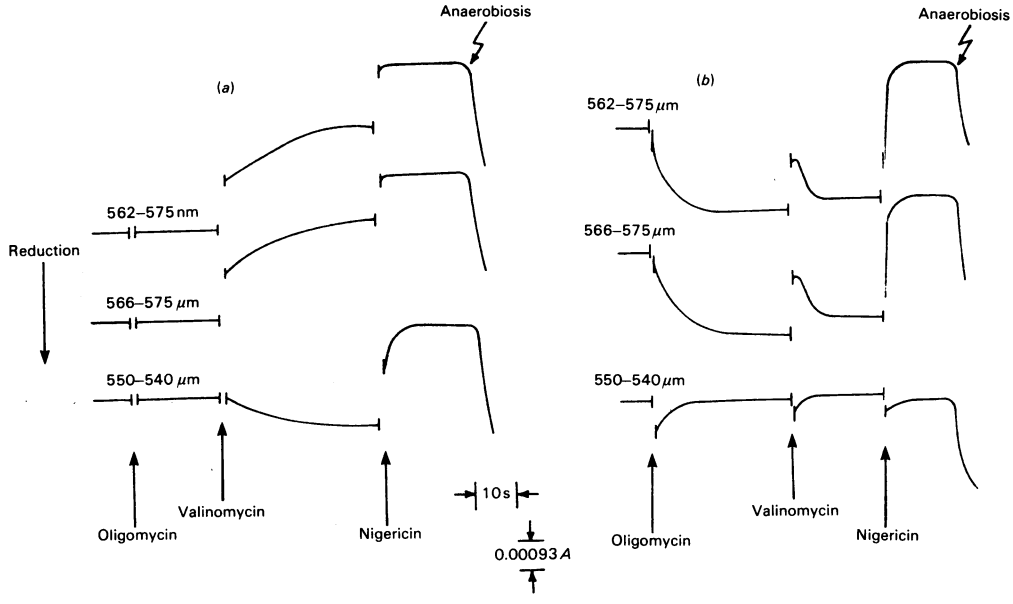


Fig. 4. *Effect of oligomycin and ionophores on the aerobic steady-state redox level of b and c cytochromes in ox heart mitochondria (a) and submitochondrial particles (b)*

The reaction mixture contained 200mM-sucrose, 20mM-KCl, 5mM-Tris/HCl, 10mM-potassium succinate, rotenone (1μg/mg of protein) 0.2mg of purified catalase/ml and ox heart mitochondria or EDTA-treated submitochondrial particles (2mg of protein/ml). Respiration was activated by adding 15μl of 3% H_2O_2 to anaerobic mitochondria or submitochondrial particles. Where indicated, oligomycin (2μg/mg of protein), valinomycin (0.4μg/mg of protein) and nigericin (0.4μg/mg of protein) were added. The final volume was 1.5ml and the final pH was 6.9; the temperature was 20°C. Oxidoreduction of *c* cytochromes was monitored at 550–540nm.

In Fig. 6 the steady-state reduction levels of cytochromes, before the addition of ionophores, in respiring mitochondria and submitochondrial particles and in the pH range 6.2–8.3, are presented. The specific reduction levels of cytochrome b_{566} and b_{562} (calculated as described in the legend to Fig. 2) did not differ much from the reduction levels obtained from the overall absorbance changes recorded at 566–575 and 562–575nm.

Fig. 7 shows the pH-dependence of the respective transitions of cytochromes b_{566}, b_{562} and *c* induced by valinomycin, expressed as mV changes in actual redox potential. Both in mitochondria and in submitochondrial particles, collapse of $\Delta\psi$ by valinomycin caused substantial oxidation of cytochrome b_{566} (increase of E_h). Cytochrome b_{562} was also oxidized, but to a much lower extent.

The fact that the pattern of cytochrome *b* response to valinomycin was qualitatively similar in mitochondria and 'inside-out' submitochondrial particles verifies that their response was due to an effect of $\Delta\psi$ collapse on the redox poise rather than to effect of internal pH changes on the E_m.

It can be noted that valinomycin produced a greater oxidation of *b* cytochromes in mitochondria than in submitochondrial particles. In submitochondrial particles, collapse of $\Delta\psi$ by valinomycin depends on efflux of K^+ (Papa *et al.*, 1973*c*) and could be limited by the intravesicular concentration of K^+.

Both in mitochondria and in submitochondrial particles, the valinomycin-induced oxidation of cytochrome b_{566} decreased as the pH of the medium was raised from neutrality to alkaline values. Concomitantly, *c* cytochromes, which at acidic pH values exhibited a small reduction, showed, at alkaline pH values, oxidation on valinomycin addition. The low oxidation of cytochrome b_{562} caused by valinomycin was, on the other hand, practically unaffected by changing the external pH. It should be mentioned that, at all the pH values tested, valinomycin left cytochromes b_{566} and b_{562} still, in part, reduced. A similar pattern of E_h changes was obtained when these were directly calculated from the overall absorbance changes recorded at 566–575nm and 562–575nm. The differences in the response of cytochromes b_{566} and b_{562} to valinomycin, indicated by these absorbance changes, were smaller, as expected from the spectral overlapping of *b* cytochromes, but still significant.

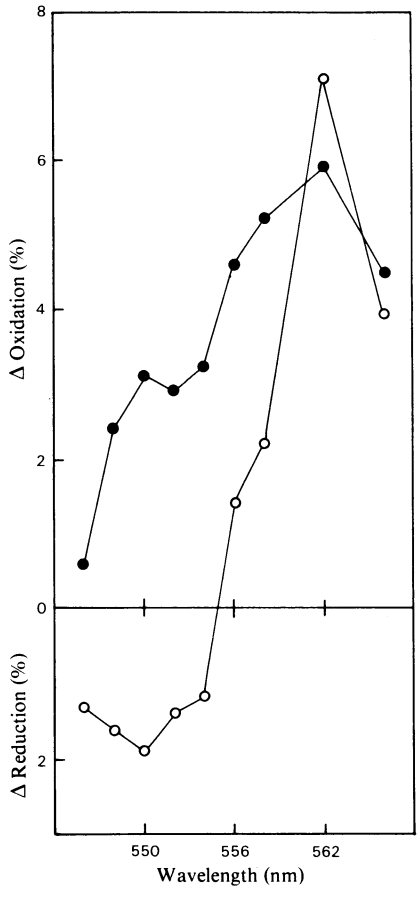


Fig. 5. *Spectra of the redox transitions of b and c cytochromes caused by nigericin addition to respiring ox heart mitochondria* (●) *and submitochondrial particles* (○)

The experimental conditions were those described in the legend to Fig. 4. The absorbance changes caused by nigericin addition were measured with the dual-wavelength spectrophotometer (see the Materials and methods section) on separate samples at the wavelengths indicated. The reference wavelength was kept constant at 540nm for measuring wavelengths varying from 546 to 558nm, and at 575nm for measuring wavelengths varying from 562 to 566nm. The redox transitions presented in the Figure are expressed as a percentage of the total absorbance increase measured at the given wavelengths on addition of solid dithionite to fully oxidized organelles. Submitochondrial particles were supplemented with oligomycin (2μg/mg of protein).

In other experiments the aerobic steady-state $\Delta\psi$ and transmembrane ΔpH were directly measured. $\Delta\psi$ was determined by the distribution of either ^{86}Rb, in the presence of valinomycin and absence of added K^+, or [^{3}H]methyltriphenylphosphonium bromide in the absence of valinomycin and in the reaction mixture used for monitoring oxidoreduction of cytochromes. The same $\Delta\psi$ values were found under the two conditions (Table 1). Respiration generated a steady-state $\Delta\mu_{H^+}$, which at pH7 amounted to 165mV, of which 86mV were contributed by $\Delta\psi$ and 79mV by ΔpH. At pH8, no change of $\Delta\psi$ was observed as compared with pH7, but ΔpH decreased by 30%. Submitochondrial particles also exhibited a $\Delta\psi$ of 90mV, which was unaffected by changing the pH from 7 to 8. Other controls showed that, both in mitochondria and in submitochondrial particles, valinomycin, added in the presence of nigericin, caused at pH8 the same stimulation of succinate respiration as at pH7.

Thus change of the external medium from neutrality to alkalinity does not cause *per se* any impairment of the capacity of mitochondria to conserve aerobic $\Delta\psi$, or of the control exerted by $\Delta\psi$ on overall electron flow from succinate to oxygen. The pH-dependence of the effect exerted by valinomycin on oxidation of cytochrome b_{566} results from a specific pH-dependence of the response of this cytochrome to $\Delta\psi$ collapse.

In Fig. 8 the effect of nigericin on the aerobic steady-state redox level of cytochromes is shown. Both in mitochondria and in submitochondrial particles, nigericin produced, at acidic and neutral pH, a significant shift of cytochrome b_{562} to a more oxidized state. Also, cytochrome b_{566} underwent oxidation, but to a smaller extent. In mitochondria the nigericin-induced oxidation of cytochrome b_{562} decreased as the pH was raised from 6.2 to 8.3. The same pattern was observed in submitochondrial particles, except that there was an increase of this effect from pH6.2 to 6.9. Nigericin caused oxidation of *c* cytochromes in mitochondria that declined as pH was raised, but reduction of *c* cytochromes in submitochondrial particles, which was augmented with increasing pH.

Fig. 9 shows that when ^1H_2O was replaced by ^2H_2O, valinomycin addition to respiring submitochondrial particles had clearly a different effect on the specific redox level of cytochrome b_{566} and b_{562}. Though cytochrome b_{566} underwent a significant oxidation, cytochrome b_{562} was almost unaffected. The shift in ^2H_2O of the peak of cytochrome b_{566} oxidation towards alkalinity with respect to that in ^1H_2O can be ascribed to the decreasing effect of ^2H_2O on the ionization constant of acidic groups in cytochrome b. In the ^2H_2O medium, nigericin caused net oxidation of *c* cytochromes over all the p^{2}H range.

Discussion

The results presented show that the $\Delta\psi$ and ΔpH components of aerobic transmembrane $\Delta\bar{\mu}_{H^+}$ exert

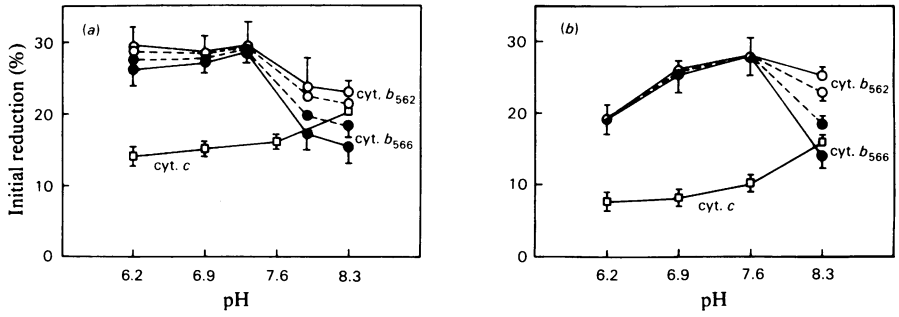


Fig. 6. *pH-dependence of the aerobic steady-state reduction levels of b and c cytochromes (cyt.) in respiring mitochondria (a) and submitochondrial particles (b)*

Both mitochondria and submitochondrial particles were supplemented with 10mM-succinate; to the latter, oligomycin (2μg/mg of protein) was also added. The stationary reduction levels of *b* cytochromes, expressed as a percentage of the total amount of dithionite-reducible cytochromes, were calculated either directly from the observed absorbance changes at 566–575 and 562–575nm (– – – –) or from the specific absorbance changes of cytochrome b_{566} and b_{562} (——), obtained from the former as described in the legend to Fig. 2. For experimental conditions, see the legend to Fig. 4. The Figures for the reduction levels of cytochromes in mitochondria and submitochondrial particles represent the means ± S.E.M. for eight experiments. O, Cytochrome b_{562}; ●, cytochrome b_{566}; □, cytochrome *c*.

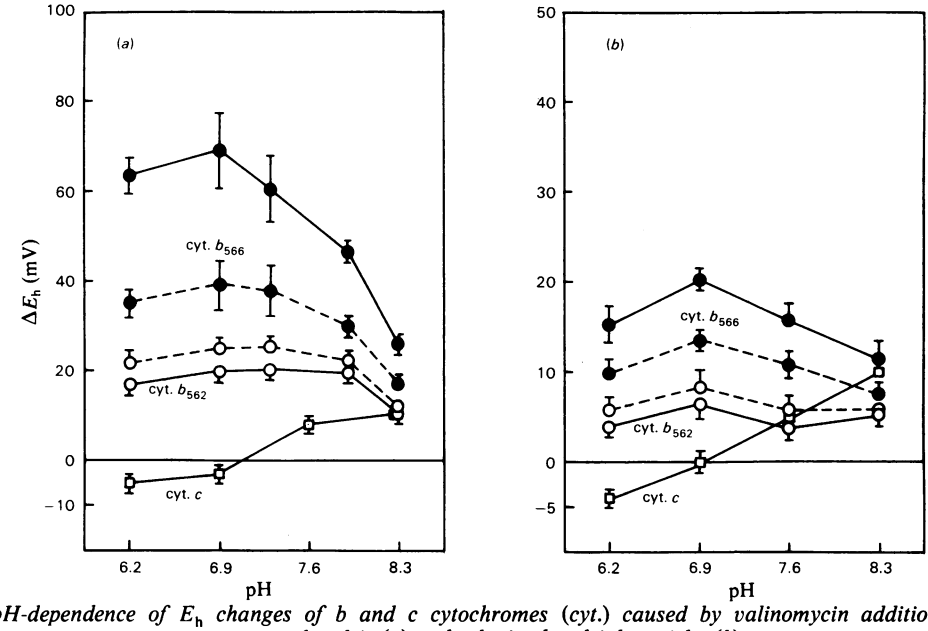


Fig. 7. *pH-dependence of E_h changes of b and c cytochromes (cyt.) caused by valinomycin addition to respiring mitochondria (a) and submitochondrial particles (b)*

For procedure, experimental conditions and calculations, see the legends to Figs. 2, 4 and 6. ΔE_h values refer to the difference in the redox level of cytochromes measured on the same sample after and before addition of valinomycin, either directly from the recorded absorbance changes at 566–575 and 562–575nm (– – – –) or from the specific changes of cytochromes b_{566} and b_{562} (——) calculated from the former as described in the legend to Fig. 2. The values for ΔE_h presented are the mean values (± S.E.M.) for four separate experiments. ●, Cytochrome b_{566}; O, cytochrome b_{562}; □, cytochrome *c*.

specific effects on different steps of the cytochrome system.

$\Delta\psi$ exerts a pH-dependent control on forward electron flow from cytochrome b_{566} to cytochrome b_{562}. This is documented by the following observations. (1) In mitochondria, as well as submitochondrial particles (Papa *et al.*, 1972), at pH values around neutrality, aerobic oxidation of

Table 1. *Steady-state membrane potential and transmembrane ΔpH in respiring ox heart mitochondria and submitochondrial particles*

Mitochondria (1.5mg of protein/ml) were incubated for 5min in a reaction mixture containing 200mM-sucrose, 5mM-Tris/HCl, 10mM-succinate, rotenone (1μg/mg of protein) and oligomycin (2μg/mg of protein). In expts. (*a*) the mixture was adjusted to the indicated pH values with 0.1M-NaOH, and 10μM-^{86}RbCl (10μCi/ml) plus valinomycin (0.4μg/mg of protein) were added. In expts. (*b*) the mixture was adjusted to the indicated pH values with 0.1M-KOH; in addition 20mM-KCl and 10μM-[^{3}H]methyltriphenylphosphonium bromide (2μCi/ml) were also present. Transmembrane ΔpH and matrix ^1H_2O were measured for both expts. (*a*) and (*b*) by adding to separate samples, in the absence of radioactive cations, ^3H_2O (10μCi/ml), [^{14}C]sucrose (10μCi/ml) and 20μM-[^{14}C]dimethyloxazolidinedione (2μCi/ml). Submitochondrial particles (2mg of protein/ml) were incubated in a reaction mixture containing 200mM-sucrose, 20mM-KCl, 50μM-KSCN, 5mM-Tris/HCl, 10mM-sodium succinate, rotenone (0.5μg/mg of protein) and oligomycin (2μg/mg of protein). The temperature was 25°C. The values represent means (±S.E.M.). ΔpH was not determined in submitochondrial particles. For experimental procedure and details, see the Materials and methods section.

	Parameter		pH7	pH8
Mitochondria	$\Delta\psi$ (mV)	(*a*)	86±5	87±4
		(*b*)	86±6	91±6
	$-59^*\,\Delta$pH (mV)	(*a*)	75±5	52±5
		(*b*)	83±3	65±4
Submitochondrial particles	$\Delta\psi$ (mV)		95±7	94±6

$^* = \dfrac{-2.303RT}{F}$

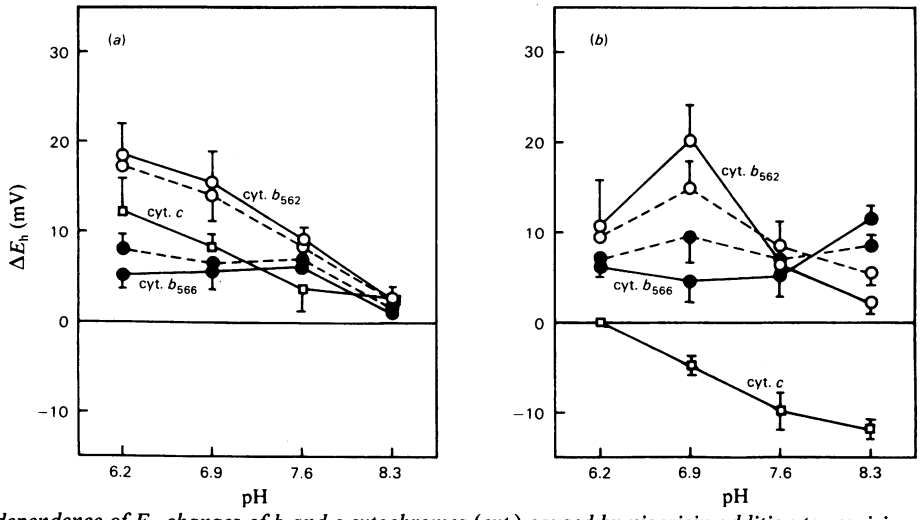


Fig. 8. *pH-dependence of E_h changes of b and c cytochromes (cyt.) caused by nigericin addition to respiring mitochondria (a) and submitochondrial particles (b)*

For procedure, experimental conditions and calculations, see the legends to Figs. 2 and 5. ΔE_h values refer to the difference in the redox level of cytochromes measured on the same sample after and before the addition of nigericin, either directly from the recorded absorbance changes at 566–575 and 562–575nm (----) or from the specific changes of cytochromes b_{566} and b_{562} (——) calculated from the former as described in the legend to Fig. 2. The values for ΔE_h presented are the mean values (± S.E.M.) for four separate experiments. O, Cytochrome b_{562}; ●, cytochrome b_{566}; □, cytochrome *c*.

cytochrome b_{562} was faster than that of cytochrome b_{566}. (2) In the presence of antimycin the oxygen-induced reduction of cytochrome b_{566} was, at pH6.5, much faster than that of cytochrome b_{562} (Fig. 3). (3) Collapse of $\Delta\psi$ by valinomycin: (*a*) accelerated aerobic oxidation of cytochrome b_{566} and decreased the net rate of cytochrome b_{562} oxidation; (*b*) promoted the oxygen/antimycin-dependent reduc-

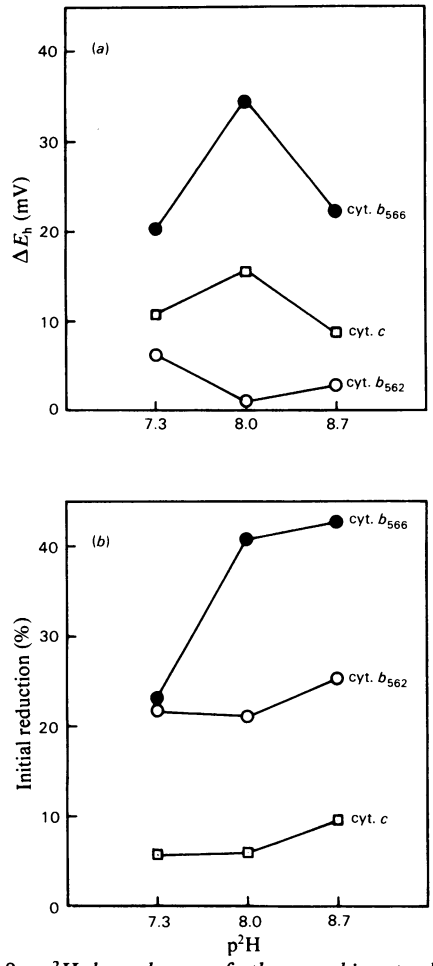


Fig. 9. *p^2H-dependence of the aerobic steady-state reduction levels (b) and E_h changes (a) of b and c cytochromes (cyt.) caused by valinomycin addition to respiring submitochondrial particles in ^2H_2O medium*
The reaction mixture was the same as that described in the legend to Fig. 4, with ^1H_2O replaced by ^2H_2O. Final p^2H was calculated from the pH-meter reading calibrated with buffers in ^1H_2O, by using the following equation: p^2H = pH-meter reading + 0.4. Temperature was 20°C. ΔE_h values refer to the difference in redox level of cytochromes measured from the specific absorbance changes of cytochromes b_{566} and b_{562} (see the legends to Figs. 2 and 7). For experimental conditions, see the legend to Fig. 4 and the Materials and methods section.

tion of cytochrome b_{562} and decreased that of cytochrome b_{566}; (*c*) at pH values around neutrality caused a preferential positive shift (oxidation) of the E_h of cytochrome b_{566} in respiring mitochondria or submitochondrial particles with respect to that of cytochrome b_{562}. ΔpH collapse by nigericin caused, on the contrary, preferential oxidation of cytochrome b_{562} with respect to cytochrome b_{566}. (4) The stimulatory effects exerted by $\Delta\psi$ collapse on aerobic oxidation of cytochrome b_{566} (and reduction of cytochrome b_{562} in the presence of antimycin) appears to be related to the pH-dependence of the E_m of *b* cytochromes (Straub & Colpa-Boonstra, 1962; Urban & Klingenberg, 1969; von Jagow *et al.*, 1978). In fact, these effects clearly evident at pH values where *b* cytochromes act essentially as pure electron carriers, tended to disappear as (increasing the pH above neutrality) *b* cytochromes changed to effective hydrogen carriers (Urban & Klingenberg, 1969; Papa, 1976).

Thus aerobic $\Delta\psi$ specifically impedes the transfer of electrons from cytochrome b_{566} to cytochrome b_{562}. It is possible that electrons passing from cytochrome b_{566} to b_{562} move across the membrane against the electrical field, i.e. from the outer (C-side, positive) to the inner side (M-side, negative).

The present observations, showing occurrence of forward electron transfer from cytochrome b_{566} to b_{562}, substantiate previous proposals (Mitchell, 1972, 1976; Papa *et al.*, 1975*b*) that *b* cytochromes are arranged in a linear sequence with b_{566} located at the C-side of the membrane and towards the substrate and b_{562} at the M-side towards oxygen. It seems, on the other hand, difficult to explain mechanisms that visualize cytochrome b_{566} as located closer to *c* cytochromes than is b_{562} (Chance *et al.*, 1970; Chance, 1972) or a mechanism, proposed by Malvija *et al.* (1980), that questions the occurrence of significant electron transfer from cytochrome b_{566} to b_{562}.

We further specify the characteristics of vectorial electron transfer from cytochrome b_{566} to b_{562} by proposing that when electrons are accepted by the haem iron of cytochrome b_{566} at the C-side of the membrane, protons are taken up at this side by the cytochrome and then transferred and released at the M-side, as electrons pass to b_{562}. Thus, at alkaline pH values, transmembrane electron flow would be replaced by effective hydrogen transfer so that the electric field control of the flow of reducing equivalents along the *b* cytochrome disappears.

The vectorial proton translocation that could result would take place in the direction opposite to that of the respiratory proton pump. It might contribute, by introducing a partial and pH-dependent back-flow of protons, to control the conversion of redox energy into transmembrane $\Delta\bar{\mu}_{H^+}$.

The present investigation shows control by transmembrane ΔpH of electron flow on the oxygen side of both *b* cytochromes. ΔpH collapse by nigericin caused in respiring mitochondria and submitochondrial particles a greater oxidation of cytochrome b_{562} relative to cytochrome b_{566}. Nigericin caused also *c*-cytochrome oxidation in mitochondria but *c*-cytochrome reduction in

submitochondrial particles. This difference is likely due to the fact that, in 'inside-out' particles, the oxygen protonation site of cytochrome oxidase, exposed in intact mitochondria to the matrix aqueous phase (Mitchell, 1976; Papa, 1976), becomes exposed to the outer space (Papa *et al.*, 1974*a*). Respiration produces significant pH increase in the matrix of mitochondria and pH decrease in the inner space of submitochondrial particles, with no appreciable pH change of the external medium.

In mitochondria, alkalinization of the matrix will decrease the rate of oxygen reduction to ^1H_2O by cytochrome *c* oxidase. ΔpH collapse by nigericin produces stimulation of cytochrome *c* oxidase. The opposite redox transitions of cytochrome b_{562} (oxidation) and *c* cytochromes (reduction), caused by nigericin in submitochondrial particles, can be ascribed to a collapse of the transmembrane ΔpH, which appears to exert a crossover effect between these two components.

The pH-dependent increase of the nigericin-induced reduction of *c* cytochrome, observed in submitochondrial particles, probably reflects the decrease of turnover of cytochrome *c* oxidase at alkaline pH values (Nicholls & Chance, 1974). This, as well as decrease of transmembrane ΔpH (Table 1; see also Rottenberg, 1978), can explain the decline at alkaline pH values of the nigericin-induced oxidation of cytochrome b_{562}, observed both in mitochondria and submitochondrial particles, and of the oxidation of *c* cytochromes in mitochondria.

Collapse of $\Delta\psi$ accelerates aerobic oxidation of cytochrome *c* (Papa *et al.*, 1974*b*). Thus $\Delta\psi$ exerts two 'crossover effects' on electron flow between cytochromes b_{566} and b_{562}, and between cytochrome *c* and oxygen. ^2H_2O, by decreasing the activity of a proton-translocating redox step between cytochromes b_{562} and *c* (Baum *et al.*, 1967; Leung & Hinkle, 1975; Papa, 1976; Papa *et al.*, 1977; cf. Mitchell, 1976), isolates cytochrome b_{562} from the pulling effect on forward electron flow exerted by $\Delta\psi$ collapse at the level of cytochrome *c* oxidase (see Fig. 9).

In conclusion, the present work, besides providing a description of specific effects exerted by pH, $\Delta\psi$ and transmembrane ΔpH on oxidoreductions in the *b–c* segment of the respiratory chain, offers a new insight into the possible implications that linkage between the redox state of the metal of *b* cytochromes, as well as of other electron carriers (Dutton & Wilson, 1972; Prince & Dutton, 1976; van Gelder *et al.*, 1977), and protolytic equilibria in these enzymes, might have for their function (Papa, 1976).

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References

Baum, H., Rieske, J. S., Silman, H. I. & Lipton, S. H. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 798–805

Berden, J. A., Opperdoes, F. R. & Slater, E. C. (1972) *Biochim. Biophys. Acta* **256**, 594–599

Chance, B. (1972) *FEBS Lett.* **23**, 3–19

Chance, B. & Williams, J. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134

Chance, B., De Vault, D., Legallais, V., Mela, L. & Yonetani, T. (1967) in *Fast Reactions and Primary Processes in Chemical Kinetics (Nobel Symp. 5th)* (Claesson, S., ed.), pp. 437–464, Interscience, New York

Chance, B., Wilson, D. F., Dutton, P. L. & Erecinska, M. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 1175–1182

Dutton, P. L. & Wilson, D. F. (1974) *Biochim. Biophys. Acta* **346**, 165–212

Dutton, P. L., Erecinska, M., Sato, N., Mukai, Y., Pring, M. & Wilson, D. F. (1972) *Biochim. Biophys. Acta* **267**, 15–24

Engel, W. D., Schägger, H. & von Jagow, G. (1979) in *Metalloproteins* (Weser, V., ed.), pp. 185–193, Thieme Verlag, Stuttgart

Erecinska, M., Chance, B., Wilson, D. F. & Dutton, P. L. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 50–54

Erecinska, M., Wilson, D. F. & Miyata, Y. (1976) *Arch. Biochem. Biophys.* **177**, 133–143

Kell, D. B., John, P., Sorgato, M. C. & Ferguson, S. J. (1978) *FEBS Lett.* **86**, 294–298

Lawford, H. G. & Garland, P. B. (1973) *Biochem. J.* **136**, 711–720

Lee, C. P. & Ernster, L. (1968) *Eur. J. Biochem.* **3**, 391–400

Leung, K. H. & Hinkle, P. C. (1975) *J. Biol. Chem.* **250**, 8467–8471

Löw, H. & Vallin, I. (1963) *Biochim. Biophys. Acta* **456**, 39–84

Malvija, A. N., Nicholls, P. & Elliott, W. B. (1980) *Biochim. Biophys. Acta* **589**, 137–149

Mitchell, P. (1972) *Proc. FEBS Meet. 8th, Amsterdam* **28**, 353–370

Mitchell, P. (1976) *J. Theor. Biol.* **62**, 327–367

Nicholls, D. G. (1974) *Eur. J. Biochem.* **50**, 305–315

Nicholls, P. & Chance, B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 479–534, Academic Press, London and New York

Papa, S. (1976) *Biochim. Biophys. Acta* **456**, 39–84

Papa, S., Guerrieri, F., Rossi Bernardi, L. & Tager, J. M. (1970) *Biochim. Biophys. Acta* **197**, 100–103

Papa, S., Scarpa, A., Lee, C. P. & Chance, B. (1972) *Biochemistry* **11**, 3091–3098

Papa, S., Guerrieri, F., Lorusso, M. & Simone, S. (1973*a*) *Biochimie* **55**, 703–716

Papa, S., Guerrieri, F., Simone, S., Lorusso, M. & Larosa, D. (1973*b*) *Biochim. Biophys. Acta* **292**, 20–38

Papa, S., Storey, B. T., Lorusso, M., Lee, C. P. & Chance, B. (1973*c*) *Biochem. Biophys. Res. Commun.* **52**, 1395–1402

Papa, S., Guerrieri, F. & Lorusso, M. (1974*a*) *Biochim. Biophys. Acta* **357**, 181–192

Papa, S., Guerrieri, F. & Lorusso, M. (1974*b*) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M. E., Quagliariello, E. & Siliprandi, N., eds.), pp. 177–186, North-Holland, Amsterdam

Papa, S., Lorusso, M. & Guerrieri, F. (1975*a*) *Biochim. Biophys. Acta* **397**, 425–440

Papa, S., Lorusso, M., Guerrieri, F. & Izzo, G. (1975*b*) in *Electron-Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. & Siliprandi, N., eds.), pp. 317–327, North-Holland, Amsterdam

Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., Boffoli, D. & Capuano, F. (1977) in *Biochemistry of Membrane Transport* (Semenza, G. & Carafoli, E., eds.), pp. 504–519, Springer-Verlag. Berlin and Heidelberg

Papa, S., Guerrieri, F. & Izzo, G. (1979) *FEBS Lett.* **105**, 213–216

Prince, R. C. & Dutton, P. L. (1976) *FEBS Lett.* **65**, 117–119

Rieske, J. S. (1976) *Biochim. Biophys. Acta* **456**, 195–274

Rottenberg, H. (1978) in *Mechanism of Proton and Calcium Pumps* (Azzone, G. F., Avron, M., Metcalfe, J. C., Quagliariello, E. & Siliprandi, N. eds.), vol. 2, pp. 125–135, Elsevier/North-Holland, Amsterdam

Schuldiner, S. & Kaback, H. R. (1975) *Biochemistry* **14**, 5451–5459

Straub, J. P. & Colpa-Boonstra, J. P. (1962) *Biochim. Biophys. Acta* **60**, 650–652

Urban, P. F. & Klingenberg, M. (1969) *Eur. J. Biochem.* **9**, 519–525

van Gelder, B. F., van Rijn, J. L. M. L., Schilder, G. J. A. & Wilms, J. (1977) in *Structure and Function of Energy-Transducing Membranes* (van Dam, K. & van Gelder, B. F., eds.) (*BBA Libr.* **14**), pp. 61–68, Elsevier, Amsterdam

von Jagow, G. (1975) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. & Siliprandi, N., eds.), pp. 23–30, North-Holland, Amsterdam

von Jagow, G., Schägger, H., Engel, W. D., Hackenberg, H. & Kolb, H. G. (1978) in *Energy Conservation in Biological Membranes* (*Mosbach Colloq.*) (Schäfer, G. & Klingenberg, M., eds.), pp. 200–209, Springer-Verlag, Berlin

Wainio, W. W. (1977) *J. Bioenerg. Biomembr.* **9**, 321–335

Weiss, H. (1976) *Biochim. Biophys. Acta* **456**, 291–313

Wikström, M. K. F. (1973) *Biochim. Biophys. Acta* **301**, 155–193

Wikström, M. K. F. & Lambowitz, A. M. (1974) *FEBS Lett.* **40**, 149–153

Wilson, D. F. & Erecinska, M. (1975) *Arch. Biochem. Biophys.* **167**, 116–128

Wilson, D. F., Lindsay, J. C. & Brockleurst, E. S. (1972) *Biochim. Biophys. Acta* **256**, 277–286

Wyman, J. (1968) *Q. Rev. Biophys.* **1**, 35–81