The mechanism of proton translocation by the cytochrome system of mitochondria

Characterization of proton-transfer reactions associated with oxidoreductions of terminal respiratory carriers

Sergio PAPA, Ferruccio GUERRIERI and Gianfranco IZZO

Institute of Biological Chemistry, Faculty of Medicine and Centre for the Study of Mitochondria and Energy Metabolism, C.N.R., University of Bari, Bari, Italy

(Received 23 March 1983/Accepted 21 June 1983)

1. A direct kinetic analysis is presented of rapid proton-releasing reactions at the outer or C-side of the membrane, in ox heart and rat liver mitochondria, associated with aerobic oxidation of reduced terminal respiratory carriers in the presence of antimycin. 2. Valinomycin plus K^+ enhances the rate of cytochrome *c* oxidation and the rate and extent of H^+ release. In the presence of valinomycin the $\rightarrow H^+/e^-$ ratio, computed on the basis of total electron flow from respiratory carriers to oxygen, varies with pH, remaining always lower than 1, and is unaffected by *N*-ethylmaleimide. 3. 2-Heptyl-4-hydroxyquinoline *N*-oxide and 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole, at concentrations which inhibit in the presence of antimycin the oxygen-induced reduction of *b* cytochromes, cause also a marked depression of the H^+ release associated with aerobic oxidation of terminal respiratory carriers. 4. Aerobic oxidation of the cytochrome system in mitochondria and of isolated *b*-*c*₁ complex and cytochrome *c* oxidase results in scalar proton release from ionizable groups (redox Bohr effects). In mitochondria and submitochondrial particles, about 70% of the oxidoreductions of the components of the cytochrome system are linked to scalar proton transfer by ionizable groups. In isolated *b*-*c*₁ complex scalar proton transfer, resulting from redox Bohr effect, amounts to $0.9 H^+$ per Fe-S protein ($190 \mu T$). In isolated cytochrome *c* oxidase, Bohr protons amount to 0.8 per haem *a* + *a*₃. 5. The results presented indicate that the H^+ release from mitochondria during oxidation of terminal respiratory carriers derives from residual antimycin-insensitive electron flow in the quinone–cytochrome *c* span and from redox Bohr effects in the *b*-*c*₁ complex and cytochrome *c* oxidase. There is no sign of proton pumping by cytochrome oxidase during its transition from the reduced to the active ‘pulsed’ state and the first one or two turnovers.

Electron flow along the cytochrome system of mitochondria results in the generation of transmembrane thermodynamic potential difference of Protons ($\Delta\bar{\mu}_{H^+}$). The mechanism by which $\Delta\bar{\mu}_{H^+}$ is generated is, however, still unsolved (Mitchell, 1976, 1979; Papa, 1976*a*, 1982*a*; Papa *et al.* 1979*a*; Wikström & Krab, 1979; Wikström *et al.*, 1981).

As a direct approach to this problem, kinetic analysis has been developed and directed to: (i) resolve the proton-transfer reactions associated with individual components of the system; (ii) localize the sites in the membrane where these take place; (iii)

define the kinetics and stoichiometric relationship of individual proton and electron-transfer reactions (Papa *et al.*, 1974*a*, 1975, 1978*b*). Investigations along these lines provided evidence showing that protons, consumed in the reduction of dioxygen to H_2O , reach cytochrome oxidase (EC 1.9.3.1) from the inner or M-side of the mitochondrial membrane (Papa *et al.*, 1974*a,b*; Papa, 1976*a*; cf. Mitchell, 1969; Mitchell & Moyle, 1967, 1970).

Another category of proton-transfer reactions, relevant here, are those arising from co-operative linkage between redox transitions of the metals of components of the cytochrome system and protonation of ionizable groups in the apoproteins (Papa *et al.*, 1973; Papa, 1976*a*). The occurrence of

Abbreviation used: HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

these linkage phenomena, which by analogy to the Bohr effects of haemoglobin (Wyman, 1968; Kil-martin & Rossi-Bernardi, 1973) are denominated 'redox Bohr effects' (Chance, 1972; Papa, 1976a), is indicated by the pH-dependence of the midpoint redox potential observed for cytochromes and non-haem iron proteins (Clark, 1960; Dutton & Wilson, 1974; Prince & Dutton, 1976).

Redox Bohr effects in the cytochrome system of mitochondria have been verified by means of direct measurement of scalar proton transfer associated with redox transitions of electron carriers (Papa *et al.*, 1979c). Mechanisms have also been put forward, on the basis of which redox Bohr effects could acquire vectoriality in the membrane and could participate in proton pumping by the cytochrome system (Papa, 1976a; Papa *et al.*, 1973, 1977a; Von Jagow & Engel, 1980; Wikström *et al.*, 1981; Papa, 1982a).

We present here a study of the kinetics, H^+/e^- stoichiometry, nature and possible role of proton-transfer reactions associated with aerobic oxidation of components of the cytochrome system.

Materials and methods

Chemicals

Antimycin A, valinomycin, oligomycin, rotenone, HOQNO, *N*-ethylmaleimide and bovine serum albumin were purchased from Sigma Chemical Co. 5-(*n*-Undecyl)-6-hydroxy-4,7-dioxobenzothiazole was kindly provided by Dr. B. L. Trumpower, Dartmouth Medical School, Hanover, NH, U.S.A. All other reagents were of the highest purity grade available.

Preparation of mitochondria and submitochondrial particles

Ox-heart mitochondria were prepared as described by Löw & Vallin (1963). Rat liver mitochondria were prepared as described by Myers & Slater (1957). Submitochondrial particles were prepared by exposing ox heart mitochondria to ultrasonic energy in the presence of EDTA as described by Lee & Ernster (1968). The inverted orientation of the mitochondrial inner membrane in submitochondrial particles was determined from cytochrome *c* stimulation of succinate and NADH respiration by using the relationship:

$$\text{Percentage inversion} = \frac{\text{Non-stimulated respiratory rate}}{\text{Stimulated rate}} \times 100$$

which is based on inaccessibility of cytochrome *c* to its reaction site in 'inside-out' vesicles (Harmon *et al.*, 1974). The percentage inversion was found to range, from preparation to preparation, from 96% to 100% (see also Harmon *et al.*, 1974; Huang *et al.*, 1973).

Isolation of the *b-c*₁ complex and cytochrome *c* oxidase

The *b-c*₁ complex was prepared from ox heart mitochondria as described by Rieske *et al.* (1964). The isolated enzyme contained about 7 nmol of cytochromes *b* and 3.5 nmol of cytochrome *c*₁ per mg of protein (Rieske *et al.*, 1964; Nelson & Gellerfors, 1978). Cytochrome *c* oxidase was isolated from ox heart mitochondria as described by Errede *et al.* (1978). The isolated enzyme contained 10–11 nmol of haem *a* + *a*₃ per mg of protein.

Incubation procedure

Mitochondria or submitochondrial particles were incubated in a basal reaction mixture containing sucrose, KCl, potassium succinate and potassium malonate at the concentrations given in the legends to Figures and Tables with the other additions there indicated. The pH was adjusted by addition of 0.1 M-KOH to the values indicated in the legends to Figures and Tables. Samples of the same suspension were transferred to thermostatically controlled measurement cells for simultaneous determination of the rate of electron flow and proton translocation. Other details are specified in the legends to Figures and Tables.

Flow measurements

Rapid aerobic oxidation of cytochromes and ubiquinol were monitored with a dual-wavelength spectrophotometer equipped with a regenerative stopped-flow apparatus (mixing ratio 1:61) (Chance *et al.*, 1967), as previously described (Papa *et al.*, 1980a).

Continuous-flow pH measurements

These were performed with a Roughton-type repetitive continuous-flow pH-meter (mixing ratio 1:60) with a resolution time of 2 ms as previously described (Papa *et al.*, 1979b, 1980a).

Determination of redox Bohr effects

Experiments were carried out as described by Papa *et al.* (1979c) and Guerrieri *et al.* (1981). For experimental details, see the legends to Fig. 6 and Table 6 below.

Protein

Mitochondrial protein was determined by the biuret method, with bovine serum albumin as standard and correcting for residual KCN absorbance (Szarkowska & Klingenberg, 1963).

Results

Redox-linked proton-transfer reactions in intact mitochondria

Fig. 1 shows flow analysis of redox events and proton translocation elicited by oxygenation of ox

Fig. 1. Effect of valinomycin on the kinetics of oxidation of respiratory carriers and of respiration-driven H^+ release in antimycin-inhibited ox heart mitochondria

Mitochondria (2 mg of protein/ml) were incubated in a basal reaction mixture containing 200 mM-sucrose, 30 mM-KCl, 5 mM-potassium succinate, 5 mM-potassium malonate, $2 \mu\text{g}$ of oligomycin/mg of protein, $0.5 \mu\text{g}$ rotenone/mg of protein and $1 \mu\text{g}$ of antimycin A/mg of protein. The final pH was 6.8 and the temperature 25°C . After anaerobiosis, oxygenation was brought about by repetitive additions of the following medium (contained in the side syringe): 200 mM-sucrose, 30 mM-KCl and 5 mM-potassium malonate, pH 6.8. Additions: expt. (a) none; expt. (b) $0.1 \mu\text{g}$ of valinomycin $\cdot (\text{mg of protein})^{-1}$. For other details, see the Materials and methods section. The numbers on the traces are the t_1 values for the reactions expressed in ms. Ubiquinol (QH_2) oxidation was monitored at 280–289 nm. This narrow wavelength difference minimized light-scattering and stray-light artefacts. In addition, a 10 mm thickness of a saturated nickel sulphate/cobalt sulphate solution was used as light filter and placed between the observation chamber and the phototube (Kröger & Klingenberg, 1966). Abbreviation used: cyt., cytochrome.

heart mitochondria, brought to anaerobiosis by succinate oxidation and supplemented with malonate, rotenone and antimycin. Upon oxygenation, cytochromes a and a_3 were almost completely oxidized during the continuous-flow phase (6 ms interval). The oxidation of cytochrome c [t_1 (half reaction time) 9 ms] was accompanied by a small residual oxidation of ubiquinone, proton release and reduction of b cytochromes (cf. Erecinska *et al.*, 1972; Papa *et al.*, 1972). Valinomycin, added at a

concentration of $0.1 \mu\text{g} \cdot (\text{mg of protein})^{-1}$, practically doubled the rate of oxidation of cytochrome c and ubiquinone and the rate of reduction of b cytochromes (cf. Papa *et al.*, 1974b, 1981). These effects of redox reactions were accompanied by a twofold enhancement of the rate and extent of proton release (Fig. 1, curve b). It can be noted that the proton-release reaction, with t_1 of 17 and 9 ms in the absence and presence of valinomycin respectively, was slower than the oxidation of cytochrome

oxidase and *c* cytochromes, but practically synchronous with the residual antimycin-insensitive oxidation of ubiquinol ($t_{1/2}$ of 15 and 9 ms in the absence and presence of valinomycin respectively).

The H^+/e^- stoichiometry and the factors affecting this quotient were analysed in a series of experiments similar to that presented in Fig. 1. The H^+/e^- ratio was routinely measured from the extent of electron flow and proton release at an interval of 50 ms after oxygenation. In fact at this interval the redox reactions and proton release were almost completed and the H^+/e^- ratio reached the maximal value, which then remained constant up to 200–300 ms after oxygenation.

Table 1 illustrates the effect of valinomycin on the H^+/e^- ratio at pH 7.2. Total electron flow was computed by summing up the nmol of cytochrome $a + a_3$ oxidized and an equivalent amount of copper atoms (Nicholls & Chance, 1974), the nmol of cytochromes $c + c_1$ and of the Fe–S protein of the $b-c_1$ complex oxidized (the latter is taken as equivalent to half the amount of *c* cytochromes oxidized) and half the nmoles of quinol oxidized. It is, in fact, generally agreed that of the two reducing equivalents donated by a quinone molecule to the $b-c_1$ complex, one is transferred via an antimycin-insensitive pathway to the Rieske Fe–S protein and cytochrome c_1 , and the second is transferred to *b* cytochromes and from this to the Fe–S protein and

cytochrome c_1 via an antimycin-sensitive pathway (Wikström & Berden, 1972; Erecinska *et al.*, 1972; Mitchell, 1976; Papa, 1976a; Wikström *et al.*, 1981). In the experiments of Table 1 the oxygen-induced reduction of *b* cytochromes was also measured and found to correspond practically to half the quinol oxidized. Since for technical reasons it is, however, easier and more accurate to measure cytochrome *b* reduction rather than the small antimycin-insensitive ubiquinol oxidation, in subsequent experiments (Tables 2–5) the oxygen-induced reduction of *b* cytochromes was directly measured and taken as a measure of the e^- equivalents flowing, in the presence of antimycin, from quinol to oxygen.

The extent of proton release at 50 ms was almost doubled by $0.5 \mu\text{g}$ of valinomycin per mg of protein; overall electron transfer was enhanced by 40%. The result was that the H^+/e^- ratio, which amounted to 0.43 in the control, was raised by valinomycin to 0.58. The fact that $0.5 \mu\text{g}$ of valinomycin per mg of protein caused a much greater enhancement of the extent of proton ejection than that of electron transport indicates that, in the absence of the ionophore, there was electrophoretic proton back-flow even within 50 ms of oxygenation. To be sure that the effective potassium conductance induced by valinomycin was high enough to prevent any loss by electrophoretic back-flow of the proton released,

Table 1. *Stoichiometry of H^+ release and oxidation of redox carriers caused by oxygen pulses of anaerobic antimycin-inhibited ox heart mitochondria: effect of valinomycin*

The experimental conditions and procedures are those described in the legend of Fig. 1, except for the pH, which was 7.2. The values reported in the Table refer to 50 ms after oxygenation. The amount (nmol) of haems $a + a_3$ was calculated with a $\Delta\epsilon_{\text{mm}}$ of 14 from the absorbance changes at 605–630 nm (Nicholls & Kimelberg, 1972). The amount (ng-atoms) of copper oxidized was taken as equivalent to the amount (nmol) of haems $a + a_3$ oxidized (Nicholls & Chance, 1974). The amount (nmol) of *c* cytochromes was calculated with a $\Delta\epsilon_{\text{mm}}$ of 19.1 from the absorbance changes at 550–540 nm (Chance & Williams, 1956). The amount (nmol) of Fe–S protein ($190 \mu\text{T}$) was taken as equivalent to half the amount of *c* cytochromes oxidized. The amount (nmol) of ubiquinol oxidized was calculated with a $\Delta\epsilon_{\text{mm}}$ of 8.8 from the absorbance changes at 280–289 nm (Kröger & Klingenberg, 1966). The amount (nmol) of *b* cytochromes reduced, calculated with a $\Delta\epsilon_{\text{mm}}$ of 20 from the absorbance changes at 562–575 (Hatefi *et al.*, 1961), was found to be equivalent to half the amount of ubiquinol oxidized. Additions: (a) none; the data are means $\pm$ S.E.M. for three experiments; (b) $0.5 \mu\text{g}$ of valinomycin/mg of protein; data are means $\pm$ S.E.M., $n = 5$; (c) $1.5 \mu\text{g}$ of valinomycin/mg of protein; data are means $\pm$ S.E.M., $n = 5$.

	(a)	(b)	(c)
H^+ released [ng-ions $\cdot$ (mg of proteins) $^{-1}$]	0.78 ± 0.06	1.49 ± 0.30	1.74 ± 0.10
Respiratory carriers oxidized [nmol $\cdot$ (mg of proteins) $^{-1}$]			
$a + a_3$	0.63 ± 0.01	0.82 ± 0.00	0.89 ± 0.05
Cu	0.63 ± 0.01	0.82 ± 0.09	0.89 ± 0.05
$c + c_1$	0.29 ± 0.02	0.48 ± 0.09	0.66 ± 0.02
Fe–S ($190 \mu\text{T}$)	0.14 ± 0.01	0.24 ± 0.04	0.33 ± 0.01
$\frac{1}{2}\text{QH}_2$	0.12 ± 0.01	0.21 ± 0.04	0.26 ± 0.01
Σe^- flow	1.81 ± 0.06	2.57 ± 0.33	3.03 ± 0.13
$H^+/\Sigma e^-$ flow	0.43 ± 0.02	0.58 ± 0.04	0.57 ± 0.05

measurements were repeated in the presence of 1.5 μg of valinomycin per mg of protein. At this concentration valinomycin caused a significant further enhancement of oxidation of *c* cytochromes at 50 ms of oxygenation as compared with that found with 0.5 μg of valinomycin per mg of protein. Also, proton release was further enhanced. This stimulation was, however, smaller than that observed for *c* cytochromes. The H^+/e^- ratio remained unchanged. It is evident that, although concentrations of valinomycin higher than 0.5 μg per mg of protein were needed to develop an effective potassium conductance high enough to fully collapse the aerobic $\Delta\psi$ in a 50 ms interval, the conductance induced by 0.5 μg of valinomycin was already sufficient to prevent any loss of H^+ release by proton back-flow.

Table 2. *Stoichiometry of H^+ release and oxidation of redox carriers caused by oxygen pulses of anaerobic antimycin-inhibited ox heart mitochondria: effect of exogenous cytochrome *c**

The experimental conditions and procedures are those described in the legend to Fig. 1, except that 0.5 μg of valinomycin/mg of protein was also present. The reduction of *b* cytochromes was measured from the absorbance changes at 562–575 nm ($\Delta\epsilon_{\text{nm}}=20$). The e^- equivalent to cytochrome *b* reduction was included in the calculation of total electron flow to oxygen, as representing as estimate of the electron transferred in the presence of antimycin, from ubiquinol to oxygen (see the text). The other calculations were carried out as described in the legend to Table 1. Additions: Expt. (a) none; expt. (b) 2.2 μM -cytochrome *c*; expt. (c) 6 μM -cytochrome *c*. Bovine heart cytochrome *c* type IV was purchased from Sigma Chemical Co. The turnovers undergone by cytochrome oxidase were calculated as:

$$\frac{\sum c + c_1 + \text{Fe-S} + \text{reduced cytochrome } b \text{ (nmol)}}{\sum a + a_3 + \text{Cu (mol)}} + 0.5$$

(this half turnover corresponds to the initial transition of the redox centres of the oxidase from the reduced to the oxidized state). The values reported in the table refer to 50 ms after oxygenation.

	Expt.	...	(a)	(b)	(c)
H^+ released [ng-ions · (mg of protein) ⁻¹]			1.52	1.68	1.68
Respiratory carriers oxidized [nmol · (mg of protein) ⁻¹]					
<i>a</i> + <i>a</i> ₃			0.77	0.76	0.60
Cu			0.77	0.76	0.60
<i>c</i> + <i>c</i> ₁			0.36	0.97	1.01
Fe-S (190 μT)			0.18	0.18	0.19
e^- equivalent to cytochrome <i>b</i> reduced			0.17	0.12	0.29
Σe^- flow			2.25	2.79	2.69
$\text{H}^+/\Sigma \text{e}^-$ flow			0.68	0.60	0.63
Turnovers of cytochrome oxidase			0.96	1.33	1.74

It can be noted (see Table 3) that enhancement of the external potassium concentration above 50 mM, which was the concentration generally used in the present experiments, had, in the presence of excess valinomycin, no further stimulatory effect on the oxidation of electron carriers and proton release. It can be concluded that the effective potassium conductance developed by valinomycin was, under the prevailing experimental conditions, sufficient to measure the maximal release of protons associated with antimycin-insensitive aerobic oxidation of terminal respiratory carriers.

In the experiments so far described, electron flow was practically limited to oxidation of endogenous respiratory carriers on the oxygen side of the antimycin-inhibition site. It has been pointed out (Wikström & Krab, 1979) that, under these circumstances, the available amount of electrons outside cytochrome oxidase were not enough to reduce completely the four redox centres of the oxidase after they were oxidized upon oxygenation. In order to verify the situation arising when the

Table 3. *Stoichiometry of H^+ release and oxidation of redox carriers caused by oxygen pulses of anaerobic antimycin-inhibited ox heart mitochondria: effect of ionic strength*

The experimental conditions and procedures are those described in the legend to Fig. 1, except that 0.5 μg of valinomycin/mg of protein was also present and the incubation of mitochondria was carried out in the incubation medium described below. After anaerobiosis, oxygenation was brought about by repetitive additions of medium (i) or (ii) with the addition of 5 mM-potassium malonate. The pH was 6.8. The amount (nmol) of *b* cytochromes reduced was measured at 562–575 nm (see legend to Table 2). Calculations were carried out as described in the legend to Table 1. Incubation medium: (i) 100 mM-sucrose + 80 mM-KCl; (ii) 150 mM-KCl. Additions: (I) none; (II) + 6 μM -cytochrome *c*. The values reported in the Table refer to 50 ms after oxygenation.

	I		II	
	(i)	(ii)	(i)	(ii)
H^+ released [ng-ions · (mg of protein) ⁻¹]	1.2	1.3	1.82	1.76
Respiratory carriers oxidized [nmol · (mg of protein) ⁻¹]				
<i>a</i> + <i>a</i> ₃	0.68	0.75	0.69	0.72
Cu	0.68	0.75	0.69	0.72
<i>c</i> + <i>c</i> ₁	0.32	0.24	1.13	1.35
Fe-S (190 μT)	0.16	0.11	0.16	0.12
e^- equivalent to cyt. <i>b</i> reduced	0.17	0.19	0.38	0.34
Σe^- flow	2.01	2.04	3.05	3.25
$\text{H}^+/\Sigma \text{e}^-$ flow	0.60	0.64	0.60	0.54
Turnovers of cytochrome oxidase	0.98	0.87	1.71	1.76

oxidase undergoes one full turnover or more, the amount of electrons entering the oxidase was increased to the required level by adding, to the mitochondrial suspension, exogenous ferricytochrome *c*. This was completely reduced in anaerobiosis and rapidly oxidized upon oxygenation. It can be seen that addition of exogenous cytochrome *c* caused some enhancement of proton release, but this was smaller than the enhancement of total electron flow to oxygen (Table 2). Thus increase of the amount of electrons entering the oxidase to a level sufficient for a full turnover or more did not cause any enhancement of the $\rightarrow\text{H}^+/\text{e}^-$ ratio; rather, a small decrease was apparent as compared with the control, where the oxidase underwent a little less than one turnover.

More recently Wikström & Penttilä (1982) have reported that vectorial proton translocation could be caused by aerobic oxidation of externally added ferrocytochrome *c* in rat liver mitochondria provided that the osmolarity of the medium was contributed by 120 mM-KCl. The experiments presented in Table 3 show, however, that enhancement of the K^+ concentration in the medium from 50 mM (see Table 2) to 100 and 170 mM had no stimulatory effect on the oxidation of respiratory carriers or on H^+ release from ox heart mitochondria in the absence or presence of externally added cytochrome *c*.

Fig. 2 illustrates the pH-dependence of the $\rightarrow\text{H}^+/\text{e}^-$ ratio for proton release associated with aerobic oxidation of terminal respiratory carriers. Various points emerge from these experiments: (i) the $\rightarrow\text{H}^+/\text{e}^-$ ratio exhibited a peculiar pH-dependence: it first decreased as the pH was enhanced from 6.8 to 8.2, then increased as the pH was further raised to 9.1; (ii) the $\rightarrow\text{H}^+/\text{e}^-$ ratio was, over the pH range 6.8–9.1, always lower than 1; (iii) $\rightarrow\text{H}^+/\text{e}^-$ ratio was unaffected by *N*-ethylmaleimide at a concentration sufficient to block H^+ /anion symports (Papa, 1976b; Tischler *et al.*, 1976). Thus, in the short interval monitored (50 ms), there was no significant effect of proton back-flow, through H^+ /anion symport, on the $\rightarrow\text{H}^+/\text{e}^-$ stoichiometry.

Effect of HOQNO and 5-(n-undecyl)-6-hydroxy-4,7-dioxobenzothiazole

The experiment of Fig. 3 shows that HOQNO, at a concentration of $10 \text{ nmol} \cdot (\text{mg of protein})^{-1}$, caused a marked decrease in the rate of reduction of *b* cytochromes, leaving the oxidation of cytochrome oxidase and *c* cytochromes unaffected. This was accompanied by a significant depression of the rapid H^+ release induced by oxygenation. The result was that the $\rightarrow\text{H}^+/\text{e}^-$ ratio fell down from 0.51 in the control with antimycin alone to 0.34 in the presence of antimycin plus HOQNO (see Table 4) (cf. Papa *et al.*, 1978c). Fig. 4 shows that the titre for

Fig. 2. pH-dependence of $\rightarrow\text{H}^+/\text{e}^-$ ratio for antimycin-insensitive aerobic H^+ release from ox heart mitochondria

The experimental conditions and procedures are those described in the legend to Fig. 1, except that the pH was adjusted to the values shown in the Figure and $0.5 \mu\text{g}$ of valinomycin $\cdot (\text{mg of protein})^{-1}$ was always present. Calculations were carried out as described in the legend to Table 1. The bars indicate s.e.m. and the number of experiments was five. Symbols: ●, control experiments; ○, 30 nmol of *N*-ethylmaleimide $\cdot (\text{mg of protein})^{-1}$ was added to the reaction mixture in addition to the components present in the control experiments (results are means of two experiments).

HOQNO inhibition of antimycin-insensitive oxygen-induced H^+ release was practically the same as that exhibited for depression of oxygen-induced cytochrome *b* reduction. Fig. 5 shows that the depression of H^+ release brought about by HOQNO was in part counteracted by raising the concentration of antimycin in the system.

The effect of 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole on the antimycin-insensitive aerobic oxidation of respiratory carriers and associated H^+ release was also tested. This compound inhibits the *b*-*c*₁ complex at a site different from that inhibited by antimycin (Trumpower & Haggerthy, 1980; Trumpower, 1981). It can be seen (Table 4) that $2 \mu\text{M}$ -5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole had effects similar to HOQNO. It in fact depressed specifically the aerobic reduction of *b* cytochromes, leaving the oxidation of *c* cytochromes and cytochrome oxidase unaffected, and caused a marked depression of H^+ release, so that the $\rightarrow\text{H}^+/\text{e}^-$ fell from 0.56 to 0.24.

The experiments so far reported were all carried out with ox heart mitochondria that were kept frozen for weeks before being thawed for use. Although direct measurements (Papa *et al.*, 1974a) showed that the rate of passive back-diffusion of protons in

Fig. 3. Effect of HOQNO on H^+ release and oxidation-reduction of redox carriers caused by oxygen pulses of anaerobic antimycin-inhibited ox heart mitochondria

Mitochondria (3 mg of protein/ml) were incubated in the reaction mixture described in the legend to Fig. 1, with the addition of $0.5 \mu\text{g}$ of valinomycin $(\text{mg of protein})^{-1}$; the pH was 7.2. After anaerobiosis, oxygenation was brought about by repetitive additions of the medium described in the legend to Fig. 1, except that the pH was adjusted to 7.2. Additions: expt. (a) none; expt. (b) $10 \text{ nmol of HOQNO} \cdot (\text{mg of protein})^{-1}$. The numbers on the traces represent $t_{1/2}$ values for the reactions expressed in ms. Abbreviation used: cyt., cytochrome.

these mitochondria was negligible with respect to rapid redox-linked proton release in the short-time intervals explored ($t_{1/2} \approx 17$ and 9 ms in the absence and presence of valinomycin respectively), experiments were carried out on freshly isolated rat liver mitochondria. The results presented in Table 5 show that, in these mitochondria, rapid aerobic oxidation of endogenous respiratory carriers plus added cytochrome *c* in the presence of antimycin was associated with proton release at a stoichiometry of $0.7 H^+/e^-$. As observed with ox heart mitochondria, $2 \mu\text{M}$ -5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole lowered the H^+/e^- ratio to 0.25.

Redox Bohr effects in the cytochrome system

To measure redox Bohr effects, ox heart mitochondria and submitochondrial particles were treated with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to abolish vectorial proton translocation, and the net extents of electron-carrier

oxidation and proton consumption, observed upon transition of the system from anaerobiosis to the aerobic steady state, were measured. Under these conditions, aerobic oxidation of electron carriers on the oxygen side of the antimycin block resulted, in both mitochondria and submitochondrial particles, in net proton consumption. Upon exhaustion of added oxygen, re-reduction of these components by succinate produced the release of the same amount of protons taken up upon oxygenation. Proton uptake and release were, however, considerably lower than the sum of the electron carriers undergoing a cycle of oxidation and reduction. This difference, which is due to proton release from ionizable groups of terminal respiratory carriers associated with their oxidation and proton uptake upon their re-reduction, provides an overall estimate of Bohr effects in this segment of the respiratory chain.

The numbers of Bohr protons per electron transferred to oxygen, calculated in this way, are

Fig. 4. Titration of the effect of HOQNO on the first-order-kinetics constant of cytochrome *b* reduction (a) and H⁺ release (b), induced by oxygenation of anaerobic antimycin inhibited ox heart mitochondria
For experimental conditions see the legend to Fig. 3. (a) First-order kinetics constant of reduction of *b* cytochromes: O, measurements at 566–575 nm; ●, measurements at 562–575 nm; (b) ●, H⁺ release at 30 ms after oxygenation of antimycin-treated ox heart mitochondria.

Table 4. Effect of HOQNO and 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole on H⁺ release and oxidation of redox carriers caused by oxygen pulses of anaerobic antimycin-inhibited ox heart mitochondria

For experimental conditions and procedures, see the legend to Fig. 3. Calculations were carried out as described in the legends to Tables 1 and 2. Two different batches of ox heart mitochondria were used for expts. I and II. Additions: expt. (a) none; expt. (b) 10 nmol of HOQNO · (mg of protein)⁻¹; expt. (c) none; expt. (d) 2 μM-5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole. The values reported in the Table refer to 50 ms after oxygenation.

	I		II	
	(a)	(b)	(c)	(d)
H ⁺ released [ng-ions · (mg of protein) ⁻¹]	1.10	0.7	1.48	0.67
Respiratory carriers oxidized [nmol · (mg of protein) ⁻¹]				
<i>a</i> + <i>a</i> ₃	0.63	0.62	0.79	0.89
Cu	0.63	0.62	0.79	0.89
<i>c</i> + <i>c</i> ₁	0.41	0.40	0.55	0.56
Fe-S (190 μT)	0.20	0.20	0.28	0.28
e ⁻ equivalent to cytochrome <i>b</i> reduced	0.27	0.20	0.24	0.20
Σe ⁻ flow	2.14	2.04	2.65	2.82
H ⁺ /Σe ⁻ flow	0.51	0.34	0.56	0.24

Fig. 5. Effect of antimycin A concentration on the inhibition by HOQNO of H⁺ release induced by oxygenation of anaerobic antimycin-treated ox heart mitochondria
For experimental conditions and procedures, see the legend to Fig. 3. The pH was 6.8. The experimental points refer to H⁺ release at 30 ms after oxygenation. Symbols: ●, 0.5 μg of antimycin A · (mg of protein)⁻¹; O, 1 μg of antimycin A · (mg of protein)⁻¹.

presented in Fig. 6. The same H⁺/e⁻ coupling numbers were obtained in mitochondria and sub-mitochondrial particles. They amounted to about

0.70 and were in both cases practically pH-independent, except for a small rise when the pH was increased from 8.2 to 9.1. This ratio indicates that, at physiological pH values, 70% of oxidoreductions

Table 5. *Stoichiometry of H⁺ release and oxidation of redox carriers caused by oxygen pulses of anaerobic antimycin-inhibited rat liver mitochondria: effects of 5-(n-undecyl)-6-hydroxy-4,7-dioxobenzothiazole*

Rat liver mitochondria (2 mg/ml) were incubated in the reaction mixture described in Fig. 1, with 0.5 μ g of valinomycin/mg of protein. The pH was 7.2. Calculations were carried out as described in Tables 1 and 2; the amount of Fe-S protein (190 μ T) was taken as equivalent to half the amount of *c* cytochromes oxidized in 50 ms in the absence of exogenous cytochrome *c*. Additions: (a) 6 μ M-cytochrome *c*; (b) 6 μ M-cytochrome *c* + 2 μ M-5-(n-undecyl)-6-hydroxy-4,7-dioxobenzothiazole.

	(a)	(b)
H ⁺ released [ng-ions · (mg of protein) ⁻¹]	1.16	0.50
Respiratory carriers oxidized [nmol · (mg of protein) ⁻¹]		
<i>a</i> + <i>a</i> ₃	0.36	0.40
Cu	0.36	0.40
Fe-S (<i>g</i> = 1.90 μ T)	0.14	0.14
<i>c</i> + <i>c</i> ₁	0.73	0.97
e ⁻ equivalent to cytochrome <i>b</i> reduced	0.05	0.05
Σ e ⁻ flow	1.64	1.96
H ⁺ / Σ e ⁻ flow	0.71	0.25

of the components of the cytochrome system are linked to proton transfer by ionizable groups.

Redox Bohr effects were also measured in isolated complexes of the respiratory chain (Papa *et al.*, 1980b; Guerrieri *et al.*, 1981). Measurement of redox Bohr effects in isolated *b*-*c*₁ complex is presented in Table 6. Making use of the contamination of succinate dehydrogenase and traces of purified cytochrome *c* oxidase and cytochrome *c* added to the system, we produced oxidation cycles of the *b*-*c*₁ complex by pulsing with oxygen the enzyme brought to anaerobic reduction by succinate respiration. The control experiment (Table 6, *a*) shows that H⁺ taken up upon aerobic oxidation of the redox centres of the *b*-*c*₁ complex, and subsequently released upon their re-reduction by succinate, were less than the sum of electron carriers oxidized. The midpoint redox potential (*E*_m) of cytochrome *c*₁ is pH-independent (Von Jagow & Engel, 1980). Thus the deficit of proton transfer has to be referred to redox transition of *b* cytochromes and Rieske Fe-S protein and gives an overall H⁺/e⁻ coupling number of 1.3. In Expt. (*b*) of Table 6 a concentration of antimycin A that prevented net redox change of *b*-cytochromes was added. Also, under these conditions H⁺ uptake was less than the sum of electron carriers oxidized. The deficit of proton transfer gave, when referred to net redox transition of the Rieske Fe-S protein, an H⁺/e⁻

Fig. 6. *pH-dependence of the H⁺/e⁻ stoichiometry number for redox Bohr effects in the cytochrome system in ox heart mitochondria and submitochondrial particles*

Mitochondria or submitochondrial particles (2 mg of protein/ml) were incubated with 200 mM-sucrose, 30 mM-KCl, 5 mM-potassium succinate, 2 μ g of oligomycin/mg of protein, 0.5 μ g of rotenone/mg of protein, 1 μ g of antimycin/mg of protein, 3 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine and 0.01 μ g of purified catalase (Boehringer)/ml. The temperature was 25°C. Oxygenation was brought about by repetitive additions of 0.5 μ l of 0.1% H₂O₂ to anaerobic mitochondria or submitochondrial particles. The suspension was made anaerobic by succinate oxidation under a stream of nitrogen. Antimycin was then added and the suspension repetitively pulsed with H₂O₂. The H⁺/e⁻ values were calculated as the ratios between Bohr H⁺ released upon oxidation and taken up on reduction of respiratory carriers and the sum of electron carriers oxidized by oxygen and re-reduced in anaerobiosis by succinate. Bohr H⁺ values were computed by subtracting from the sum of electron carriers undergoing oxidation and reduction the net amount of H⁺ consumed upon oxygenation and released upon anaerobiosis (these two values were exactly equal; cf. Papa *et al.*, 1979c). Total oxidation of electron carriers was obtained by summing up the haems *a* + *a*₃ oxidized, plus an equivalent amount of copper, cytochromes *c* + *c*₁ and the Fe-S protein (*g* = 1.90 μ T), taken as equivalent to half the amount of cytochromes *c* oxidized. Bohr H⁺ values were corrected for H⁺ transfer associated with oxygen-induced reduction of *b* cytochromes (see Papa *et al.*, 1977b). The points represent the means of three experiments. Symbols: ●, ox heart mitochondria; ○, submitochondrial particles.

coupling number of 1.6. HOQNO (Table 6, Expt. *c*) depressed the oxidation of cytochrome *c*₁ and to a greater extent the deficit of H⁺ uptake. The result was that the H⁺/e⁻ coupling number referred to redox transition of the Rieske Fe-S protein was lowered to 1.4.

The *E*_m of the Rieske Fe-S protein has been found to decrease by 60 mV per pH unit increase above pH 7.9 (Prince & Dutton, 1976). According to this, at pH values around neutrality the H⁺/e⁻ ratio for Bohr protons in the Fe-S protein should be lower than 1 (Papa, 1976a). In the oxidation of the *b*-*c*₁

Table 6. Analysis of scalar proton-transfer reactions associated with redox transitions of respiratory carriers in isolated b - c_1 complex and cytochrome c oxidase

Complex b - c_1 ($0.43 \text{ mg} \cdot \text{ml}^{-1}$) (expts. a , b and c) was incubated with 200 mM -sucrose, 30 mM -KCl, $0.3 \mu\text{g}$ of purified complex IV, $0.24 \mu\text{M}$ -cytochrome c and $9 \mu\text{g}$ of purified catalase. The pH was 7.2, and the total volume was 2.25 ml . The incubation was carried out in a stoppered spectrophotometric cuvette for 20 min under a continuous stream of argon to lower the concentration of dissolved oxygen, then 1 mM -potassium succinate was added. Anaerobiosis was reached in about 10 min. Under these conditions cytochrome c_1 was reduced by about 80%, b cytochromes by about 50%. Oxygenation was brought about by repetitive additions of $1 \mu\text{l}$ of 0.1% H_2O_2 , in the presence of 0.5 mM -potassium malonate. Experiments with the addition of $1 \mu\text{M}$ -carbonyl cyanide p -trifluoromethoxyphenyl hydrazone or 0.2% Emasol produced the same results. The extent of oxidation of electron carriers and of proton consumption was measured in conventional incubation cells at an interval of 10–15 s after oxygenation, when the steady state was clearly reached. The amount (nmol) of cytochrome c_1 was calculated from the absorbance changes at 552–540 nm, with a $\Delta\epsilon_{\text{nm}}$ of 17.5 (King, 1979), corrected for the contribution of cytochrome c oxidoreduction at this wavelength couple ($\Delta\epsilon_{\text{nm}}$ of 14); the Fe-S protein ($190 \mu\text{T}$) was taken as equivalent to cytochrome c_1 . The amount (nmol) of cytochrome b oxidized was calculated from the absorbance changes at 562–575 nm with a $\Delta\epsilon_{\text{nm}}$ of 20. Purified cytochrome c oxidase (0.41 mg/ml) (expt. d) was incubated with 200 mM -sucrose, 30 mM -KCl, $10 \mu\text{g}$ of purified catalase, $0.2 \mu\text{M}$ -phenazine methosulphate (PMS), 10 mM -ethanol and 30 units of alcohol dehydrogenase (Boehringer). The pH was 7.2 and the volume 2.25 ml . The incubation was carried in a stoppered spectrophotometric cuvette for 20 min under a continuous stream of argon. Then $0.5 \mu\text{M}$ -NAD $^+$ was added. Anaerobiosis was reached in $\sim 5 \text{ min}$. Under these conditions haems a and a_3 were practically completely reduced. Oxygenation was brought about by addition of $1 \mu\text{l}$ of 0.10% H_2O_2 . The amount (nmol) of haem $a + a_3$ oxidized was calculated from the absorbance changes at 605–630 nm with a $\Delta\epsilon_{\text{nm}}$ of 14 (Nicholls & Kimelberg, 1972). The amount of copper oxidized was taken as equivalent to haems $a + a_3$. The values are expressed in ng-ions or nmol $\cdot$ (mg of protein of b - c_1 complex or cytochrome c oxidase) $^{-1}$. Expt. (a) b - c_1 complex; (b) b - c_1 complex + $2.3 \mu\text{g}$ of antimycin A/mg of protein; (c) b - c_1 complex + 6 nmol of HOQNO/mg of protein; (d) cytochrome c oxidase. Abbreviation used: cyt., cytochrome.

	(a)	(b)	(c)		(d)
H $^+$ uptake	2.50	1.60	1.74	H $^+$ uptake	13.5
cyt. b	1.68	—	—	$a + a_3$	11.80
cyt. c_1	3.71	2.96	2.09	Cu	11.80
Fe-S ($g = 1.90$)	3.71	2.96	2.09	cyt. c_1	1.40
cyt. c	<u>0.56</u>	<u>0.56</u>	<u>0.56</u>	PMS	<u>0.49</u>
Σe^- flow	9.66	6.48	4.74		25.49
ΔH^+ uptake	7.16	4.88	3.00	ΔH^+	11.99
$\frac{\Delta\text{H}^+}{\text{cyt. } b + \text{Fe-S}}$	1.33	$\frac{\Delta\text{H}^+}{\text{Fe-S}}$	1.39	$\frac{\Delta\text{H}^+}{a + a_3}$	1.01
$\frac{\text{Bohr H}^+}{\text{cyt. } b + \text{Fe-S}}$	0.84	$\frac{\text{Bohr H}^+}{\text{Fe-S}}$	0.74	$\frac{\text{Bohr H}^+}{a + a_3}$	0.84

complex, net H $^+$ release could, however, also derive from aerobic oxidation of endogenous ubiquinol (Nelson & Gellerfors, 1978) to protein-stabilized ubisemiquinone (Yu & Yu, 1980; Yu *et al.*, 1978; Ohnishi & Trumpower, 1980) according to the following equation:

H $^+$ release in eqn. (1) was calculated by assuming a content for endogenous ubiquinone of 0.7 mol/mol of cytochrome c_1 and a pK of 6 for semiquinone (Guerrieri *et al.*, 1981). When the deficit of H $^+$ consumption was corrected for H $^+$ release in eqn. (1), the H $^+$ /e $^-$ coupling number for redox Bohr effects in b cytochromes and the Fe-S protein was 0.8. In the presence of antimycin the H $^+$ /e $^-$ coupling number for the Fe-S protein was 0.96. This quotient decreased to 0.7 in the presence of HOQNO.

In Table 6, Expt. (d), measurements of scalar proton-transfer reactions associated with redox transitions of isolated cytochrome oxidase are shown. Also, in this case, proton consumption was smaller than the sum of the redox centres oxidized, from which an H $^+$ /e $^-$ ratio of 0.8 could be calculated for Bohr effects in the oxidase linked to oxidoreduction of haems a and a_3 . It should be recalled that the E_m of the copper in the oxidase is pH-independent (Dutton & Wilson, 1974).

Discussion

There seems to exist a consensus on an anisotropic arrangement in the membrane of the reduction of dioxygen to H $_2\text{O}$ by cytochrome c oxidase, whereby electrons are delivered from cytochrome c located externally to the insulating layer of the membrane and protons derive from the matrix aqueous phase (Mitchell & Moyle, 1970; Papa *et al.*,

Table 7. *Experimental and simulated H⁺/e⁻ ratios for aerobic oxidation of terminal respiratory carriers in antimycin-supplemented ox heart mitochondria*

The experimental H⁺/e⁻ ratios were measured at 50 ms after oxygenation (see Table 1 and Fig. 2). The predicted H⁺/e⁻ ratios represent quotients between expected H⁺ release from mitochondria and experimentally determined electron flow. (I) Vectorial H⁺ release from mitochondria was calculated as equal to the sum of electrons flowing through the *b-c*₁ complex (half quinol oxidized) and those flowing through cytochrome *c* oxidase [c cytochromes, Fe-S protein (190 μT) and half quinol oxidized]. Scalar H⁺ release from mitochondria was taken as equivalent to the sum of net H⁺ released for ubiquinol oxidation, H⁺ release for redox Bohr effects in cytochrome *c* oxidase as equivalent to nmol of haems *a* + *a*₃ oxidized multiplied by the coupling factor of 0.9 (see Table 6 and Guerrieri *et al.*, 1981) and H⁺ release for redox Bohr effect in *b-c*₁ complex equivalent to half the cytochrome *c* oxidized multiplied by the coupling factor of 1.5 (see Table 6 and Guerrieri *et al.*, 1981). (II) Vectorial H⁺ translocation was in this case taken as equivalent to electron flow through the *b-c*₁ complex. Scalar H⁺ transfer was equal to that calculated in case (I). (III) Vectorial H⁺ translocation was equivalent to electron flow through *b-c*₁ complex. Scalar H⁺ transfer was calculated as in (I) and (II), except that Bohr H⁺ of cytochrome *c* oxidase were assumed to be released in the matrix and thus not to appear in the medium. Values are means ± S.E.M. for eight experiments.

H ⁺ /e ⁻ (pH 7.2)				
	Measured	Predicted		
		(I)	(II) Control	(III)
H ⁺ released/Σe ⁻	0.52	1.06	0.69	0.37
S.E.M.	±0.05	±0.04	±0.02	±0.02
Student's <i>t</i>		9.44	2.43	3.73
<i>P</i>		<i>P</i> < 0.001	0.02 < <i>P</i> < 0.05	0.01 < <i>P</i> < 0.01

1974a,b; Papa, 1976a; Wikström & Saari, 1977; Wikström, 1982; Papa, 1982a). This arrangement is sufficient to explain Δμ_{H⁺} generation by the oxidase in mitochondria.

Wikström and co-workers (Wikström, 1977; Wikström & Saari, 1977; Wikström & Krab, 1979; Wikström, 1982) and others (Sigel & Carafoli, 1978, 1980; Alexandre *et al.*, 1978; Azzone *et al.*, 1979; Casey *et al.*, 1979; Sone & Hinkle, 1982) have, however, produced data that are taken as evidence for the existence in cytochrome *c* oxidase of a proton pump, which, in addition to vectorial electron flow from the C to the M side of the membrane, would transport 1 H⁺/e⁻ (Wikström & Krab, 1979; Sigel & Carafoli, 1980) or 2 H⁺/e⁻ (Alexandre *et al.*, 1978; Azzone *et al.*, 1979) from the matrix to the outer aqueous phase.

Mitchell (1979, 1980) (see also Moyle & Mitchell, 1978; Mitchell *et al.*, 1978), Papa *et al.* (1978a,b,c, 1980a) and Lorusso *et al.* (1979) have extensively looked for a proton pump in cytochrome oxidase, but their results appear to exclude the likelihood that the oxidase in the native functional state in the membrane of mitochondria operates as a proton pump [see, for reviews, Mitchell (1980) and Papa (1982b)].

The present study affords a direct kinetic analysis of proton translocation associated with oxygen pulses of the fully reduced cytochrome *c* oxidase in the mitochondrial membrane. Under these conditions the oxidase reacts with oxygen and is converted into the active or pulsed state in an interval of

few milliseconds (Bonaventura *et al.*, 1978; Brunori *et al.*, 1979). The ensuing oxidation of respiratory carriers results, in the presence of antimycin, in a rapid proton release. The oxidase is fully competent in generating a transmembrane protonmotive force, as documented by twofold stimulation of the rate of cytochrome *c* oxidation produced by valinomycin plus K⁺ (see Fig. 1 and Table 1).

The proton release appears to have a composite nature, judging from the peculiar pH-dependence of the →H⁺/e⁻ ratio. The acidification process could represent the net result of: (a) the residual oxidation of ubiquinol (see Fig. 1); (b) deprotonation of acidic groups involved in redox Bohr effects in the cytochrome system; and (c) antimycin-insensitive proton pumping associated to electron flow in the cytochrome system.

The kinetic analysis shows that the rapid proton release, associated with aerobic oxidation of respiratory carriers in the presence of antimycin and valinomycin, is, with a *t*₁ of 9 ms, definitely slower than the oxidation of cytochrome oxidase (*t*₁ < 2 ms) and *c* cytochromes (*t*₁ 4 ms), but practically synchronous with the residual oxidation of ubiquinol (*t*₁ 9 ms) and reduction of *b* cytochromes (*t*₁ 9 ms).

Residual antimycin-insensitive aerobic oxidation of ubiquinol is apparently associated with oxidant-induced reduction of *b* cytochromes (Erecinska *et al.*, 1972; Wikström & Berden, 1972; Papa *et al.*, 1972; Mitchell, 1976). HOQNO, besides inhibiting, like antimycin, electron flow between cytochromes *b* and *c* (Chance & Williams, 1956; Brandon *et al.*,

1972), inhibits also the oxidant- and antimycin-promoted reduction of *b* cytochromes (Eisenbach & Gutman, 1975; Izzo *et al.*, 1978; Papa *et al.*, 1982b). It is shown here that the inhibition by HOQNO of cytochrome *b* reduction is accompanied by substantial depression of aerobic antimycin-insensitive H^+ release.

HOQNO enhances proton conductivity in the mitochondrial membrane (Wikström, 1978; Krab & Wikström, 1980; Lorusso *et al.*, 1979). This effect can hardly apply to the present situation (see also Lorusso *et al.*, 1979), where H^+ release is very rapid and its depression by HOQNO is observed at an interval of 50ms after oxygenation. It has been shown that, in these short intervals, even a very effective protonophore like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone fails to affect active vectorial H^+ translocation (Guerrieri *et al.*, 1976; Papa, 1976a).

That the inhibition of H^+ release is a consequence of the inhibitory action of HOQNO on the *b*-*c*₁ complex is, on the other hand, supported by the correspondence of the titre for HOQNO inhibition of antimycin-insensitive aerobic H^+ release to that for depression of oxygen-induced cytochrome *b* reduction, as well as by the observation that the depression of H^+ release brought about by HOQNO is counteracted by raising the antimycin concentration (Fig. 5) (cf. Von Ark & Berden, 1977).

Further definite evidence that the rapid proton release associated with aerobic oxidation of respiratory carriers in the presence of antimycin derives in large part from oxygen-induced electron flow in the *b*-*c*₁ complex is provided by the observation that this acidification process was also inhibited by 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole, a specific inhibitor of electron flow in the *b*-*c*₁ complex which acts at a site different from antimycin (Trumpower & Haggerthy, 1980; Trumpower, 1981).

It can also be mentioned that Papa *et al.* (1982b) have found that the inhibitory action exerted by HOQNO on the antimycin-insensitive H^+ release is additive with that of 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole.

The finding that under no conditions was the oxidation of terminal carriers accompanied by synchronous proton disappearance from the external medium provides further definite evidence that the protons consumed in the reduction of dioxygen to H_2O derive from the inner aqueous space (matrix) of mitochondria.

The occurrence of redox Bohr effects in the cytochrome system *in situ* [Fig. 6; see also Papa *et al.* (1979c)] as well as in isolated cytochrome *c* oxidase and *b*-*c*₁ complex has been directly verified by measuring scalar proton transfer associated with redox transitions of respiratory carriers [Fig. 6 and

Table 6; see also Papa *et al.* (1979c, 1980b) and Guerrieri *et al.* (1981)].

By using the H^+/e^- coupling numbers determined for redox Bohr effects in isolated cytochrome *c* oxidase and *b*-*c*₁ complex it is possible to compare the H^+/e^- quotients measured experimentally during rapid aerobic oxidation of terminal electron carriers in mitochondria with the ratios that can be predicted in the cases where Bohr protons are transferred at the C or the M side of the membrane and do or do not contribute to proton pumping.

In Table 7 such a comparison is presented for three cases. In the first it is assumed that there exist proton pumps in the *b*-*c*₁ complex and in cytochrome oxidase and that electrons cause a 1:1 stoichiometric pumping of protons when passing through each of the two complexes. In the case of proton pumps in the *b*-*c*₁ complex and in the oxidase, it is assumed that Bohr protons are released, upon oxidation of electron carriers, at the C surface of the membrane. The data presented in Table 7 show that the H^+/e^- ratios expected in this case are twice as high as the experimental values.

In the second and third case the H^+/e^- ratios are presented that would be expected in the absence of a proton pump in cytochrome *c* oxidase and when Bohr protons in the oxidase are respectively released at the C or at M side. The experimental H^+/e^- quotients measured fall in between these two predicted ratios. This would mean that Bohr protons of haem *a* and *a*₃ are released in part at the M side and in part at the C side (cf. Konstantinov & Rungen, 1977).

In conclusion the present results show that cytochrome *c* oxidase *in situ*, in ox heart and rat liver mitochondria, in the transition from the anaerobic reduced state to the active pulsed state and during one or two turnovers does not display any proton-pumping activity. There are also: (i) observations (Mitchell, 1980; Papa, 1982b) showing that the H^+ release observed upon pulses with artificial reductants of the fully oxidized 'resting' oxidase in intact mitochondria results from re-reduction of the oxidized form of the reductant by hydrogenated donors, and (ii) measurements, based on accurate spectrophotometric measurement of the rate of electron flow (Papa *et al.*, 1982a) showing at the aerobic steady state an H^+/O ratio for succinate oxidation of 4, which is equivalent to the $H^+/2e^-$ of 4 for proton release from mitochondria observed for electron flow from quinol to cytochrome (Wikström & Krab, 1979; Papa, 1982b). All this allows one to conclude that cytochrome *c* oxidase *in situ* in the mitochondrial membrane does not function as a proton pump. Thus the generation of transmembrane $\Delta\bar{\mu}_{H^+}$ associated with aerobic oxidation of cytochrome *c* by the oxidase derives only from anisotropic arrangement of the reduction

of dioxygen to H_2O , whereby the electrons are donated by cytochrome *c*, located at the outer surface of the membrane, and protons derive from the matrix aqueous space.

The present study and conclusions would suggest that the acidification process observed upon activation of electron flow in cytochrome *c* oxidase reconstituted in phospholipid vesicles [see, for review, Wikström & Krab (1979), Wikström & Saari (1977) and Wikström (1982)] is not related to the capacity displayed by the enzyme in its native environment to convert redox energy into transmembrane $\Delta\mu_{\text{H}^+}$.

Redox Bohr effects in cytochrome *c* oxidase, i.e. co-operative proton transfer in the enzyme linked to redox transitions of haems *a* and *a*₃, may facilitate translocation of the protons, needed for protonation of reduced oxygen to H_2O , from the matrix aqueous space to the reaction centre of the oxidase (Papa *et al.*, 1982c), which is apparently displaced towards the outer side of the membrane (Chance, 1981).

The redox Bohr effect of *b* cytochromes (Papa *et al.*, 1973; Papa, 1976a) and of the Rieske Fe-S protein of the *b*-*c*₁ complex (Papa, 1982a) may, on the other hand, be involved in the protonmotive activity of the *b*-*c*₁ complex. They could act in series or in parallel, with direct proton translocation by quinones (Papa, 1982a). This would be supported by the observation that HOQNO, which depresses redox Bohr effects in isolated *b*-*c*₁ complex (Table 6), lowers the $\rightarrow\text{H}^+/\text{e}^-$ ratio for antimycin-insensitive proton release from mitochondria.

The skilful technical assistance of Mr. M. Minuto and Mrs. A. Carlone is gratefully acknowledged.

References

- Alexandre, A., Reynafaje, B. & Lehninger, A. L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5296–5300
- Azzone, G. G., Pozzan, T. & Di Virgilio, F. (1979) *J. Biol. Chem.* **254**, 10206–10212
- Bonaventura, C., Bonaventura, J., Brunori, M. & Wilson, M. T. (1978) *FEBS Lett.* **85**, 30–34
- Brandon, J. R., Brockleurst, J. R. & Lee, C. P. (1972) *Biochemistry* **11**, 1150–1154
- Brunori, M., Colosimo, A., Rainori, G., Wilson, M. T. & Antonini, E. (1979) *J. Biol. Chem.* **254**, 10769–10775
- Casey, R. P., Chappell, J. B. & Azzi, A. (1979) *Biochem. J.* **183**, 149–156
- Chance, B. (1972) *FEBS Lett.* **23**, 3–20
- Chance, B. (1981) in *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P. & Hinkle, P. L., eds.), p. 981, Addison Wesley Publishing Co., Reading, MA
- Chance, B. & Williams, G. 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. 5) (Claesson S., ed.), pp. 437–464, Interscience, New York
- Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Systems*, Weverly Press, Baltimore, MD
- Dutton, P. L. & Wilson, D. F. (1974) *Biochim. Biophys. Acta* **346**, 165–212
- Eisenbach, M. & Gutman, M. (1975) *Eur. J. Biochem.* **59**, 223–230
- Erecinska, M., Chance, B., Wilson, D. F. & Dutton, P. L. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 50–54
- Errede, B., Kamen, M. D. & Hatefi, Y. (1978) *Methods Enzymol.* **53**, 40–47
- Guerrieri, F., Lorusso, M., Pansini, A., Ferrarese, V. & Papa, S. (1976) *J. Bioenerg. Biomembr.* **8**, 131–142
- Guerrieri, F., Izzo, G., Maida, I. & Papa, S. (1981) *FEBS Lett.* **125**, 261–265
- Harmon, J. H., Hall, J. D. & Crane, F. L. (1974) *Biochim. Biophys. Acta* **344**, 119–155
- Hatefi, Y., Haavik, A. G. & Griffiths, D. E. (1961) *J. Biol. Chem.* **237**, 1681–1685
- Huang, C. H., Keyhani, E. & Lee, C. P. (1973) *Biochim. Biophys. Acta* **305**, 455–473
- Izzo, G., Guerrieri, F. & Papa, S. (1978) *FEBS Lett.* **93**, 320–322
- Kilmartin, J. V. & Rossi-Bernardi, L. (1973) *Physiol. Rev.* **53**, 836–889
- King, T. E. (1979) *Methods Enzymol.* **53**, 181–191
- Konstantinov, A. A. & Rungen, E. K. (1977) *FEBS Lett.* **81**, 137–141
- Krab, K. & Wikström, M. (1980) *Biochem. J.* **186**, 637–639
- Kröger, A. & Klingenberg, M. (1966) *Biochem. Z.* **344**, 317–336
- Lee, C. P. & Ernster, L. (1968) *Eur. J. Biochem.* **3**, 391–400
- Lorusso, M., Capuano, F., Boffoli, D., Stefanelli, R. & Papa, S. (1979) *Biochem. J.* **182**, 133–147
- Löw, H. & Vallin, I. (1963) *Biochim. Biophys. Acta* **69**, 361–374
- Mitchell, P. (1969) in *The Molecular Basis of Membrane Function* (Tosteson, D. C., ed.), pp. 483–518, Prentice-Hall, Englewood Cliffs, NJ
- Mitchell, P. (1976) *J. Theor. Biol.* **62**, 327–367
- Mitchell, P. (1979) *Eur. J. Biochem.* **95**, 1–20
- Mitchell, P. (1980) *Ann. N.Y. Acad. Sci.* **341**, 564–584
- Mitchell, P. & Moyle, J. (1967) in *Biochemistry of Mitochondria* (Slater, E. C., Kaniuga, Z. & Wojtczak, L., eds.), pp. 55–74, Academic Press/PWN, London and Warsaw
- Mitchell, P. & Moyle, J. (1970) in *Electron Transport and Energy Conservation* (Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C., eds.), pp. 575–587, Adriatica Editrice, Bari
- Mitchell, P., Moyle, J. & Mitchell, R. (1978) in *Frontiers of Biological Energetics: from Electrons to Tissues* (Dutton, P. L., Leigh, J. & Scarpa, A., eds.), vol. 1, pp. 349–402, Academic Press, New York
- Moyle, J. & Mitchell, P. (1978) *FEBS Lett.* **88**, 268–272
- Myers, D. K. & Slater, E. C. (1957) *Biochem. J.* **67**, 558–568
- Nelson, B. D. & Gellerfors, P. (1978) *Methods Enzymol.* **53**, 80–91
- Nicholls, P. & Chance, B. (1974) in *Molecular Mechanism of Oxygen Activation* (Hayaishi, O., ed.), pp. 479–534, Academic Press, New York

- Nicholls, P. & Kimelberg, H. K. (1972) in *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E. & Siliprandi, N., eds.), pp. 17–32, Academic Press, New York
- Ohnishi, T. & Trumpower, B. L. (1980) *J. Biol. Chem.* **225**, 3278–3284
- Papa, S. (1976a) *Biochim. Biophys. Acta* **456**, 39–84
- Papa, S. (1976b) in *Cell Biology; Biological Handbooks* (Altman, P. L. & Katz, D. D., eds.), vol. 1, pp. 155–159, FEBS, Bethesda, MD
- Papa, S. (1982a) *J. Bioenerg. Biomembr.* **14**, 69–86
- Papa, S. (1982b) in *Membranes and Transport* (Martonosi, A., ed.), pp. 363–368, Plenum Press, New York
- Papa, S., Scarpa, A., Lee, C. P. & Chance, B. (1972) *Biochemistry* **11**, 3091–3097
- Papa, S., Guerrieri, F., Lorusso, M. & Simone, S. (1973) *Biochimie* **55**, 703–716
- Papa, S., Guerrieri, F. & Lorusso, M. (1974a) *Biochim. Biophys. Acta* **357**, 181–192
- Papa, S., Guerrieri, F. & Lorusso, M. (1974b) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M., Quagliariello, E. & Siliprandi, N., eds.), pp. 177–186, North-Holland Publishing Co., Amsterdam
- Papa, S., Lorusso, M. & Guerrieri, F. (1975) *Biochim. Biophys. Acta* **387**, 425–440
- Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., Boffoli, D. & Capuano, F. (1977a) in *Biochemistry of Membrane Transport* (Semenza, G. & Carafoli, E., eds.), pp. 504–519, Springer-Verlag, Berlin and Heidelberg
- Papa, S., Lorusso, M., Guerrieri, F., Boffoli, D., Izzo, G. & Capuano, F. (1977b) in *Bioenergetics of Membranes* (Packer, L., Papageorgiou, G. & Trebst, A., eds.), pp. 377–388, Elsevier/North-Holland, Amsterdam
- Papa, S., Guerrieri, F., Lorusso, M., Capuano, F., Izzo, G. & Boffoli, D. (1978a) in *Frontiers of Biological Energetics* (Dutton, P. L., Leigh, J. & Scarpa, A., eds.), vol. 1, pp. 367–374, Academic Press, New York
- Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., Boffoli, D. & Stefanelli, R. (1978b) in *Membrane Proteins* (Nicholls, P., Møller, J. V., Leth Jørgensen, P. & Moody, A. L., eds.), pp. 37–48, Pergamon Press, New York and Oxford
- Papa, S., Lorusso, M., Guerrieri, F., Izzo, G. & Capuano, F. (1978c) in *The Proton and Calcium Pumps* (Azzone, G. F., Avron, M., Metcalfe, J. C., Quagliariello, E. & Siliprandi, N., eds.), pp. 227–237, Elsevier/North-Holland, Amsterdam
- Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., Capuano, F. & Boffoli, D. (1979a) in *Function and Molecular Aspects of Biomembrane Transport* (Quagliariello, E., Palmieri, F., Papa, S. & Klingenberg, M., eds.), pp. 197–207, Elsevier/North-Holland, Amsterdam
- Papa, S., Guerrieri, F. & Rossi-Bernardi, L. (1979b) *Methods Enzymol.* **55**, 614–627
- Papa, S., Guerrieri, F. & Izzo, G. (1979c) *FEBS Lett.* **105**, 213–216
- Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., Boffoli, D., Capuano, F., Capitanio, N. & Altamura, N. (1980a) *Biochem. J.* **192**, 203–218
- Papa, S., Guerrieri, F. & Lorusso, M. (1980b) *1st EBEC Short Report*, pp. 19–20, Patron Editore, Bologna
- Papa, S., Lorusso, M., Izzo, G. & Capuano, F. (1981) *Biochem. J.* **194**, 395–406
- Papa, S., Guerrieri, F., Lorusso, M., Izzo, G. & Capuano, F. (1982a) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., ed.), pp. 527–539, Academic Press, New York
- Papa, S., Izzo, G. & Guerrieri, F. (1982b) *FEBS Lett.* **145**, 93–98
- Papa, S., Lorusso, M. & Guerrieri, F. (1982c) in *Cell Function and Differentiation*, part B (Akoyunoglou, G., Evangelopoulos, A. E., Georgatson, J., Palaiologos, G., Trakatellis, A. & Tsiganos, C. P., eds.) (*FEBS Symp.* **65**), pp. 423–437, Alan R. Liss, New York
- Prince, R. C. & Dutton, P. L. (1976) *FEBS Lett.* **65**, 117–119
- Rieske, J. S., Zougg, W. S. & Hansen, R. E. (1964) *J. Biol. Chem.* **239**, 3023–3031
- Sigel, E. & Carafoli, E. (1978) *Eur. J. Biochem.* **89**, 119–123
- Sigel, E. & Carafoli, E. (1980) *Eur. J. Biochem.* **111**, 299–306
- Sone, N. & Hinkle, P. C. (1982) *J. Biol. Chem.* **257**, 12600–12604
- Szarkowska, L. & Klingenberg, M. (1963) *Biochem. Z.* **338**, 674–697
- Tischler, M. E., Land, J. M. & Williamson, J. R. (1976) in *Cell Biology: Biological Handbooks* (Altman, P. L. & Katz, D. D., eds.), vol. 1, pp. 195–207, Plenum Press, New York
- Trumpower, B. L. (1981) *J. Bioenerg. Biomembr.* **13**, 1–34
- Trumpower, B. L. & Haggerthy, J. G. (1980) *J. Bioenerg. Biomembr.* **12**, 151–164
- Von Ark, G. & Berden, J. A. (1977) *Biochim. Biophys. Acta* **459**, 119–137
- Von Jagow, G. & Engel, W. D. (1980) *FEBS Lett.* **111**, 1–5
- Wikström, M. (1977) *Nature (London)* **266**, 271–273
- Wikström, M. (1978) in *The Proton and Calcium Pumps* (Azzone, G. F., Avron, M., Metcalfe, J. C., Quagliariello, E. & Siliprandi, N., eds.), pp. 215–226, Elsevier/North-Holland, Amsterdam
- Wikström, M. (1982) in *Membranes and Transport*, (Martonosi, A. N., ed.), vol. I, pp. 357–362, Plenum Press, New York
- Wikström, M. & Berden, J. A. (1972) *Biochim. Biophys. Acta* **283**, 403–420
- Wikström, M. & Krab, K. (1979) *Biochim. Biophys. Acta* **549**, 177–222
- Wikström, M. & Penttilä, T. (1982) *FEBS Lett.* **144**, 183–189
- Wikström, M. & Saari, H. T. (1977) *Biochim. Biophys. Acta* **462**, 347–361
- Wikström, M., Krab, K. & Saraste, M. (1981) *Annu. Rev. Biochem.* **50**, 623–655
- Wyman, J. (1968) *Q. Rev. Biophys.* **1**, 35–81
- Yu, C. A. & Yu, L. (1980) *Biochem. Biophys. Res. Commun.* **96**, 286–292
- Yu, C. A., Nagaoka, S. & King, T. E. (1978) *Biochem. Biophys. Res. Commun.* **82**, 1070–1078