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The H^+/e^- stoicheiometry of respiration-linked proton translocation in the cytochrome system of mitochondria

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1. The $\rightarrow H^+/e^-$ quotients for proton release from mitochondria associated with electron flow from succinate and duroquinol to O_2, ferricyanide or ferricytochrome *c*, and from *NNN'N'*-tetramethyl-*p*-phenylenediamine + ascorbate to O_2, were determined from rate measurements of electron flow and proton translocation. 2. Care was taken to avoid, or to take into account, unrelated electron flow and proton translocation, which might take place in addition to the oxido-reductions that were the subject of our analysis. Spectrophotometric techniques were chosen to provide accurate measurement of the rate of consumption of oxidants and reductants. The rate of proton translocation was measured with fast pH meters with a precision of 10^{-3} pH unit. 3. The $\rightarrow H^+/O$ quotient for succinate or duroquinol oxidation was, at neutral pH, 4, when computed on the basis of spectrophotometric determinations of the rate of O_2 consumption or duroquinol oxidation. Higher $\rightarrow H^+/O$ quotients for succinate oxidation, obtained from polarographic measurements of O_2 consumption, resulted from underestimation of the respiratory rate. 4. The $\rightarrow H^+/2e^-$ quotient for electron flow from succinate and duroquinol to ferricyanide or ferricytochrome *c* ranged from 3.9 to 3.6. 5. Respiration elicited by *NNN'N'*-tetramethyl-*p*-phenylenediamine + ascorbate by antimycin-inhibited mitochondria resulted in extra proton release in addition to that produced for oxidation of ascorbate to dehydroascorbate. Accurate spectrophotometric measurement of respiration showed that the $\rightarrow H^+/e^-$ ratio was only 0.25 and not 0.7–1.0 as obtained with the inadequate polarographic assay of respiration. Proton release was practically suppressed when mitochondria were preincubated aerobically in the absence of antimycin. Furthermore, the rate of scalar proton consumption for water production was lower than that expected from the stoicheiometry. Thus the extra proton release observed during respiration elicited by *NNN'N'*-tetramethyl-*p*-phenylenediamine + ascorbate is caused by oxidation of endogenous hydrogenated reductants. 6. It is concluded that (i) the $\rightarrow H^+/O$ quotient for the cytochrome system is, at neutral pH, 4 and not 6 or 8 as reported by others; (ii) all the four protons are released during electron flow from quinol to cytochrome *c*; (iii) the oxidase transfers electrons from cytochrome *c* to protons from the matrix aqueous phase and does not pump protons from the matrix to the outer aqueous phase.

Mitochondrial respiration is compulsorily linked to proton extrusion from the matrix to the outer space (Mitchell, 1976; Papa, 1976). The resulting transmembrane thermodynamic potential difference of protons ($\Delta\bar{\mu}_{H^+}$) acts as intermediate force in oxidative phosphorylation and active ion transport across the mitochondrial membrane (Boyer *et al.*, 1977).

The molecular mechanism by which $\Delta\mu_{H^+}$ is generated and utilized in mitochondria, as well as in other coupling membranes, is however yet unsolved (Boyer *et al.*, 1977). One important aspect of this problem is represented by the stoicheiometric relationship between proton translocation and electron transport.

Abbreviations used: HbO_2, oxyhaemoglobin; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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It should be recalled that the chemiosmotic protonmotive model of the respiratory chain as proposed by Mitchell (1966, 1979) requires a fixed stoicheiometry of two protons released from mitochondria for every two electrons traversing an effective protonmotive redox loop or energy conserving site, i.e. a $\rightarrow H^+/2e^-$ (per site) quotient of 2 (Mitchell, 1966, 1979). According to the conformational type of mechanism (Papa, 1976; Boyer *et al.*, 1977), which can be conceived as being based on co-operative linkage between transfer of reducing equivalents by the catalytic centres and proton translocation by ionizable groups in the apoproteins (Papa *et al.*, 1973; Papa, 1976), the $\rightarrow H^+/2e^-$ (per site) quotient may be different from 2 and may be variable.

Experimental determinations of the $\rightarrow H^+/2e^-$ and $\rightarrow H^+/ATP$ stoicheiometries have produced contrasting results (Papa, 1976; Brand, 1977; Lehninger, 1977; Azzone *et al.*, 1978; Mitchell, 1979; Wikström & Krab, 1979).

As far as the cytochrome system of mitochondria is concerned, it is reported that the $\rightarrow H^+/O$ quotient for succinate or quinol respiration (Sites 2 + 3) is either 4 (Mitchell *et al.*, 1978; Papa *et al.*, 1978*a*; Mitchell, 1979) or 6 (Brand, 1977; Brand *et al.*, 1978; Wikström & Krab, 1979), or 8 (Reynafarje *et al.*, 1976; Lehninger, 1977; Azzone *et al.*, 1978).

Mitchell (1979) and Papa *et al.* (1978*a*) (see also Lorusso *et al.*, 1979) maintain that vectorial proton translocation by the cytochrome system is confined to the bc_1 complex. Four protons would be released from mitochondria; two of them originating from succinate or quinol, the other two, and hence two positive charges, being translocated from the internal to the external aqueous phase as two electrons flow from quinol to cytochrome *c*. Two positive charges, but no protons, would effectively be translocated from inside to outside, as two electrons are transported across the insulating barrier of the membrane from cytochrome *c*, located at the outer side, to protons, which enter cytochrome oxidase from the inner side of the membrane (Mitchell & Moyle, 1970; Papa *et al.*, 1974; Papa, 1976).

According to others, in addition to these vectorial processes, two protons (Wikström, 1977; Sigel & Carafoli, 1978; Wikström & Krab, 1979) or four protons (Alexandre *et al.*, 1978; Azzone *et al.*, 1979*a*) would be pumped by cytochrome oxidase from inside to outside for every two electrons flowing from cytochrome *c* to O_2.

In this paper we present measurements of the H^+/e^- stoicheiometry in the cytochrome system, based on accurate spectrophotometric determination of the rate of electron flow and potentiometric measurements of the acidification of the external phase.

It is unequivocally shown that (i) the $\rightarrow H^+/O$ quotient for the cytochrome system is 4 and not 6 or 8 and (ii) vectorial proton translocation in the cytochrome system is confined to the bc_1 complex and no proton pump is operative in cytochrome *c* oxidase.

Materials and methods

Chemicals

Antimycin A, valinomycin, oligomycin, rotenone, *N*-ethylmaleimide, bovine serum albumin and TMPD of high purity grade were purchased from Sigma. Duroquinone and potassium ferricyanide were from BDH. FCCP was a gift from Dr. C. P. Lee, Wayne State University, Detroit, MI, U.S.A. All the other reagents were of the highest purity grade available. Duroquinol was obtained by reduction of duroquinone with KBH_4 in methanol, excess KBH_4 being eliminated by acidification with 0.1 M-HCl. The methanolic solution, diluted with the appropriate reaction mixture at about pH 7, was kept at 0°C and used within 1 h. The mixture of TMPD and ascorbate used was prepared by first dissolving the ascorbic acid in 150 mM-LiCl/1 mM-KCl/3 mM-Hepes (lithium salt). The solid TMPD was added, the pH was adjusted with LiOH to 7.0 ± 0.005 and the solution was brought to volume to avoid possible formation of oxidation products of TMPD in aqueous solutions exposed to air (Miller *et al.*, 1979). The mixture was kept in the dark at 0°C and used within 2–3 h. Recrystallized TMPD gave the same results obtained without this step.

Preparation of mitochondria

Rat liver mitochondria were isolated as described by Myers & Slater (1957) and were suspended in 0.25 M-sucrose at a protein concentration of about 60 mg/ml. Bovine heart mitochondria were prepared as described by Löw & Vallin (1963) and suspended in 0.25 M-sucrose at a protein concentration of about 20 mg/ml.

Incubation procedure

Rat liver and beef heart mitochondria were incubated in the reaction mixtures described in the legends to the Figures and Tables. Samples of the same mitochondrial suspension, adjusted to pH 7.2 or 7.0 (± 0.005) were transferred to thermostatically controlled (25 ± 0.01°C) measurement cells for simultaneous determination of the rates of electron flow and proton translocation. Other details are given in the legends to the Figures and Tables.

Polarographic measurement of O_2 consumption

The mitochondrial suspension was transferred to an all-glass reaction cell, sealed at the top with a

glass plug with 3 cm-long thin channels, filled with the mitochondrial suspension, for insertion of the glass electrode and microsyringe needles. This cell excluded detectable O_2 diffusion. The oxygen electrode was immersed through an O-ring-sealed side hole into the mitochondrial suspension which was vigorously stirred by a magnetic follower. When desired the concentration of dissolved O_2 was reduced by blowing a stream of Ar (or N_2) on to the surface of the mitochondrial suspension.

The oxygen electrode, of the Clark type (YSI 4004; Yellow Spring Instrument Co., Yellow Spring, OH, U.S.A.) was coated either with a standard membrane (YSI 5775) or a high-sensitivity membrane (YSI 5776). The electrode coated with the standard membrane responded to immediate O_2 consumption caused by addition of solid sodium dithionite after a lag of 3 s with a first-order process with a $t_{\frac{1}{2}}$ of 3 s; when the electrode was coated with the high-sensitivity membrane the lag was 1.5 s and the $t_{\frac{1}{2}}$ 1 s (*cf.* Wikström & Krab, 1979; Capuano *et al.*, 1980). O_2 concentration was calibrated by setting the null point after dissolving dithionite into the reaction mixture and taking the signal displayed at equilibrium with atmospheric O_2 at 25°C and 10^5Pa as corresponding to 250μM-O_2 in 150mM-KCl (Hodgman, 1961). Calibration was also checked by aerobic oxidation of duroquinol by mitochondria (Capuano *et al.*, 1980).

Spectrophotometric determination of respiratory rate

The spectrophotometric assay of respiration with haemoglobin was carried out by the method of Barzu (1978) as described by Capuano *et al.* (1980). The concentration of human haemoglobin, prepared as described by Hultborn (1972), was estimated, on a haem basis, with an ε at 577nm of 15.4mM·cm^{-1} (Barzu, 1978). Deoxygenation of HbO_2 was monitored as the absorbance decrease at 577–568nm or the absorbance increase at 558–568nm (Capuano *et al.*, 1980) with a Johnson Foundation dual-wavelength spectrophotometer. The rate of O_2 consumption in ng-atoms·min^{-1} was obtained multiplying the rate of HbO_2 deoxygenation (nmol of haem), by 2 and by a correction factor, *f*. This factor, which depends on the haemoglobin concentration and the relative affinity for O_2 of haemoglobin and mitochondria and may vary slightly from sample to sample of human blood, was directly calculated both polarographically and spectrophotometrically (see Barzu, 1978; Capuano *et al.* 1980) for each haemoglobin preparation used.

When desired the concentration of dissolved O_2 was decreased by blowing Ar on to the surface of the mitochondrial suspension kept in the measuring cell under magnetic stirring. The cell was then sealed with a glass plug with 3 cm long thin channels, filled with the incubation mixture, for insertion of microsyringe needles. This cell excluded detectable O_2 diffusion, as shown by the absorbance trace of incubation mixtures whose O_2 concentration had been decreased to less than 50μM with Ar (Figs. 1 and 6).

Measurement of ferricyanide reduction

Ferricyanide reduction by mitochondria was directly monitored at the wavelength couple 420–500nm by using the dual-wavelength spectrophotometer, in a 1cm cuvette. The absorbance changes were converted into ferricyanide equivalents by addition to the mitochondrial suspension of standard ferricyanide solution (final concentration 50μM).

Flow measurement of fast redox processes

Rapid aerobic oxidation of duroquinol, and reduction of ferricytochrome *c* or ferricyanide by duroquinol in KCN-inhibited mitochondria, were measured with the dual-wavelength spectrophotometer equipped with a regenerative stopped-flow apparatus (mixing ratio 1:60) (Chance *et al.*, 1967). The flow cell was surrounded by a water bath thermostatted at 25 ± 0.01°C. The amplifier output was displayed, in parallel, on a potentiometric strip-chart recorder (Speedomax XL 681, Leeds and Northrup Italiana, Milano, Italy) and on a storage oscilloscope (type 564B, Tektronix Inc., Beaverton, OR 97077, U.S.A.). A mercury arc (Ultraviolet Strahler type ST-75, Original Quarz-Lampen GmbH, Hanau, Germany) was used as the u.v. light source. Duroquinol oxidation was measured at 270–285nm assuming a $\Delta\varepsilon$ of 16mM^{-1}·cm^{-1}, which was directly obtained by measuring the reduction with borohydride of small amounts of a standard solution of duroquinone. The narrow wavelength differences used minimized light-scattering and stray-light artefacts. In addition, a 10mm thickness of a saturated $NiSO_4/CoSO_4$ solution placed between the observation cell and the phototube was used as a light filter (Kröger & Klingenberg, 1966). The optical path of the flow cell was 0.6cm.

The reduction of ferricytochrome *c* was measured at 550–540nm by using a $\Delta\varepsilon$ of 19.1mM^{-1}·cm^{-1} (Chance & Williams, 1956). Reduction of ferricyanide was determined at 420–500nm and the absorbance changes were converted into equivalents of ferricyanide as previously described.

The reaction time at the point of observation in the stopped-flow spectrophotometer (continuous-flow phase) ranged, from experiment to experiment, between 6 and 9ms, depending upon the speed of the discharge (the flow trace in the Figures indicates the duration of the flow phase, determined by the N_2

pressure applied to push the pistons as well as by the distance that the pistons travel).

Measurement of proton translocation

pH changes were measured potentiometrically either on the stirred mitochondrial suspension in the standard reaction cell or, when dealing with particularly rapid reactions, by a continuous-flow method.

Standard pH measurement. A combination glass electrode (no. 10405328, Ingold AG, Zurich, Switzerland) was immersed in the mitochondrial suspension, kept under stirring in the same reaction cell used for polarographic determination of O_2 consumption. The electrode was connected to an electrometer amplifier (model 604, Keithley Instruments, Cleveland, OH, U.S.A.). The output of the electrometer was fed into a two-channel Leeds and Northrup potentiometric pen recorder. The time for overall increase (10–90% change) of the pH recording system was 0.4 s, the noise level was less than 5×10^{-4} pH unit, and the precision was 10^{-3} pH unit (Papa *et al.*, 1979*c*). Potential changes were converted into proton equivalents by double titration with standard solutions of HCl and KOH.

Continuous-flow pH measurement. This was performed with a Roughton-type, repetitive, continuous-flow pH meter (mixing ratio 1:60) with a resolution time of 2 ms (Papa *et al.*, 1979*c*). The electrodes used were a 50–100 MΩ Ingold glass electrode and a calomel electrode connected to the incubation mixture in the measuring cell of the apparatus through a satd.-KCl bridge. The electrodes were connected to a Vibron precision electrometer (model 62A, Electronic Instrument, Richmond, Surrey, U.K.) set to a sensitivity at the output of 24 V/pH unit. The input capacitance of the electrometer was 1 pF. The electrometer output was displayed on a Honeywell strip-chart recorder, model Electronik 194. The sensitivity of the recorder was adjusted to give a 5 cm deflection/V. The circuit used allowed the pH to be measured with a precision of 10^{-3} pH unit. Potential changes were converted into proton equivalents by double titration with standard solutions of KOH and HCl. These titrations were performed by following the pH changes that occurred within 30 ms after the addition, to aerobic or KCN-inhibited aerobic mitochondria, of standard solutions of HCl and KOH. The short time interval used avoids titration of the interior of the particles. The syringes of the continuous-flow pH meter, the mixing chamber and the measurement compartment were surrounded by a water bath thermostatted at 25 ± 0.01 °C. The reaction times at the measuring tip of the glass electrode during the continuous-flow phase (see the pH traces in Figs. 2, 4 and 5) were selected by changing the speed of the flow and the distance between the mixing chamber and the electrode tip (Papa *et al.*, 1979*c*).

Protein

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

Results

From succinate and quinol to O_2

The $\rightarrow H^+/O$ stoicheiometry of mitochondrial respiration has been measured in various laboratories using either the respiratory pulse method (Mitchell & Moyle, 1967) or the steady-state rate method (Reynafarje *et al.*, 1976). Numerous determinations by the respiratory pulse method produced, with succinate as substrate, an $\rightarrow H^+/O$ quotient of 4 (Mitchell, 1976; Papa, 1976; Haddock & Jones, 1977). More recently, however, an $\rightarrow H^+/O$ quotient of 6 has been obtained for succinate with this method (Brand, 1977; Brand *et al.*, 1976*b*) and its validity has become a matter of controversy (Papa, 1976; Brand, 1977; Lehninger, 1977; Pozzan *et al.*, 1979; Mitchell *et al.*, 1979) (see, however, the Discussion section).

The steady-state rate method, introduced to avoid the difficulties that might arise in the respiratory-pulse method, produced an H^+/O quotient of 8 with succinate (Reynafarje *et al.*, 1976; Lehninger, 1977). This method, which is based upon activation of respiration by rapid addition of succinate, or another reductant, to aerobic mitochondria, depends on accurate determination of the initial rate of respiration elicited by the substrate and that of proton ejection. In all the reports that produced an $\rightarrow H^+/O$ quotient of 8 the respiratory rate elicited by succinate was measured polarographically with a Clark oxygen electrode. Recently, however, direct comparison of the polarographic determination and the spectrophotometric assay with haemoglobin of respiratory rates showed that the Clark electrode is inadequate to measure accurately initial respiratory rates in rapid functional transitions of respiratory systems (Capuano *et al.*, 1980; Papa *et al.*, 1980). On the other hand, the spectrophotometric method with haemoglobin appears to be the method of choice for these measurements (Capuano *et al.*, 1980; Papa *et al.*, 1980).

The experiment shown in Fig. 1 shows that addition of succinate to a suspension of rat liver mitochondria whose O_2 concentration had been pre-lowered so to cause 50% deoxygenation of 25 μM-human HbO_2 added to the system, resulted in an immediate respiratory burst, as signalled by the abrupt absorbance decrease at 577−568 nm associated with HbO_2 deoxygenation. The Clark

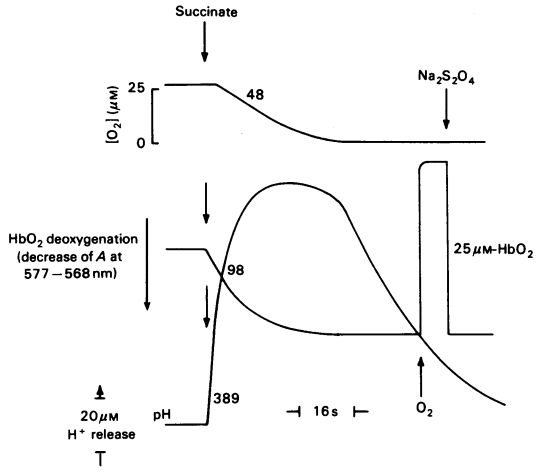

Fig. 1. *Respiration and proton release elicited by succinate addition to rat liver mitochondria*

Mitochondria (2.5 mg of protein/ml) were suspended in a reaction mixture containing 130 mM-LiCl, 1 mM-KCl, 3 mM-Hepes (lithium salt) 0.1 μg of valinomycin/mg of protein, 0.5 μg of rotenone/mg of protein and 30 nmol of *N*-ethylmaleimide/mg of protein. The final pH was 7.2. The suspension in the spectrophotometric cell was supplemented with 25 μM-HbO_2. Ar was blown onto the surface of the mitochondrial suspension for 4 min until the concentration of dissolved O_2 in the sample in the polarographic cell had decreased to 27 μM. The same effective O_2 concentration (dissolved O_2 + O_2 contributed by HbO_2) was obtained in the sample in the spectrophotometric cell, where HbO_2 was 50% deoxygenated by the Ar stream (Capuano *et al.*, 1980). After a further 1 min preincubation for temperature equilibration to 25°C, 1 mM-lithium succinate was rapidly injected into the mitochondrial suspension. The Clark-type electrode (see the Materials and methods section) was coated with a standard membrane (YSI 5775). The absorbance decrease at 577−568 nm, associated with HbO_2 deoxygenation, was converted into nmol of haem by using the absorbance increase at this wavelength couple caused by the oxygenation of the mitochondrial suspension made anaerobic by succinate respiration. Addition of solid sodium dithionite, which caused immediate complete deoxygenation of HbO_2, returned the absorbance trace to the level reached with anaerobiosis. The rate of O_2 consumption (ng-atoms·min^{-1}) was obtained by multiplying the rate of HbO_2 deoxygenation, in nmol of haem, by 2 and by the correction factor (*f*). This was calculated from polarographic and spectrophotometric measurements and amounted, for rat-liver mitochondria and 25 μM-haemoglobin, to 2.41 (Barzu, 1978; Capuano *et al.*, 1980). Separate control experiments showed that addition of succinate to the HbO_2-containing suspension equilibrated with air (haemoglobin fully oxygenated) did not cause any absorbance changes at 577−568 nm. Furthermore, in the absence of HbO_2, no absorbance change was observed at this wavelength couple when mitochondria became anaerobic, or when anaerobic mitochondria were oxygenated and then sodium dithionite was added. The numbers on the traces are rates expressed as ng-atoms of O_2 consumed and ng-ions of H^+ translocated/min per mg of protein.

electrode coated with the conventional membrane responded to respiration elicited by succinate only after a lag of 3 s. When the electrode was coated with the high-sensitivity membrane the lag was 1.5 s (results not shown). The respiratory rate measured either polarographically or spectrophotometrically was not constant and declined with time. Furthermore, the initial respiratory rate measured spectrophotometrically was considerably higher than that measured polarographically, with either the conventional or the high-sensitivity membrane. The results of a series of these experiments, presented in Table 1, show that (i) the initial rate of succinate respiration measured spectrophotometrically was twice as high as that measured polarographically with the standard membrane and 25% higher than that measured with the high-sensitivity membrane; (ii) the H^+/O quotient for succinate respiration was 8 (cf. Reynafarje *et al.*, 1976; Lehninger, 1977; Azzone *et al.*, 1979*b*) when obtained from the polarographic measurement of respiration with the standard membrane, 4.1 with the high-sensitivity membrane and 4 with the spectrophotometric measurement of O_2 consumption (see also Papa *et al.*, 1980). In these and the following measurements of $\rightarrow H^+/O$ quotients, haemoglobin, which was added to the sample of the mitochondrial suspension used to measure the respiratory rate, was omitted from the sample used to monitor the O_2 consumption polarographically and the pH changes (see legends to Figs. 1 and 6). This avoided a contribution to respiratory pH changes from those caused by HbO_2 deoxygenation as a result of the Bohr effect of this haemoprotein (Kilmartin & Rossi Bernardi, 1973).

Separate control experiments showed that haemoglobin *per se* had no effect on the initial rates of O_2 consumption, measured polarographically, and proton release when the reductant was added to air saturated mitochondria (Papa *et al.*, 1980). On the other hand, the pH changes caused by respiration were so small, approx. 10^{-2} pH unit, that they could not produce any significant change of the affinity of haemoglobin for O_2 (Kilmartin & Rossi Bernardi, 1973).

It should be noted that in these experiments mitochondria were supplemented with valinomycin + K^+ and *N*-ethylmaleimide, so to allow maximal electrogenic proton ejection and minimize back-diffusion of protons through the membrane (*cf.* Reynafarje *et al.*, 1976; Lehninger, 1977). It has,

Table 1. *Statistical analysis of the initial rates and stoicheiometric relationships of proton release and respiration in succinate- and duroquinol-pulse experiments*

The values are the means ± S.E.M. for the number of experiments indicated. The values for the $\rightarrow H^+/O$ and the $\rightarrow H^+/2e^-$ ratios are the means ± S.E.M. of the internal ratios measured in different experiments. (i) Succinate oxidation by rat liver mitochondria; $n = 22$. For experimental conditions see the legend to Fig. 1 and the Materials and methods section; (ii) duroquinol oxidation by beef heart mitochondria; $n = 12$. For experimental conditions see the legend to Fig. 2 and the Materials and methods section.

		O_2 consumption (ng-atoms/min per mg of protein)			$\rightarrow H^+/O$ calculated from		
	Method ...	Polarographic		Spectrophotometric (25 μM-haemoglobin)			
(i) H^+ release (ng-ions/min per mg of protein)	Membrane ...	Standard (*a*)	High-sensitivity (*b*)	(*c*)	(*a*)	(*b*)	(*c*)
415 ± 15		52.1 ± 2.5	83.4 ± 6.3	104 ± 3.8	7.96 ± 0.11	5.10 ± 0.12	4.0 ± 0.05

(ii) H^+ release from 273 to 459 ms (ng-ions/mg of protein)	Duroquinol oxidation from 273 to 459 ms (nmol/mg of protein)	$\rightarrow H^+/2e^-$
1.79 ± 0.15	0.5 ± 0.07	3.6 ± 0.15

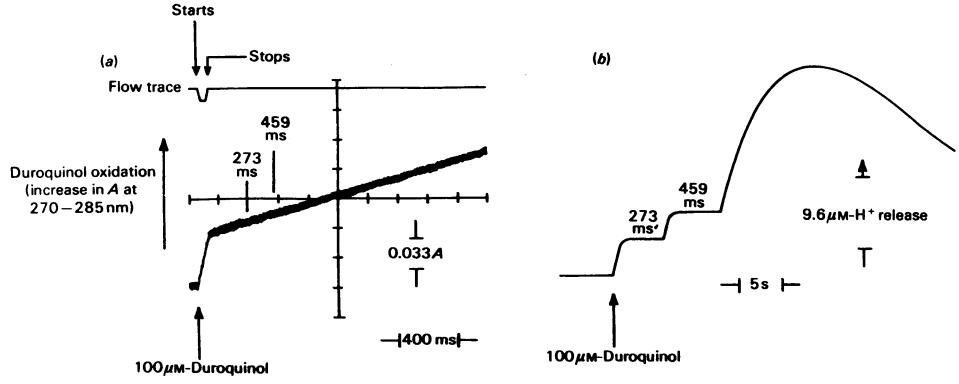

Fig. 2. *Duroquinol oxidation (a) and associated proton release (b) in beef heart mitochondria*

Mitochondria (2 mg of protein/ml) were preincubated for 5 min in the main syringes of the flow apparatus in an air-saturated medium containing 150 mM-KCl, 5 mM-potassium malonate, 0.5 μg of valinomycin/mg of protein, 0.5 μg of rotenone/mg of protein and 2 μg of oligomycin/mg of protein. The final pH was 7.2. Mitochondria were then pulsed with methanolic 6 mM-duroquinol (final concentration in the mitochondrial suspension, 100 μM). The reaction time during the two continuous steps, which were separated by an interval of about 5 s, needed to change the distance between the mixing chamber and the electrode tip, were 273 and 459 ms respectively (see Papa *et al.*, 1979*c*). For the measurement of the rate of duroquinol oxidation and proton release, as well as for other experimental details, see the Materials and methods section.

however, to be considered that, when respiration is activated by added succinate, the carrier-mediated diffusion of this substrate to succinate dehydrogenase at the matrix side of the membrane, in exchange for endogenous P_i and anionic substrates, might result in proton release or uptake (Papa *et al.*, 1970) that must be added or subtracted from the proton ejection directly associated with electron flow through Sites 2 and 3 (Papa *et al.*, 1979*b*). To check this, succinate was replaced by duroquinol, since this reductant can diffuse to the cytochrome system and initiate respiration without the intervention of proton-coupled transport systems. Another advantage of using duroquinol is that its oxidation can be directly measured spectrophotometrically.

Small amounts of duroquinol, which has a K_m of 28 μM for the cytochrome system (Lawford & Garland, 1973), were rapidly oxidized by beef-heart mitochondria. Conventional spectrophotometric and potentiometric measurements in stirred suspensions

of mitochondria turned out to be inadequate to measure accurately the initial rates of duroquinol oxidation and proton release, since (i) duroquinol oxidation exhibited a very rapid initial phase which lasted only 2s (see Fig. 2); (ii) the addition of the methanolic solution of duroquinol to the mitochondrial suspension caused a potentiometric artifact that added itself to the initial acidification caused by duroquinol oxidation and (iii) the duroquinol solution always contained traces of oxidized quinone.

These difficulties were overcome by measuring the initial rate of duroquinol oxidation with the stopped-flow spectrophotometer and that of acidification with the continuous-flow pH meter (see the Materials and methods section). With these instruments, which have a resolution time of less than 5ms, duroquinol was rapidly injected into the mitochondrial suspension and the rates of duroquinol oxidation and proton release were obtained from the progress of the reaction from 273 to 459ms after the addition of the reductant (Fig. 2). The $H^+/2e^-$ quotient so measured was 3.6 (Table 1) (*cf.* Lawford & Garland, 1973; Papa *et al.*, 1975).

From succinate and quinol to ferricytochrome c

The $\rightarrow H^+/2e^-$ quotient for proton release associated with the span from the dehydrogenase terminus of the cytochrome system to cytochrome *c* was determined by activating electron flow in KCN-inhibited rat liver or beef heart mitochondria in one of the following ways: (i) succinate pulse of mitochondria equilibrated with ferricyanide; (ii) ferricytochrome *c* pulse of mitochondria equilibrated with

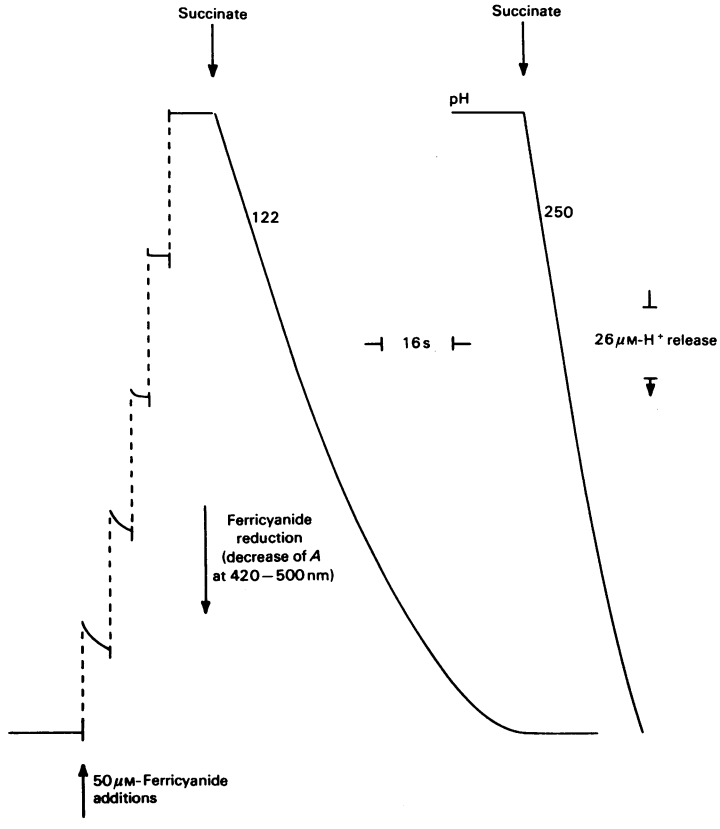

Fig. 3. *Proton release associated with reduction of ferricyanide by succinate in KCN-treated rat liver mitochondria*
Mitochondria (2.5mg of protein/ml) were suspended in the reaction mixture described in the legend to Fig. 1. KCN (1mM) was also present. Ferricyanide (250μM) was added in 50μM-aliquots. The first aliquots of ferricyanide underwent partial reduction by endogenous reductants. The absorbance changes caused by the last two aliquots of ferricyanide, which did not undergo further reduction, were equivalent and were used to convert the absorbance decrease at 420–500nm caused by succinate addition into nmol of ferricyanide reduced. Reduction of ferricyanide and proton release were initiated by the addition of 1mM-lithium succinate. For other experimental details see the Materials and methods section. The numbers on the traces are initial rates expressed as ng-ions of H^+ translocated and nmol of ferricyanide reduced/min per mg of protein.

duroquinol and (iii) duroquinol pulse of mitochondria equilibrated with ferricyanide. Mitochondria were supplemented with valinomycin + K^+, and *N*-ethylmaleimide was added except in type-(ii) experiments.

Fig. 3 illustrates type-(i) experiments. Addition of 1 mM-succinate to a stirred suspension of rat liver mitochondria, supplemented with rotenone and KCN, resulted in immediate reduction of ferricyanide, measured spectrophotometrically from the absorbance decrease at 420–500nm, and rapid proton ejection. The $\rightarrow H^+/2e^-$ quotient obtained from the initial rates of ferricyanide reduction and proton release was 3.9 (see Table 2) (*cf.* Alexandre *et al.*, 1978; Pozzan *et al.*, 1979).

Fig. 4 shows type-(ii) experiments. Beef-heart mitochondria were supplemented with KCN, and malonate + rotenone were also present to inhibit succinate and NADH dehydrogenases. An excess of duroquinol was then added and, after a short preincubation to allow redox equilibration, mitochondria were rapidly pulsed with exogenous 8 μM-cytochrome *c*. The speed of electron flow from duroquinol to cytochrome *c* required application of flow methods to measure the initial rate of cytochrome *c* reduction and proton release from mitochondria.

The stopped-flow double-beam trace (Fig. 4) shows that ferricytochrome *c* was rapidly and completely reduced with a $t_{\frac{1}{2}}$ of 80ms. Cytochrome *c* reduction exhibited a small antimycin-insensitive fast phase that amounted to about 1ng-equiv. of electrons per mg of protein. This roughly corresponds to the molar content of the known redox carriers of Complex III on the O_2 side of the antimycin inhibition site (Erecinska *et al.*, 1976). The remaining slower phase of cytochrome *c* reduction was 95% inhibited by antimycin.

The antimycin-sensitive electron flow from duroquinol to cytochrome *c* was accompanied by rapid proton release from mitochondria, which was measured with the continuous-flow pH meter (see Fig. 4). To avoid any significant loss of active proton ejection through back-flow, an interval of 45ms was chosen to compute the $H^+/2e^-$ ratio for antimycin-sensitive proton release. The $\rightarrow H^+/2e^-$ quotient, so measured, was 3.6 (see Table 2).

In the experiment shown in Fig. 5 beef heart mitochondria, treated as in Fig. 4, were equilibrated with ferricyanide, then duroquinol was rapidly injected into the suspension and the rate of ferricyanide reduction and proton release was measured by flow methods. The progress of the reaction from 273 to 459ms was used to compute the $\rightarrow H^+/2e^-$ quotient, which was 3.6 (see Table 2).

From cytochrome c to O_2

It has been reported (Wikström, 1977; Wikström & Saari, 1977; Wikström & Krab, 1979; Sigel & Carafoli, 1978; Alexandre *et al.*, 1978; Azzone *et al.*, 1979*a*) that, when specific precautions are taken to prevent proton back-flow across the membrane, aerobic oxidation of artificial reductants of cytochrome *c* by antimycin-inhibited mitochondria results in proton release, which is in excess of the acidification eventually associated with the conversion of the reductant into its oxidized product. Papa *et al.* (1978*a*,*b*,*c*), Moyle & Mitchell (1978*a*),

Table 2. *Statistical analysis of proton release, electron flow and $H^+/2e^-$ quotient for electron flow from succinate to ferricyanide and from duroquinol to exogenous ferricytochrome c or ferricyanide*

The values are the means ± S.E.M. for the number of experiments indicated. The values for $H^+/2e^-$ ratios are means ± S.E.M. of the internal $H^+/2e^-$ ratios measured in different experiments. Experimental conditions: (i) succinate/ferricyanide in rat-liver mitochondria (see the legend to Fig. 3 and the Materials and methods section); (ii) duroquinol/ferricytochrome *c* in beef heart mitochondria, both electron flow and H^+ release were corrected for the antimycin-insensitive reaction (see the legend to Fig. 4 and the Materials and methods section); (iii) duroquinol/ferricyanide in beef-heart mitochondria (see the legend to Fig. 5 and the Materials and methods section). In beef-heart mitochondria the rate of ferricytochrome *c* reduction (in nmol·min^{-1}) that can be computed from the progress of the reaction in 45ms (Expt. ii) is considerably higher than the rate of ferricyanide reduction computed from the progress of the reaction from 273 to 459msec (Expt. iii). This reflects a rapid decay of the rate of electron flow in the bc_1 complex (see Figs. 4 and 5) as well as a reaction of ferricytochrome faster than that elicited by ferricyanide.

Conditions	No. of expts.	H^+ release (ng-ions/mg of protein)	Ferricyanide or ferricytochrome *c* reduction (nmol/mg of protein)	$\rightarrow H^+/2e^-$
(i) Succinate/ferricyanide	4	250 ± 12*	129 ± 5*	3.9 ± 0.09
(ii) Duroquinol/ferricytochrome *c*	10	4.4 ± 0.4†	2.51 ± 0.18†	3.6 ± 0.1
(iii) Duroquinol/ferricyanide	6	3.5 ± 0.2‡	1.95 ± 0.12‡	3.6 ± 0.04

* Initial rate/min.
† In the first 45ms
‡ From 273 to 459ms.

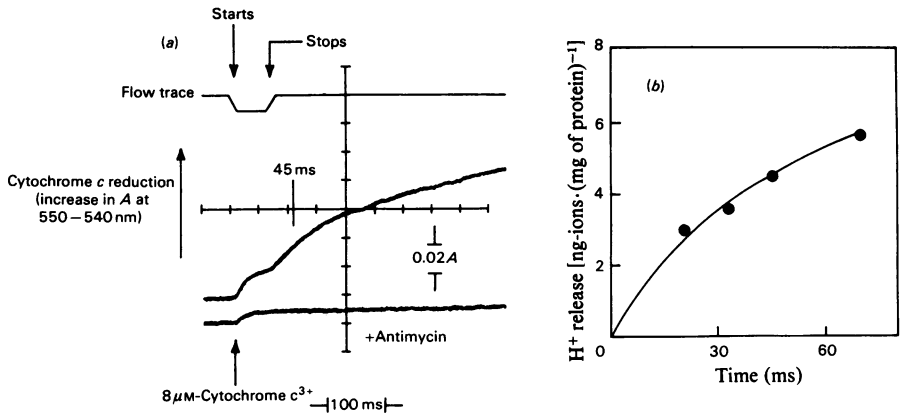

Fig. 4. *Reduction of exogenous ferricytochrome c by duroquinol (a) and associated proton release (b) in KCN-inhibited beef heart mitochondria*

Mitochondria (1 mg of protein/ml) were incubated for 5 min in the main syringes of the flow apparatus in the same medium described in the legend to Fig. 2, which contained also 1 mM-KCN. After incubation, a methanolic solution of duroquinol was added directly in the main syringes at a final concentration of 100 μM. After 2 min the mitochondrial suspension was pulsed with ferricytochrome *c*, present in the side syringes as an 0.48 mM solution in 180 mM-KCl (the final concentration in the mitochondrial suspension was 8 μM). The H^+ release shown in the figure was corrected for a small pH change caused by ferricytochrome *c* addition to antimycin-supplemented mitochondria. For measurement of the initial rate of ferricytochrome *c* reduction and proton release as well as for other experimental details see the Materials and methods section.

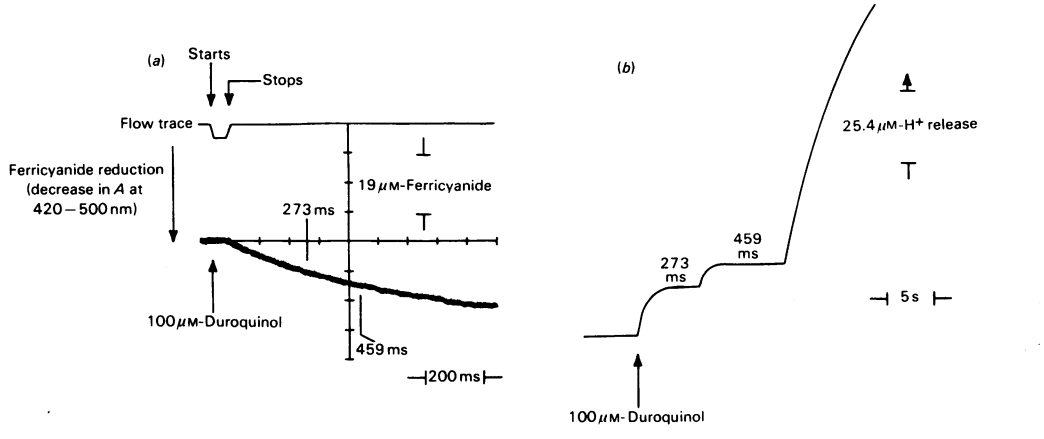

Fig. 5. *Ferricyanide reduction by duroquinol (a) and associated proton release (b) in KCN-inhibited beef heart mitochondria*

Mitochondria (2 mg of protein/ml) were incubated for 10 min in a medium containing 150 mM-KCl, 5 mM-malonate, 2 mM-glycylglycine, 30 nmol of *N*-ethylmaleimide/mg of protein, 0.5 μg of valinomycin/mg of protein and 0.5 μg of oligomycin/mg of protein. After this interval, 2 mM-KCN and 0.5 μg of rotenone/mg of protein were added and the pH was adjusted to 7.2. A 20 ml aliquot of the mitochondrial suspension was used for ferricyanide calibration. To the remaining, 250 μM-ferricyanide was added; 5 min later the mitochondrial suspension was pulsed with methanolic 6 mM-duroquinol (the final concentration in the mitochondrial suspension was 100 μM). The reaction time during the two continuous steps, which were separated by an interval of about 5 s, needed to change the distance between the mixing chamber and the electrode tip, were 273 and 459 ms respectively (see Papa *et al.*, 1979*c*). Ferricyanide calibration was obtained by subsequent additions of 50 μM-ferricyanide to the mitochondrial suspension (see Fig. 3). For measurement of the initial rate of ferricyanide reduction and proton release, as well as for other experimental details see the Materials and methods section.

Mitchell *et al.* (1978) and Lorusso *et al.* (1979) have, however, shown that proton release associated with ferrocyanide respiration results from re-reduction by endogenous hydrogenated reductants of the ferricyanide formed.

Fig. 6 illustrates the time course of oxygen

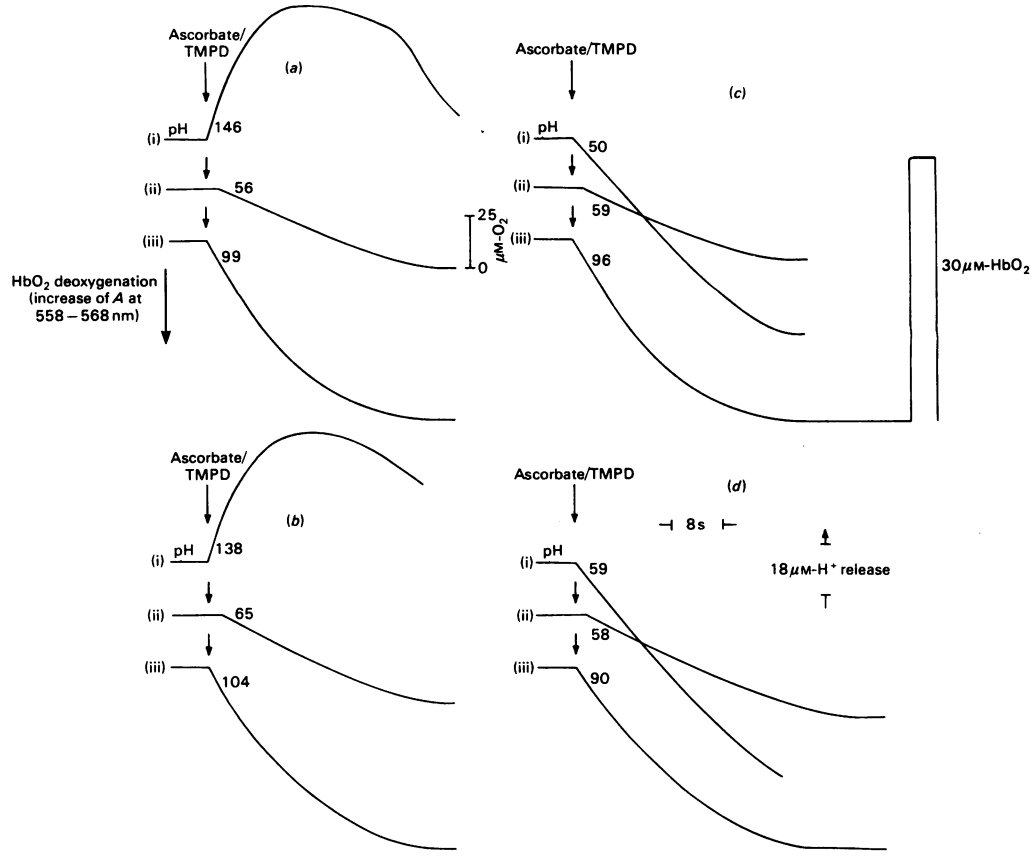

Fig. 6. *Respiration and proton translocation elicited by TMPD + ascorbate pulses of rat-liver mitochondria at a decreased O_2 concentration*

Mitochondria (3 mg of protein/ml) were suspended in a reaction mixture containing 150 mM-LiCl, 1 mM-KCl, 3 mM-Hepes (lithium salt), 0.5 mM-EDTA (lithium salt), 0.1 μg of valinomycin/mg of protein, 1 μg of rotenone/mg of protein, 1 μg of oligomycin/mg of protein and 30 nmol of *N*-ethylmaleimide/mg of protein. In addition the mixture contained 0.05 μg of antimycin/mg of protein in Expts. (*a*) and (*c*) and 6 μM-FCCP in Expts. (*c*) and (*d*). The final pH was 7.0. The suspension in the spectrophotometric cell was supplemented with 30 μM-HbO_2. Ar was blown onto the surface of the mitochondrial suspension, until the concentration of dissolved O_2 in the samples in the polarographic cell was decreased to 35–50 μM and the HbO_2, in the samples in the spectrophotometric cell, was 70% deoxygenated; the corresponding effective O_2 concentration (dissolved $O_2 + O_2$ contributed by HbO_2) was 44 μM. After a further 1 min preincubation, for temperature equilibration to 25°C, a mixture of TMPD (50 μM final concentration) + ascorbate (1 mM final concentration) adjusted to pH 7.0 ± 0.005 with LiOH, was rapidly injected into the mitochondrial suspension in Expts. (*a*) and (*c*); in Expts. (*b*) and (*d*) first 0.05 μg of antimycin/mg of protein was added, followed 15 s later by TMPD + ascorbate. The oxygen electrode was coated with a high-sensitivity membrane (YSI 5776). Deoxygenation of HbO_2 was measured as the absorbance increase at 558–568 nm (see Capuano *et al.*, 1980) instead of the absorbance decrease at 577–568 nm (see Fig. 1) since at this latter wavelength couple interference by possible Wurster Blue formation might occur. The absorbance decrease at 558–568 nm associated with HbO_2 deoxygenation was converted to nmol of haem, and the rate of O_2 consumption was calculated from that of HbO_2 deoxygenation as described in the legend to Fig. 1. The factor (*f*), calculated from polarographic and spectrophotometric measurements, was, for 30 μM-HbO_2, 1.98. Separate controls showed that, in the absence of HbO_2, no detectable absorbance change was observed at 558–568 nm upon addition of TMPD + ascorbate and when the mitochondria become anaerobic. The numbers on the traces are initial rates expressed as ng-atoms of O_2 consumed and ng-ions of H^+ translocated/min per mg of protein.

consumption and proton translocation elicited by addition of TMPD + ascorbate to rat liver mitochondria supplemented with *N*-ethylmaleimide, valinomycin and antimycin. HbO_2 (30 μM) was included in the suspension and the concentration of dissolved O_2 was pre-lowered so as to cause 70% deoxygenation of HbO_2 and to allow spectrophotometric measurement of the initial respiratory rate elicited by the reductant from the slope of the absorbance changes associated with deoxygenation of HbO_2.

The addition of TMPD + ascorbate caused an immediate respiratory burst, monitored as an abrupt deoxygenation of HbO_2 (measured in this case as the absorbance increase at 558 − 568 nm). The respiratory rate, however, rapidly declined (see Fig. 6, trace iii). The initial rapid respiratory phase, detected by the spectrophotometric method, could not be determined accurately by the oxygen electrode coated with the high-sensitivity membrane, which started to respond to the respiratory burst with a lag of 1.5 s. Thus the polarographic assay resulted in a substantial underestimation of the initial respiratory rate as compared with that measured spectrophotometrically (see Table 3).

The respiratory burst was accompanied by a rapid acidification of the external medium followed by a net change towards alkalinization, as expected from the overall stoicheiometry of the reaction:

$$\text{Ascorbate} + \tfrac{1}{2}O_2 + H^+ \rightarrow \text{dehydroascorbate} + H_2O \quad (1)$$

The $\rightarrow H^+/O$ quotient for aerobic proton release, corrected for H^+ production in the oxidation of ascorbate to dehydroascorbate, was, when computed on the basis of polarographic measurements of O_2 consumption, 1.7 (Table 3) (*cf.* Sigel & Carafoli, 1978; Krab & Wikström, 1979). The $\rightarrow H^+/O$ quotient was, however, only 0.5 when computed from the accurate spectrophotometric determination of the initial respiratory rate (Table 3).

It should be noted that separate control experiments (see Fig. 7) showed that (i) when TMPD + ascorbate were added to air-saturated mitochondria the $\rightarrow H^+/O$ quotient obtained from polarographic measurements of O_2 consumption was the same as that calculated from this method in mitochondria with a decreased concentration of dissolved O_2; (ii) deoxygenation of 30 μM-HbO_2 added to the mitochondrial suspension started when the concentration of dissolved O_2 was decreased to about 100 μM (Barzu, 1978; Capuano *et al.*, 1980). The respiratory rate obtained from the slope of the linear part of the HbO_2-deoxygenation curve was equal to that measured polarographically in the terminal respiratory phase and (iii) addition of TMPD + ascorbate to HbO_2-supplemented aerobic mitochondria and their subsequent oxidation did not

Table 3. *Statistical analysis of the initial rates and stoicheiometric relationships of proton release and respiration and net proton consumption for TMPD + ascorbate pulse experiments in rat liver mitochondria*

The values are the means ± S.E.M. for eight experiments. The values for H^+/O ratios are the means ± S.E.M. for the internal values measured in different experiments. The H^+/O quotients corrected for H^+ production in the oxidation of ascorbate to dehydroascorbate are also given. For experimental conditions see the legend to Fig. 6 and the Materials and methods section.

								+6 μM-FCCP					
			O_2 consumption (ng-atoms/min per mg of protein)		$\rightarrow H^+/O$				O_2 consumption (ng-atoms/min per mg of protein)		H^+/O	
Expt.	H^+ release (ng-ions/min per mg of protein)	Method	Polarographic (i)	Spectrophotometric (30 μM-haemoglobin) (ii)	calculated from (i)	(ii)	H^+ consumption (ng-ions/min per mg of protein)	Method	Polarographic (i)	Spectrophotometric (ii)	calculated from (i)	(ii)
(*a*)	137.2 ± 8.1		52.0 ± 3.2	95.0 ± 7.9	2.67 ± 0.01	1.48 ± 0.1	48.6 ± 1.8		57.7 ± 2.7	94.5 ± 7.5	0.85 ± 0.04	0.54 ± 0.05
	Corrected values				1.67	0.48					1.85	1.54
(*b*)	124.9 ± 7.1		55.2 ± 3.7	96.1 ± 7.1	2.29 ± 0.1	1.33 ± 0.1	55.8 ± 1.5		58.5 ± 1.5	88.7 ± 5.6	0.98 ± 0.1	0.64 ± 0.04
	Corrected values				1.29	0.33					1.98	1.64

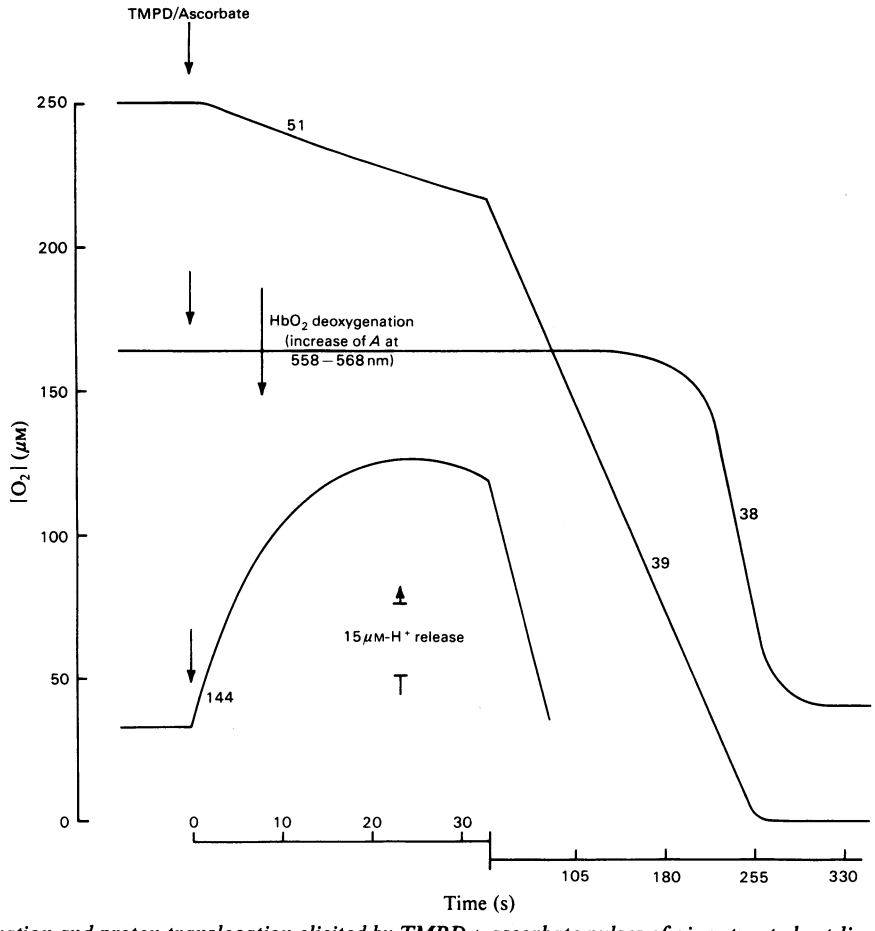

Fig. 7. *Respiration and proton translocation elicited by TMPD + ascorbate pulses of air-saturated rat-liver mitochondria*
Mitochondria (3 mg of protein/ml) were suspended in the same reaction mixture as for the experiments shown in Fig. 6. The concentration of HbO_2, added only to the suspension in the spectrophotometric cuvette, was 30 μM and the factor (*f*) was 1.98. In addition 0.05 μg of antimycin/mg of protein was also present. After 5 min preincubation at 25°C a mixture of TMPD (50 μM final concentration) + ascorbate (1 mM final concentration) was injected into the mitochondrial suspension. The oxygen electrode was coated with the high-sensitivity membrane. The numbers on the traces are rates expressed as ng-atoms of O_2 consumed and ng-ions of H^+ translocated/min per mg of protein.

cause by themselves any absorbance change at 558–568 nm.

Measurement in the presence of FCCP of the scalar proton consumption expected from reaction (1), gave, with polarographic measurements of O_2 consumption, an H^+/O ratio of 0.85 (1.85 when corrected for the acidification caused by oxidation of ascorbate to dehydroascorbate). The ratio was, however, only 0.54 (corrected value 1.54) when the respiratory rate was measured spectrophotometrically with haemoglobin. Thus the H^+/O quotient for net scalar proton consumption for O_2 reduction by ascorbate is significantly lower than the expected value of 1 (reaction 1) (the corrected value is lower than 2).

When mitochondria were preincubated aerobically for 6 min with valinomycin but in the absence of antimycin, which was then added only 15 s before TMPD + ascorbate, the deficit of scalar proton consumption versus O_2 consumption was smaller; the H^+/O quotient, in fact, increased from 0.54 to 0.64. At the same time the $\rightarrow H^+/O$ quotient for proton release fell to 1.3 when obtained by polarographic measurement of respiration, and to 0.3 by spectrophotometric assay of respiration.

Discussion

The results presented show that the $\rightarrow H^+/O$ quotient for proton release associated with electron

flow down the cytochrome system of mitochondria from succinate or quinol to O_2 is, at neutral pH, 4. All the four protons are shown to be released during electron flow from quinol to ferricytochrome *c*. It is demonstrated that no proton release takes place when electrons are further transferred from ferrocytochrome *c* to O_2.

These conclusions, which invalidate recent reports claiming $\rightarrow H^+/O$ quotients for the cytochrome system of 6 (Brand, 1977; Brand *et al.*, 1978; Wikström & Krab, 1979) or 8 (Reynafarje *et al.*, 1976; Lehninger, 1977; Azzone *et al.*, 1978) and the existence of a proton pump in cytochrome *c* oxidase (Wikström & Krab, 1979; Sigel & Carafoli, 1978; Alexandre *et al.*, 1978; Azzone *et al.*, 1979*a*) are based on the following experimental grounds.

(*a*) *Accurate determination of the initial rate of electron flow elicited by reductant pulses by independent spectrophotometric measurements of consumption of oxidants and reductants.* It is shown in this paper that the rate of electron flow elicited by the addition to mitochondria of reductants such as succinate (see also Papa *et al.*, 1980), duroquinol or TMPD + ascorbate, after an immediate burst, declines in 1–2 s or less (see Figs. 1–6), Under these circumstances the initial rate of electron flow is underestimated by the oxygen electrode, due to its response characteristics (see the Materials and methods section).

The spectrophotometric method with haemoglobin, which responds to immediate O_2 consumption by sodium dithionite with a $t_{\frac{1}{2}}$ of 0.1 s (this includes the overall response time of the spectrophotometric recording system) and without any lag, is, on the other hand, adequate to measure accurately the initial rate of respiration elicited by succinate or TMPD + ascorbate (Capuano *et al.*, 1980). Electron flows from duroquinol to O_2, ferricyanide or cytochrome *c* are even faster processes. In this case, accurate spectrophotometric measurement of the initial rates of quinol consumption and reduction of ferricyanide or ferricytochrome *c*, which decline rapidly, could be obtained by using a flow spectrophotometer (see the Materials and methods and Results sections).

(*b*) *Accurate potentiometric determinations of the rate of proton translocation.* The initial rate of proton translocation elicited by addition of succinate or TMPD + ascorbate to aerobic mitochondria could be accurately determined in stirred suspension with a conventional, rapidly responding pH meter. Faster proton translocation caused by electron flow from duroquinol to O_2, ferricyanide or ferricytochrome *c* had to be determined with a flow pH meter. It should be pointed out that measurement of the H^+/e^- stoicheiometry based upon direct measurement with flow techniques of the rapid initial phase of proton-translocating redox reactions provides a time resolution of the primary process and eliminates interferences by secondary proton redistribution across the membrane through leaks and proton-linked solute-transporting systems, such as anion/proton symporters and alkali metal/proton antiporters. The rate of these secondary proton fluxes is, in fact, negligible with respect to the high rate of electron flow and proton transfer elicited by a pulse of redox reactants (Mitchell & Moyle, 1967; Papa, 1971).

(*c*) *Precise definition of the reductants and oxidants involved in the electron transfer process.* When electron flow in a given span of the respiratory chain is activated by pulsing the aerobic system with a reductant, accurate determination of the H^+/e^- quotient from rate measurements requires that the oxidized form of the reductants is not re-reduced by endogenous substrates. This condition is apparently met when rotenone-treated mitochondria are pulsed with succinate or duroquinol (*cf.* Papa *et al.*, 1980; Lawford & Garland, 1973). The residual rate of rotenone-insensitive electron flow on the substrate side of ubiquinone is, in fact, negligible with respect to the rate of electron flow along the cytochrome system. What is presented in this paper and elsewhere (Moyle & Mitchell, 1978*a*; Lorusso *et al.*, 1979) shows, on the contrary, that, when electron flow in the cytochrome c/O_2 span of the respiratory chain is activated by a pulse of ferrocyanide or TMPD + ascorbate, the oxidized forms of these reductants can be re-reduced at a significant rate, even in the presence of antimycin, by endogenous hydrogenated reductants. This introduces unrelated electron flow and proton translocation in addition to that taking place at the third site. Thus experimental conditions that avoid this have to be used, so as to determine precisely the electron and proton transfer reactions taking place directly in the cytochrome c/O_2 span (see also Lorusso *et al.*, 1979).

The present study demonstrates that it has been failure to meet one or more of the above conditions that has produced erroneous $\rightarrow H^+/e^-$ quotients in some other laboratories (Reynafarje *et al.*, 1976; Alexandre *et al.*, 1978; Azzone *et al.*, 1978) and results that appeared to support the existence of a proton pump in cytochrome *c* oxidase (Wikström & Krab, 1979; Sigel & Carafoli, 1978; Alexandre *et al.*, 1978; Azzone *et al.*, 1979*a*).

The $\rightarrow H^+/O$ quotient for proton release elicited by succinate addition to aerobic mitochondria is 4 when computed on the basis of an accurate spectrophotometric determination with haemoglobin of the initial respiratory rate (Table 1). The higher H^+/O quotients obtained with the steady-state rate method by other investigators (Lehninger, 1977; Reynafarje *et al.*, 1976; Azzone *et al.*, 1979*b*) and reproduced in this study by using polarographic estimates of the respiratory rate (see Table 1), result from under-

estimation of the initial respiratory rate. In fact, the respiratory rate elicited by succinate oxidation is characterized, under the experimental conditions of the steady-state rate method introduced by Reynafarje *et al.* (1976), by an initial transient rapid phase (see also Papa *et al.*, 1980). This can be detected and accurately determined by the haemoglobin method but is underestimated by the polarographic method (see Table 1). The rapid phase of succinate respiration appears to be sustained by rapid succinate uptake in exchange with P_i and anionic substrates, accumulated in the matrix during preincubation with *N*-ethylmaleimide and rotenone (Papa *et al.*, 1980).

Brand *et al.* (1976*a*,*b*; see also Brand, 1977), have found that *N*-ethylmaleimide enhances the $\rightarrow H^+/2e^-$ (per site) quotient, measured with the respiratory pulse method, from 2 to 3. Hence it was concluded by these authors (see also Wikström & Krab, 1979; Pozzan *et al.*, 1979) that the $\rightarrow H^+/2e^-$ (per site) quotient of 2, previously obtained with this method, was underestimated because of proton back-flow through P_i/H^+ symport. Moyle & Mitchell (1978*b*) and Mitchell *et al.* (1979) have, on the basis of different observations, rejected this claim and proposed that the enhancement of the $\rightarrow H^+/2e^-$ quotient caused by *N*-ethylmaleimide is, in these experiments, due to activation of the H^+-translocating oxidation of endogenous NAD(P)H.

The $\rightarrow H^+/2e^-$ ratio for duroquinol respiration obtained from flow potentiometric and spectrophotometric measurements is, at pH 7.2, practically 4 (3.6 ± 0.15). An $H^+/2e^-$ quotient of 4 had also been calculated by Lawford & Garland (1973) from the observed extent of acidification and from the amount of quinols added to aerobic mitochondria and by Papa *et al.* (1975) on the basis of flow measurements of proton uptake associated with aerobic oxidation of endogenous ubiquinol in 'inside out' submitochondrial vesicles.

The $\rightarrow H^+/2e^-$ quotient for electron flow from the substrate terminus of the cytochrome system to ferricytochrome, determined by three independent approaches, ranged from 3.9 to 3.6.

The inescapable conclusion that has to be drawn from the correspondence of the $\rightarrow H^+/2e^-$ quotient for electron flow from succinate or duroquinol to O_2 and from these substrates to ferricytochrome *c* or ferricyanide is that electron flow from ferrocytochrome *c* to O_2 does not cause any proton ejection from mitochondria. Neither is the proton release from mitochondria that has been shown to accompany under particular conditions the aerobic oxidation of artificial reductants of cytochrome *c* (Wikström & Krab, 1979; Sigel & Carafoli, 1978; Alexandre *et al.*, 1978; Azzone *et al.*, 1979*a*) derived from a proton pump in cytochrome *c* oxidase.

Thus it has been shown (Papa *et al.*, 1978*a*,*b*,*c*; Moyle & Mitchell, 1978*a*; Mitchell *et al.*, 1978; Lorusso *et al.*, 1979) that proton release caused by ferrocyanide respiration results from antimycin-insensitive re-reduction by endogenous hydrogenated reductants of the ferricyanide formed. In fact, (i) when ferrocyanide respiration results in proton release, the rate of ferricyanide net formation is lower than the rate of O_2 reduction (Lorusso *et al.*, 1979; *cf.* Wikström & Krab, 1978) [thus it is erroneous to compute the $\rightarrow H^+/e^-$ quotient from the rate of net ferricyanide formation (Alexandre *et al.*, 1978)]; (ii) the rate of scalar proton consumption for reduction of O_2 to H_2O measured in the presence of FCCP is also lower, by the same measure as ferricyanide formation, than the rate of O_2 reduction (Papa *et al.*, 1978*c*; Moyle & Mitchell, 1978*a*; Lorusso *et al.*, 1979); (iii) 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (Brandon *et al.*, 1972; Izzo *et al.*, 1978) inhibits the antimycin-insensitive proton release caused by ferrocyanide respiration (Papa *et al.*, 1978*a*,*b*,*c*; Lorusso *et al.* 1979; Azzone *et al.* 1979*a*). It has been shown that this effect of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide is due to direct inhibition by this substance of re-reduction of formed ferricyanide by endogenous hydrogenated reductants (Fig. 5 of Lorusso *et al.*, 1979) in addition to its proton-conducting activity (Lorusso *et al.*, 1979; Krab & Wikström, 1980).

These observations are extended in the present paper, in that it provides evidence showing that the proton release that accompanies, in the presence of antimycin, the aerobic oxidation of TMPD + ascorbate, in addition to the acid production arising directly from oxidation of ascorbate to dehydroascorbate, is derived from oxidation of endogenous hydrogenated reductants.

First, the $\rightarrow H^+/e^-$ quotient for the corrected proton release amounts, under the most favourable conditions used by other investigators (Sigel & Carafoli, 1978), to only 0.24 when computed on the basis of the real initial respiratory rate estimated spectrophotometrically. This is much lower than the value of 1 (Sigel & Carafoli, 1978) or 2 (Alexandre *et al.*, 1978; Azzone *et al.*, 1979*a*) both calculated by using respiratory rates obtained with the oxygen electrode (*cf.* Fig. 6 and Table 3). The fact that the respiratory rate elicited by TMPD + ascorbate decays rapidly with time also invalidates the determination by Krab & Wikström (1979) of an $\rightarrow H^+/e^-$ quotient of 1 with a mathematical model based on the condition that the respiratory rate elicited by reductants of cytochrome *c* is constant.

Secondly, the rate of scalar proton consumption measured in the presence of FCCP, is, with TMPD + ascorbate, as with ferrocyanide, lower than the rate of O_2 reduction. The deficit of proton consumption is clearly evident when this is com-

pared with the respiratory rate measured spectrophotometrically. Previous investigators, measuring the initial rate of O_2 consumption polarographically, did not observe a deficit of proton consumption (Sigel & Carafoli, 1978).

Thirdly, aerobic preincubation with valinomycin, but in the absence of antimycin, which is added only a few s before TMPD + ascorbate, results in almost complete suppression of vectorial proton release (the H^+/e^- ratio is 0.1) and in a smaller deficit of proton consumption versus O_2 reduction.

These observations provide evidence that hydrogenated reductants, whose aerobic oxidation during the preincubation period is prevented by the presence of antimycin, are then rapidly oxidized upon addition of TMPD + ascorbate by some Wurster Blue formed even in the presence of ascorbate. It is, in fact, well known that TMPD/Wurster Blue bypass the antimycin inhibition site (Lee *et al.*, 1967). Reduction of positively charged Wurster Blue by endogenous hydrogenated carriers might be favoured by its binding at the surface of the membrane, whose dipoles would be oriented in the energized state with negative charges towards the outer space (Mitchell & Moyle, 1967; Azzi & Vainio, 1970).

In conclusion, the data presented show that, in intact mitochondria no vectorial proton translocation from the inner to the outer aqueous phase is associated with electron flow from cytochrome *c* to O_2. Thus there is no justification for maintaining the existence of a proton pump in cytochrome *c* oxidase.

Different explanations might be sought, as outlined elsewhere (Moyle and Mitchell, 1978*a*; Lorusso *et al.*, 1979), for other observations such as the transient proton release that can be observed upon oxidation of ferrocytochrome *c* by mitochondria or cytochrome oxidase incorporated into liposomes (Wikström & Krab, 1979; Casey *et al.*, 1979; Sigel & Carafoli, 1979), or the acidification of the internal space of 'inside out' submitochondrial vesicles caused by aerobic oxidation of TMPD + ascorbate (Sorgato & Ferguson, 1978), which have been considered as supporting the existence of a proton pump in the oxidase (but see Wrigglesworth & Nicholls, 1979; Miller *et al.*, 1979). Sigel & Carafoli (1979) have reported that in reconstituted vesicles containing cytochrome *c* oxidase the K^+/e^- ratio for valinomycin-mediated K^+ uptake caused by oxidation of TMPD exhibited a maximum value of 1.75 by using polarographic measurements of O_2 consumption. The oxygen electrode used by Sigel & Carafoli (1979) was reported to have a 90% response time of 0.4 s, which would be very close to the response time for the haemoglobin method. However, in their experiments, upon addition of TMPD + ascorbate, respiration, in particular, and K^+ and H^+ translocation started only after a lag phase of 0.4–0.8 s (see Fig. 1 of Sigel & Carafoli, 1979). This, together with the present observations (see also Wikström & Krab, 1979), leave their measurements open to uncertainty.

Cytochrome *c* oxidase, as well as other respiratory carriers, exhibits co-operative linkage between the redox state of the electron transfer centres and protolytic equilibria in the apoproteins (redox Bohr effects) (Papa, 1976; Papa *et al.*, 1979*a*). What is presented in this paper (and elsewhere; Papa *et al.*, 1978*a*, 1979*a*,*b*) appears to exclude the possibility that these redox Bohr effects in cytochrome oxidase do result in a proton-pumping activity. They probably play simply a regulatory role for the redox activity of the system (*cf.* Wrigglesworth, 1978). Elucidation of the proton-translocating function of the cytochrome system would, on the other hand, require detailed analysis of the proton-transfer reactions associated with the electron transfer steps in the ubiquinone/cytochrome *c* segment (Papa, 1976; Papa *et al.*, 1977, 1978*a*; Trumpower, 1979) where vectorial proton translocation takes place. In particular it remains to be established if this process results from vectorial proton translocation by the catalytic redox centres (Mitchell, 1976, 1979) or whether it involves alternatively to, or in conjunction with, this type of mechanism, also co-operative linkage between redox transitions of the catalytic centres and anisotropic protolytic equilibria in the apoproteins (Papa, 1976; Papa *et al.*, 1979*c*).

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