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# Steady-state $H^+/O$ stoichiometry of liver mitochondria

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We have measured the $H^+/O$ stoichiometry of rat liver mitochondria respiring in a steady-state, using a novel method. This involves measuring the initial rate of $H^+$ back-flow into mitochondria after respiratory inhibition, with the assumption that this is equal to the steady-state $H^+$-ejection rate. Division by the steady-state $O_2$-consumption rate yields the $H^+/O$ ratio. The $H^+/O$ values obtained were: $8.3 \pm 1.0$ (mean ± S.E.M.) for 3-hydroxybutyrate; $8.2 \pm 0.7$ for glutamate plus malate; $6.0 \pm 0.2$ for succinate; $4.1 \pm 0.3$ for ascorbate/tetramethylphenylenediamine and $3.0 \pm 0.1$ for ascorbate/ferrocyanide. These values correspond to $H^+/O$ stoichiometries for electron flow to oxygen from $NAD^+$-linked substrates, succinate and cytochrome *c* of 8, 6 and 2 (charge/O ratio = 4) respectively.

It has been shown that respiratory and photosynthetic redox reactions result in the generation of $\Delta\tilde{\mu}H^+$ across energy-transducing membranes (Mitchell & Moyle, 1969; Rottenberg, 1970; Nicholls, 1974). It is widely accepted that $\Delta\tilde{\mu}H^+$ is the intermediate between electron transport and ATP synthesis (e.g. Boyer *et al.*, 1977). However, despite much research on stoichiometric relationships, a controversy exists concerning the true $H^+/2e$ and $H^+/ATP$ ratios (see Wikström & Krab, 1980).

Initially, Mitchell (1966*a*, 1977) postulated the concept of a redox loop involving $2H^+/2e$ per loop, as the mechanism by which $\Delta\tilde{\mu}H^+$ is generated. However, later thermodynamic studies (e.g., Nicholls, 1974, 1977*a*; Rottenberg, 1975, 1979; Azzone *et al.*, 1978*a*; amongst others) and direct measurements of stoichiometry have shown the average $H^+$/Site ratios to be greater than 2. Thus the $H^+/O$ ratio for Site 2 + 3 (from succinate) has been found to be 4 (Mitchell & Moyle, 1967*a*; Papa *et al.*, 1980*a*,*b*), or 6 (Brand *et al.*, 1976*a*,*b*, 1978; Wikström & Krab, 1979, 1980), or 8 (Reynafarje *et al.*, 1976; Alexandre *et al.*, 1978, 1980; Vercesi *et al.*, 1978; Azzone *et al.*, 1978*b*; Alexandre & Lehninger, 1979; Pozzan *et al.*, 1979*a*,*b*).

To date most $H^+/O$ measurements have been carried out employing short bursts of respiration, usually with de-energized mitochondria (Mitchell, 1966*b*; Brand *et al.*, 1976*a*,*b*; Reynafarje *et al.*, 1976; Brand, 1977; Azzone *et al.*, 1978*b*; Papa *et al.*, 1980*a*,*b*). These short-term methods are thus far removed from the steady-state respiration that occurs naturally in mitochondria, and these methods have been criticised on mechanistic and thermodynamic grounds (e.g. Rottenberg, 1979) and for various technical reasons (e.g. Wikström & Krab, 1979, 1980; Papa *et al.*, 1980*a*,*b*). Clearly the need arises for new steady-state methods of assessing $H^+/2e$ ratios. One such method has been introduced by Brand *et al.* (1978) (see also Brand, 1979; Kunz *et al.*, 1981).

The present paper is concerned with the development of a second method to measure steady-state $H^+/O$ ratios, by perturbation of steady-state respiration. A similar experimental procedure was first used by Schwartz (1968) in the elucidation of the $H^+/e$ ratio in chloroplasts by measuring $H^+$ efflux after cessation of illumination. Wikström & Krab (1978) have briefly reported the application of the method to mitochondria. The $H^+/O$ ratios obtained in the present study correspond to $H^+/O$ values of 8, 6 and 2 for $NAD^+$-linked substrates, succinate and cytochrome *c* respectively. Preliminary reports of this work have been published previously (Al-Shawi & Brand, 1981*a*,*b*).

Abbreviations used: $\Delta\tilde{\mu}H^+$, transmembrane difference in electrochemical proton potential (protonmotive force); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $H^+/ATP$, number of protons entering the mitochondria per ATP produced in the extramitochondrial space; $H^+/2e$, number of protons ejected from mitochondria per pair of electrons transferred; $H^+/O$, number of protons ejected from mitochondria per oxygen atom consumed; NEM, *N*-ethylmaleimide; $q^+/2e$, net number of electrical charges translocated (as positive charge from the inside to the outside) per pair of electrons transferred; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine.



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## Experimental

### *Materials*

Antimycin A, valinomycin, oligomycin, NEM, carboxyatractyloside, rotenone, TMPD and EGTA were obtained from Sigma (London) Chemical Co. FCCP was a gift from Dr. P. G. Heytler of Du Pont, Wilmington, DE, U.S.A. Bovine liver catalase and standard 'C.V.S.' 0.1 M-HCl were purchased from BDH, U.K. $O_2$-free $N_2$ was from the British Oxygen Co. All other chemicals were of analytical quality or of the highest grade commercially obtainable. Water was distilled and then deionized.

### *Preparation of mitochondria*

Rat liver mitochondria with a respiratory control ratio of 5–6 with succinate were isolated by the method of Chappell & Hansford (1972). After two washes, the mitochondria were suspended in 0.25 M-sucrose/1 mM-EGTA/5 mM-Tris/HCl (pH 7.4). The final protein concentration was about 60 mg/ml as determined by a biuret method (Gornall *et al.*, 1949).

### *$O_2$-consumption measurement*

$O_2$ was measured polarographically in a Clark-type oxygen electrode chamber (Rank Bros., Bottisham, Cambridge, U.K.). The electrode was fitted with a high-sensitivity thin Teflon membrane, and was calibrated as described by Robinson & Cooper (1970). The output from a potentiometer circuit was recorded on a Bryans Southern Instruments chart recorder (model 2800).

The chamber was enclosed by a thermostatically controlled water jacket connected to a circulating water bath. The sample solution was stirred by an 8-mm-long glass-covered bar driven at high speeds by a rotating magnet. The chamber was sealed with a piston through which a pH micro-combination electrode was inserted into the cell for simultaneous pH measurements. Additions to the cell were via a 1-mm-diameter hole in the piston. $O_2$ back-diffusion into the set-up was found to be insignificant. The electrode responded 10–90% of the deflection in about 2 s. This relatively slow response was not important as a steady-state rate of $O_2$ consumption was to be measured.

### *pH measurements*

pH measurements were carried out by using a Pye–Unicam, Ingold micro-combination electrode (cat. no. 401 E7M5) in conjunction with a Philips PW 9409 digital pH meter, and the signal was displayed on the recorder. The sensitivity and response time of the electrode were increased by etching the pH-sensitive glass membrane in 0.1 M-HF and by storing the electrode in 0.1 M-HCl/3.5 M-KCl solution between experiments. The electrode was standardized by using standard buffer solutions (BDH). The system was calibrated during each experiment by the addition of known amounts of standard 0.1 M- or 0.01 M-HCl. Small acid pulses injected into the cell containing experimental medium showed that the electrode responded 10–90% of the deflection in less than 0.6 s, for a range of pH changes from 0.05 to 0.5 pH units; this response included the lag time due to mixing. Fig. 1 shows the results of a second experiment to determine the ability of the apparatus to follow fast rates of pH change. The rate of release of $H^+$ in the alcohol dehydrogenase reaction was monitored by the pH electrode, while the rate of NADH formation was measured in a spectrophotometer at 340 nm in parallel. The initial rates of $\Delta[H^+]$ and $\Delta$[NADH] depended linearly on the amount of enzyme present. Thus the point where the two curves separate indicates the maximum rate of $\Delta[H^+]$ that the pH electrode can measure accurately in the experimental buffering medium. The value found was 2.5 mM-$H^+$/min. This was greater than any of the rates measured in the present paper.

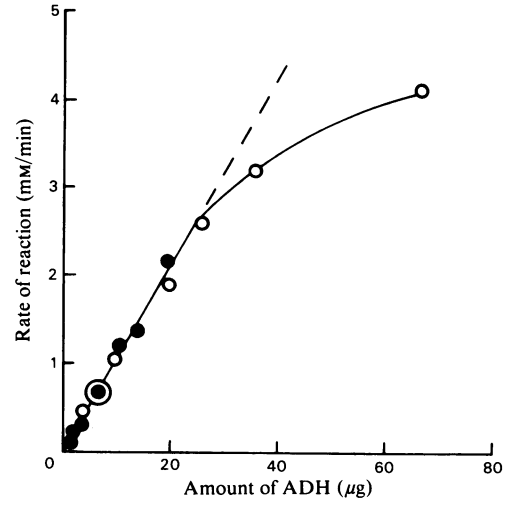


Fig. 1. *Response time of the pH electrode*
The alcohol dehydrogenase (ADH) reaction (ethanol + $NAD^+$ ⇌ acetaldehyde + NADH + $H^+$) was monitored by $[H^+]$ and [NADH]. To 3 ml of medium (0.2 M-sucrose, 20 mM-KCl, 0.9 mM-EGTA and 0.2 mM-$NAD^+$, pH 7.4, at 30°C) was added a precise amount of yeast alcohol dehydrogenase solution. The reaction was initiated by the addition of 0.16 M-ethanol and the initial rate of $H^+$ production was measured with the pH electrode and recorded. Parallel experiments were monitored in a spectrophotometer for the initial rates of NADH formation by increased absorbance at 340 nm [$\varepsilon_{340}$(NADH) = 6220 M·cm$^{-1}$]. Symbols: ●, initial rate of NADH formation; ○, initial rate of $\Delta[H^+]$.

*$H^+/O$ ratio measurements*

Mitochondria (10mg of protein) were suspended in 4.0ml of deoxygenated medium containing 0.2M-sucrose, 20mM-KCl, 0.9mM-EGTA, 5$\mu$M-rotenone and 5mM-potassium succinate (pH7.4). The medium had been deoxygenated by flushing with a stream of $O_2$-free $N_2$ before being transferred. Once the electrode chamber was sealed and checked to be anaerobic it was supplemented with 2000 i.u. bovine catalase and 0.35nmol of carboxyatractyloside/mg of protein followed by 0.5$\mu$g of oligomycin/mg of protein and then by either 10nmol of sodium mersalyl/mg or 40nmol of NEM/mg made up fresh each day. After this, valinomycin was added to 0.35$\mu$g/mg of protein, and the pH was adjusted to a value of 7.1 by small injections of 0.1M-HCl or 0.1M-KOH if needed. The mitochondria were then incubated for 2min.

The steady-state condition was set up by the injection of about 0.3$\mu$mol of $H_2O_2$ solution (50mM stock solution). During the phase of $O_2$ evolution there was a parallel acidification of the external medium, which then levelled-off to the steady-state value. This steady-state was accompanied by a steady rate of $O_2$ consumption (Fig. 2). After approx. 20s at the steady-state, respiration was abruptly inhibited by the rapid injection of 6$\mu$l of either KCN or antimycin A to a final concentration of 1.25mM or 0.17nmol/mg of protein respectively. The exponential rate of proton re-entry into the mitochondria was followed for 30s. When cyanide was used as an inhibitor, it was imperative that its pH was the same as the extramitochondrial pH of about 7.0. Thus the pH of the stoppered fresh cyanide was checked with the experimental electrode before each experiment.

After each experiment, the buffering capacity of the external medium was found by the injection of 50–100nmol of standard HCl. The deflection was recorded and the small back-decay due to titration of the inner aqueous phase of the mitochondria was accounted for by extrapolation (Mitchell & Moyle, 1967*a*).

For $NAD^+$-linked substrates, no rotenone was present, and either 3mM-potassium 3-hydroxybutyrate or 2mM-potassium malate plus 4mM-potassium glutamate were used. When potassium ascorbate (3mM) was used as respiratory substrate, in the presence of both rotenone and antimycin, either fresh 75$\mu$M-TMPD or fresh 4mM-$K_4Fe(CN)_6$ was used as redox mediator.

All results are presented as means $\pm$ S.E.M.

## Results

The rationale of the method described in the present paper is as follows. During steady-state respiration with no net pH changes, the rate of $H^+$ efflux is equal to the rate of $H^+$ influx into the mitochondria. If $H^+$ efflux is instantaneously prevented, the initial rate of $H^+$ influx will be equal to the steady-state rate of $H^+$ influx; division by the steady-state rate of $O_2$ consumption will give the $H^+/O$ ratio.

Fig. 2 shows an experimental trace obtained during the measurement of the steady-state $H^+/O$ ratio for succinate using this procedure. The rate of $H^+$ influx against time on the addition of inhibitor in the presence of valinomycin is a simple exponential as expected; this is shown as a semi-logarithmic plot in Fig. 3. The $t_{\frac{1}{2}}$ for $H^+$ re-entry was 2.3s; this is relatively fast because of the partial leakiness of the membrane induced by the experimental procedures (see below).

The maximum rate of change of $[H^+]$ was seen after 1.1s; this was taken to be the experimentally determined initial rate. This method of estimating the initial rate of influx could possibly lead to an underestimate of the true initial rate, thus leading to low $H^+/O$ values. However, the preceding 1.1s involved the summation of inhibitor injection time, mixing time, inhibitor reaction time and electrode response time, making any back-extrapolation very difficult.

An estimate of the net reaction time of the inhibitors was made by determining the kinetics of reduction of cytochrome *c* and $c_1$. The change of absorbance at the wavelength couple 551nm/540nm was investigated by using a double-beam spectrophotometer. Under normal experimental conditions, with cyanide as respiratory inhibitor, it was found that 90% of the reduction occurred within $1.4 \pm 0.1$s with a rate constant of $1.65 s^{-1}$. This indicated that, for cyanide, the inhibitor reaction time corresponded to the lag phase in proton re-entry.

For the antimycin reaction time, it was seen that within the oxygen electrode's response time of 2s, there was complete inhibition of $O_2$ consumption, indicating complete inhibition of electron flow through cytochrome *c* oxidase.

By using the maximum proton flux as the initial rate of proton re-entry, and hence equal to the steady-state rate of proton ejection, an $H^+/O$ ratio of $5.99 \pm 0.17$ was obtained for succinate when 0.17nmol of antimycin/mg of protein was the inhibitor. A corresponding value of $5.83 \pm 0.42$ was obtained when 1mM-KCN was the inhibitor (see Table 1). These values were for an electron flux of 150nmol of e/min per mg. The electron fluxes for State 4, State 3 and total uncoupling with FCCP were 45, 250 and 300nmol of e/min per mg respectively. Thus it is apparent that the experimental rates were between State 4 and 3. This indicated that the mitochondria were partially

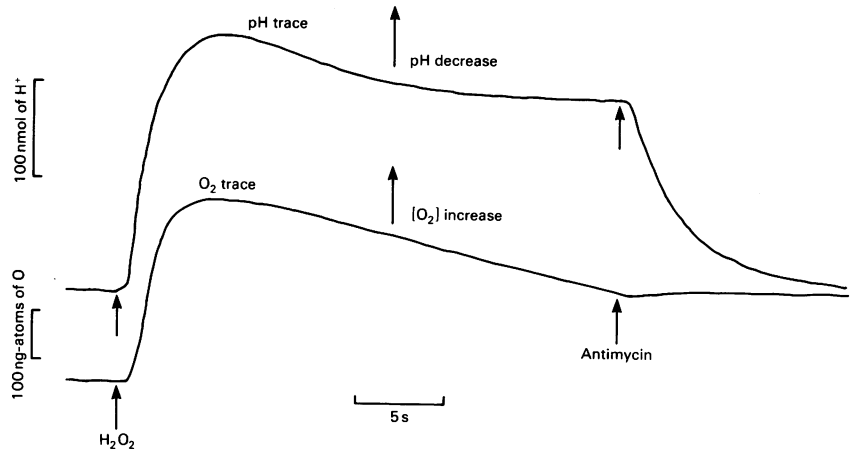


Fig. 2. *Experimental trace obtained during measurement of the $H^+/O$ ratio*
Mitochondrial (8.2 mg of protein) in 4.25 ml were treated as described under '$H^+/O$ ratio measurements'. Respiration was initiated by the injection of 0.45 $\mu$mol of $H_2O_2$ solution, and then inhibited by the injection of 0.17 nmol/mg of antimycin. This experiment gives an $H^+$ re-entry rate of 410 nmol/min per mg of protein and a steady-state $O_2$-consumption rate of 65.9 nmol of O/min per mg of protein, giving an $H^+/O$ ratio for succinate of 6.22.

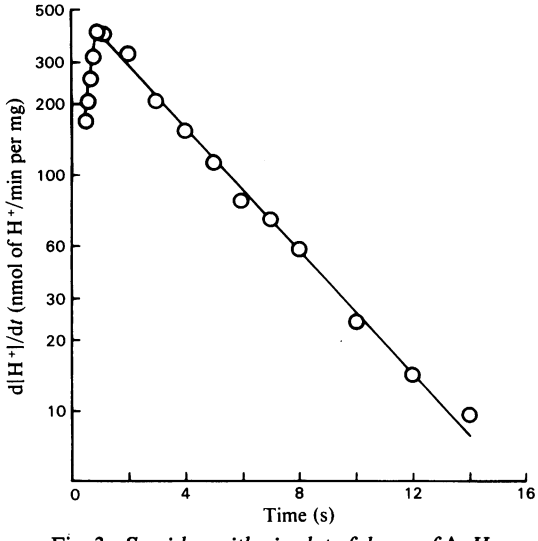


Fig. 3. *Semi-logarithmic plot of decay of $\Delta pH$*
Data are of the experiment shown in Fig. 2. At time zero, antimycin was administered. From this graph, the initial rate of proton flux was measured to be 410 nmol of $H^+$/min per mg (see the text).

uncoupled during the experiments (due to the large $\Delta pH$ generated in the presence of valinomycin). This makes it unlikely that gated proton leaks (Nicholls, 1977*b*) would be operating during the steady-state. If gated proton leaks were occurring in the steady-state, it could lead to an underestimate of the true $H^+/O$ ratio, since an initial rapid leak might not be completely detected by the electrode. The unlikelihood of gated proton leaks operating is strengthened by the fact that the decay of the pH gradient (Fig. 3) was a single monophasic exponential process.

Additional evidence for the similarity of the $H^+$-leak rate in the steady-state and after respiratory inhibition was obtained by acid-pulse experiments (Mitchell & Moyle, 1967*b*) before and after inhibition. The $t_{\frac{1}{2}}$ for $H^+$ entry to the matrix was the same in both cases (see below).

The rates of decay of pH gradients were generally higher than those of Mitchell & Moyle (1967*b*) for anaerobic non-respiring mitochondria. However, the experimental procedures do not depend on tight coupling as long as there is a measurable pH change in the external medium. This is so since uncoupling would only increase the steady-state rate of proton cycling, which would be accounted for by the increased rate of proton re-entry on inhibition. This is clearly illustrated by the linear plateau region of Fig. 4, which shows an experiment in which FCCP was added to vary the steady-state $H^+$ permeability. Thus the mitochondria that had been pre-incubated with low FCCP concentrations yielded similar $H^+/O$ values. However, for the highest uncoupled point of Fig. 4, the estimated rate of proton ejection during respiration (assuming a stoichiometry of 6 $H^+/O$) was 0.87 $\mu$mol of $H^+$/min per mg (2 mM-$H^+$/min), which, coupled with the very low steady-state $\Delta pH$ involved with this point, leads to the low apparent $H^+/O$ ratio, since the pH

Table 1. *Steady-state $H^+/O$ ratios*

Results are means ± S.E.M. for the numbers of observations shown in parentheses.

| Substrate and conditions | $H^+/O$ |
|---|---|
| Succinate (antimycin-inhibited) | 5.99 ± 0.17 (23) |
| Succinate (KCN-inhibited) | 5.83 ± 0.42 (8) |
| Succinate (+ phenylmalonate)* | 6.06 ± 0.41 (12) |
| Succinate (no NEM) | 4.69 ± 0.19 (6) |
| 3-Hydroxybutyrate | 8.27 ± 0.88 (11) |
| Glutamate/malate | 8.17 ± 0.67 (13) |
| Ascorbate/TMPD | 4.07 ± 0.30 (13) |
| Ascorbate/ferrocyanide | 3.01 ± 0.12 (4) |
| Endogenous | 9.57 ± 3.1 (2) |

* Phenylmalonate (20mM) was included at the start of the anaerobic incubation after the mitochondria had been allowed to accumulate succinate aerobically.

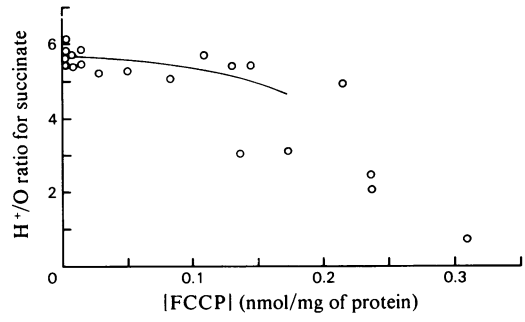


Fig. 4. *Effect of uncoupler (FCCP) on the $H^+/O$ ratio*

Experimental procedure was as in the Experimental section, except that the mitochondria were pre-incubated with various amounts of FCCP during the anaerobic phase. Antimycin was used as the respiratory inhibitor.

electrode was not sufficiently sensitive. The initial plateau region supports the view that there were no undetected gated proton leaks in the absence of FCCP, nor any undetected increased conductivity due to swelling when the mitochondria were energized in the presence of valinomycin.

Table 1 also shows the results for $NAD^+$-linked substrates. When 3-hydroxybutyrate was used as substrate, the $H^+/O$ ratio was 8.27 ± 0.88; when glutamate plus malate was used, it was 8.17 ± 0.67. A value of 9.57 ± 3.1 ($n = 2$) was obtained for endogenous substrates. In this case, the very low rates of $O_2$ consumption led to large inaccuracies.

The redox mediators TMPD and ferrocyanide are both thought to donate electrons to cytochrome *c* on the outside of the inner membrane in intact mitochondria (Jacobs & Sanadi, 1960; Wikström, 1978). These two redox mediators are taken to be pure electron donors. However, at pH 7.0, ascorbate is ionized and, when oxidized to dehydroascorbate, acts as a 2e donor and 1.08 $H^+$ donor (Krab & Wikström, 1979). The dehydroascorbate is unstable and slowly reacts with water to give a diketo acid with a net release of 1 $H^+$. By measuring the rate of re-acidification after inhibition with cyanide, the rate of dehydroascorbate hydrolysis was found to be equivalent to 0.16 ± 0.02 $H^+$ produced per 2e from ascorbate during steady-state respiration. These considerations account satisfactorily for the observed scalar steady-state alkaline drifts of 0.77 ± 0.05 $H^+/2e$ for ascorbate/TMPD and 0.76 ± 0.09 $H^+/2e$ for ascorbate/ferrocyanide.

The $H^+/O$ ratios observed for ascorbate/TMPD and ascorbate/ferrocyanide were 4.07 ± 0.30 and 3.01 ± 0.12 respectively. This is an unexpected result since theoretically they should be equivalent. However, discrepancies between these two electron-donating systems have been observed by other groups (Alexandre *et al.*, 1978; Azzone *et al.*, 1979).

The average electron fluxes were 100nmol of e/min per mg of protein for ascorbate/TMPD and 80nmol of e/min per mg of protein for ascorbate/ferrocyanide. In the presence of oxidized TMPD alone, the steady-state electron flux was 1nmol of e/min per mg, thus excluding the possibility that TMPD was being re-reduced by more reducing components of the electron-transport chain that might lead to artificially increased $H^+/O$ stoichiometries.

If cytochrome oxidase translocates $2H^+/2e$ (Wikström & Krab, 1979), the expected $H^+/O$ ratio from cytochrome *c* to $O_2$ by this method is 4. This is because the steady-state $H^+$ influx must balance the $2H^+/O$ consumed in the scalar matrix reaction producing water, plus the $2H^+/O$ translocated by the enzyme. The $H^+/O$ value of 4 with ascorbate/TMPD is therefore consistent with translocation of $2H^+/2e$ by the oxidase.

By varying the mitochondrial protein concentration while keeping the specific concentrations of inhibitors constant, it was seen that the observed stoichiometry was constant over a range of mitochondrial concentrations (4–15mg of protein), showing that under the normal experimental conditions, both the pH and oxygen electrodes were able to respond to the rates of concentration changes involved.

$H_2O_2$ is known to damage membranes. To minimize any damage, 2000 i.u. of bovine catalase was used in each experiment. $O_2$ evolution appeared to be complete in less than 6s. Furthermore it was found that varying the amount of $H_2O_2$ (0.8–4.5 μmol) had little effect on the observed stoichiometry.

Varying the succinate concentration (0.2–4.5mM) had little effect on the results except at very low

respiration rates where the measurement of $O_2$ consumption was inaccurate and any slight $O_2$ back-diffusion became significant, leading to an overestimate of the true $H^+/O$ ratios.

To illustrate that the results were not being affected by rapid succinate movements during re-energization, 20mM-phenylmalonate was included in some incubations to inhibit the decarboxylate transporter. The phenylmalonate was injected at the start of the anaerobic incubation phase, after the mitochondria had been allowed to accumulate succinate aerobically. The inclusion of phenylmalonate had little effect on the observed $H^+/O$ values.

The possibility that the proton conductance of the membrane changed after perturbation of the steady-state was investigated by using the acid-pulse technique (Mitchell & Moyle, 1967*b*). HCl (50nmol) was injected during the steady-state and again after the inhibitor had collapsed the pH gradient. By using 0.17nmol of antimycin/mg of protein as inhibitor, and succinate as substrate, an $H^+/O$ ratio of $5.83 \pm 0.27$ was obtained for six experiments. The average $t_{\frac{1}{2}}$ values for $H^+$ entry were $2.4 \pm 0.1$s during the steady-state and $2.3 \pm 0.1$s after antimycin inhibition. About 10nmol of $H^+$ entered the mitochondria after each acid pulse.

To investigate the uncoupling effects of antimycin at higher concentrations, the observed succinate $H^+/O$ quotients were measured for various concentrations of antimycin (Fig. 5). Any agent causing proton uncoupling during inhibition of respiration and not present during the steady-state would lead to an increase in the rate of proton re-entry and hence an overestimate of the proton cycling rate during the steady-state, and thus an overestimate of the true stoichiometry. The results are consistent with antimycin having a slight uncoupling effect at higher concentration, thus leading to increased measured stoichiometries. However, in the experimental range used, the uncoupling was slight, as shown above (see also Haas, 1964). The results of the cyanide titration curve (Fig. 6) support this conclusion, as the $H^+/O$ ratio did not vary for a 3-fold change in cyanide concentrations.

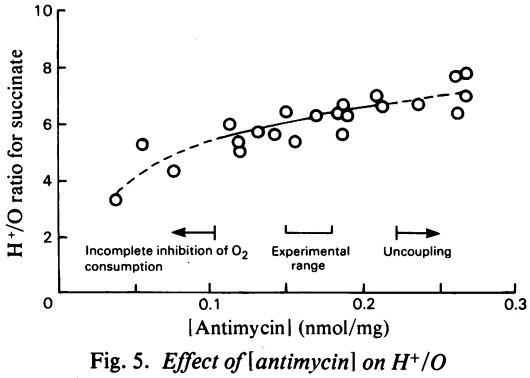


Fig. 5. *Effect of [antimycin] on $H^+/O$*
Experimental procedure was as described in the Experimental section, except that various amounts of antimycin were used as indicated. At low concentrations of antimycin, $O_2$ was still consumed after injection, indicating only partial inhibition and hence leading to underestimates of the true stoichiometry. The start of the uncoupling range was calculated from the values of Haas (1964).

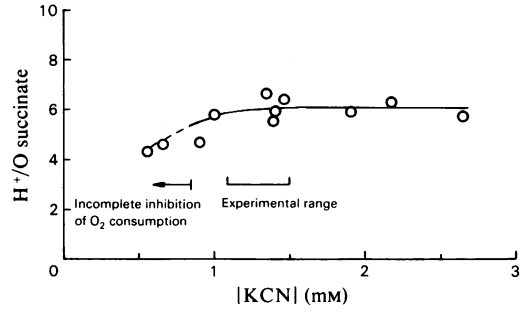


Fig. 6. *Effect of [KCN] on $H^+/O$*
The experiment was as in Fig. 5, but KCN was used as the respiratory inhibitor.

## Discussion

The results presented in this paper show the steady-state $H^+/O$ quotients for mitochondrial respiration to be close to 8, 6 and 2 ($q^+/O = 4$) for electron flow from $NAD^+$-linked substrates, succinate and cytochrome *c* respectively.

These values agree with other steady-state determinations (Brand *et al.*, 1978; and see Wikström & Krab, 1980), but are different from the results obtained with rapid kinetic methods (Alexandre *et al.*, 1978; Papa *et al.*, 1980*a*,*b*). They are also similar to those obtained using the $O_2$ pulse technique (Brand *et al.*, 1976*a*,*b*).

They are not consistent with the previous redox loop formulations described by Mitchell (1979), but provide strong support for the now well-established proton pumping function of cytochrome oxidase (Wikström & Krab, 1980).

The steady-state method described here has several advantages. It is technically straightforward and rapid, and it depends on a quite different set of assumptions from previous methods. It measures the steady-state $H^+/O$ ratio, which is most relevant to normal mitochondrial function. The chief potential drawback involves the measurement of the initial rate of $H^+$ re-entry; however, mixing time and inhibitor reaction time are not easily allowed for by

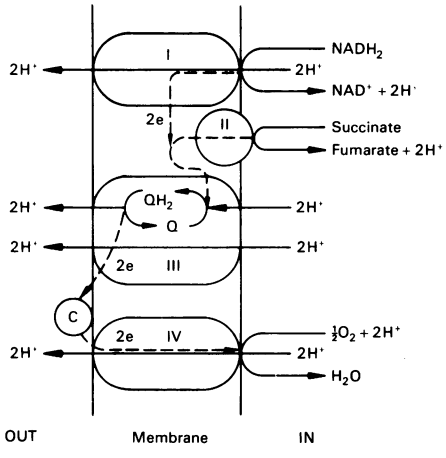


Fig. 7. *Vectorial model of the mitochondrial electron-transport chain*

See the text for details. Abbreviations: C, cytochrome *c*; Q, oxidized ubiquinone; $QH_2$, reduced ubiquinol.

any simple extrapolation procedure. If the measured initial rates underestimate the true initial rate, then our values of $H^+/O$ will be too low. That this is not a problem is suggested by the fact that the measured $H^+/O$ ratios are independent of a number of parameters, particularly the mitochondrial concentration (i.e. the absolute rate being measured).

A simple model to display our results is shown in Fig. 7. This shows complexes I, III and IV each pumping $2H^+/2e$ by mechanisms that could be redox loop or proton pump in complex I, Q cycle (Mitchell, 1976), b cycle (Wikström & Krab, 1980) or proton pump (Papa, 1976) in complex III and proton pump in complex IV (Wikström & Krab, 1979). In addition, Q is shown moving $2H^+/2e$ by the H-carrying arm of a redox loop (Mitchell, 1966*a*), which is completed by cytochrome oxidase.

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