Biochimica et Biophysica Acta 894 (1987) 209–227
Elsevier

BBA 42448

Protonmotive stoichiometry of rat liver cytochrome *c* oxidase: determination by a new rate/pulse method

A. John Moody, Roy Mitchell, Ian C. West and Peter Mitchell

Glynn Research Institute, Bodmin, Cornwall (U.K.)

(Received 8 May 1987)

Key words: Protonmotive stoichiometry; Rate/pulse method; Oxidant pulse experiments; Cytochrome *c* oxidase; Cytochrome *c* reductase; (Rat liver mitochondria)

The stoichoimetry of vectorial H^+ ejection coupled to electron flow through the cytochrome *c* oxidase (EC 1.9.3.1) of rat liver mitochondria was determined by a new rate/pulse method. This is a modification of the oxygen-pulse method. Electron flow through the oxidase is initiated by adding oxygen to suspensions of anaerobic mitochondria at a known and constant rate. Cytochrome *c* oxidase was examined directly or in combination with cytochrome *c* reductase (ubiquinol:ferricytochrome *c* oxidoreductase). In both cases the $\leftarrow H_o^+/2e^-$ ratio was found to be constant during the time-course of oxygen reduction, and thus independent of ΔpH. The stoichiometries observed were consistent with mechanistic stoichiometries of 2 and 6 for cytochrome *c* oxidase alone and cytochrome *c* oxidase together with cytochrome *c* reductase, respectively. The stoichiometry of cytochrome *c* reductase alone was also examined, by using ferricyanide in place of oxygen. The results obtained were consistent with the accepted mechanistic stoichiometry of 4 for this enzyme.

Introduction

The determination of the protonmotive stoichiometry of mitochondrial cytochrome *c* oxidase (ferrocytochrome $c:O_2$ oxidoreductase, E.C. 1.9.3.1) is a prerequisite for the elucidation of the mechanism of action of this osmoenzyme. At present there is substantial disagreement as to what the stoichiometry may be. Proton output ratios ($\leftarrow H_o^+/2e^-$) of 0 [1–6], 2 [7–14], 3 [15] and 4 [16–20] have been inferred from various types of experiment; and it has been suggested [21,22] that the spread of values may to some extent be attributable to a dependence of the mechanistic $\leftarrow H_o^+/2e^-$ ratio on the respiratory conditions. However, we now consider [14,23] that some observations in this laboratory [24–27] and in Papa's laboratory (e.g., Ref. 4) that were previously taken

Abbreviations: pH_O, the pH of the suspension medium; pH_I, the pH of the mitochondrial matrix; ΔpH, the difference between pH_O and pH_I produced by the action of osmoenzymes; ΔpH_O and ΔpH_I, respectively, changes in pH_O and pH_I; ΔH_O^+, the change in the quantity of H^+ in the suspension medium; $\Delta(\Delta pH_O)$, the difference between ΔpH_O observed at a given time and what it would have been if the proton conductance of the inner mitochondrial membrane had been infinitely large; $\Delta(\Delta H_O^+)$, the difference between ΔH_O^+ observed at a given time and what it would have been if the proton conductance of the inner mitochondrial membrane had been infinitely large; $\leftarrow H_o^+/2e^-$, the protonmotive stoichiometry, i.e., the number of protons output into the suspension medium per $2e^-$ accepted by oxidant; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Cyt, cytochrome.

Correspondence: A.J. Moody, Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, U.K.

to be compatible with a $\leftarrow H_o^+/2e^-$ ratio of 0 for cytochrome oxidase were wrongly interpreted, and are more consistent with a $\leftarrow H_o^+/2e^-$ ratio approaching 2, as argued by Wikström and coworkers [10,11].

In most experiments designed to measure protonmotive stoichiometry in mitochondria, oxygen or ferricyanide is reduced by a mitochondrial suspension, using a known reductant, under conditions allowing the movement of a charge-compensating ion through the inner membrane (usually K^+ in the presence of valinomycin). The quantity of protons output during the respiration ($\leftarrow H_o^+$) is estimated from the change in pH of the outer medium (ΔpH_O; measured using a pH electrode), scaled by the buffering power of the outer medium (obtained by measuring ΔpH_O produced by the addition of standard acid solution). The amount of oxidant reduced is estimated from the disappearance of oxygen (usually measured using an oxygen electrode), or by the disappearance of ferricyanide (measured photometrically); or it is determined by the quantity of oxidant injected and completely consumed. After application of corrections as necessary (e.g., for back flow of H^+, or for gain or loss of H^+ in the outer medium other than by the protonmotive osmoenzymes), the $\leftarrow H_o^+/2e^-$ ratio is given by the ratio of the estimated quantities of protons output and oxygen atoms or pairs of electrons taken up. This ratio has generally been obtained by two main experimental methods: the pulse method and the rate method.

In the pulse method [28,29], after the mitochondrial suspension has been equilibrated anaerobically, a known quantity of air-saturated medium or oxygen-free ferricyanide solution (generally equivalent to less than the quantity of hydrogen equivalents in the mitochondrial ubiquinone pool) is rapidly injected. This oxidant is reduced in a short burst of respiratory activity lasting a few seconds at most. The $\leftarrow H_o^+/2e^-$ ratio is calculated from the known total quantity of oxidant in the pulse and from the measured total quantity of protons output, corrected for H^+ back flow (given by the observed rate of decay of pH_O following the respiratory pulse).

In the rate method [30,18–21], after the mitochondrial suspension has been appropriately equilibrated anaerobically or aerobically, a pseudo-steady-state of respiration is initiated. This has been done by providing oxidant in the presence of reductant [18,19]; by providing reductant in the presence of oxidant [20]; or by adding an inhibitor antagonist in the presence of oxidant, reductant and inhibitor [21]. The $\leftarrow H_o^+/2e^-$ ratio is calculated from the rates of oxidant consumption and H^+ ejection, measured as indicated above.

Conflicting claims have been made about the relative merits of the pulse and rate methods [31–34]. Central to this issue is the question of whether the protonmotive stoichiometry of the mitochondrial osmoenzymes decreases as the protonmotive force increases [35,36]. Therefore, we have introduced a hybrid rate/pulse method in which the ratios of the rates and the total quantities of protons ejected and oxidant consumed can both be measured in the same experiment. This has been done by a simple modification of the oxidant pulse method.

Instead of rapidly injecting the air-saturated medium or ferricyanide solution to induce a pulse of respiration, we have used a computer-controlled motorized syringe system to inject it at a predetermined rate. After an initial lag, the rate of oxidant consumption is effectively defined by the known rate of addition of the oxidant solution. The rate and quantity of H^+ output is then estimated from measurements of pH_O.

Using the rate/pulse method, the stoichiometries calculated from the total quantities of protons output and oxidant reduced were found to be the same, within experimental error, as those calculated from the relative rates of proton output and oxidant reduction during the time-course of the rate/pulse experiments. From these results, we conclude that the protonmotive stoichiometries of cytochrome *c* reductase and cytochrome *c* oxidase are essentially invariant over the range of protonmotive force (ΔpH) encountered in these experiments. The values of the $\leftarrow H_o^+/2e^-$ ratios for oxygen reduction via cytochrome *c* oxidase (in the presence of rotenone, antimycin and myxothiazol), and for oxygen reduction via cytochrome *c* oxidase plus cytochrome *c* reductase (in the presence of rotenone) were found to be consistent with mechanistic stoichiometries of 2 and 6, respectively; and the $\leftarrow H_o^+/2e^-$ ratio for fer-

ricyanide reduction via cytochrome *c* reductase (in the presence of rotenone) was found to be consistent with the generally accepted mechanistic stoichiometry of 4 for this enzyme.

These observations help to reconfirm our acceptance of the evidence for proton translocation by cytochrome *c* oxidase with a $\leftarrow H_o^+/2e^-$ ratio of 2 [37,14], as originally found by Wikström and co-worker [38,39].

Materials and Methods

Rat liver mitochondria were isolated and protein estimations were carried out as described previously [28]. Each mitochondrial suspension was kept on ice for use on the same day. The general principles, apparatus and procedure for the rate/pulse experiments were as described for oxidant-pulse experiments in Mitchell et al. [29] and West et al. [14], with the following modifications.

The oxidant solution was added using an all-glass 'Agla' micrometer syringe (Burroughs Wellcome, Beckenham, U.K.), fitted with a fine glass needle (see Ref. 20) and driven, via an extensible coupling, by the stepping motor of an LKB 2132 Microperpex peristaltic pump (LKB Produkter AB, Bromma, Sweden). The pump (in its uncalibrated state, with the step rate set at maximum) was controlled via its 'remote' socket using one of the 'annunciator' outputs of an Apple IIe microcomputer (Apple Computer Inc., Cupertino, CA, U.S.A.). One step was equivalent to 50 nl.

The signal from the glass pH electrode, after amplification, was sampled using the Apple IIe microcomputer via an AI13 A/D converter (12 bit precision, programmable gain, Interactive Structures Inc., Bala Cynwyd, PA, U.S.A.). A single 6502 microprocessor machine code routine, accessed by an Applesoft BASIC program, was used to control the pump and to collect the data. The routine was designed so that activation of the pump did not affect the sampling rate. By varying the delay produced by two pairs of nested loops in this routine, the pH sampling rate or the oxidant delivery rate were changed independently of each other. After completion of the sampling the pH_O data were compressed by averaging successive blocks of 16.

Other specific experimental details are given in the appropriate figure legends.

Reagents were supplied either by Sigma (Dorset, U.K.) or by BDH (Dorset, U.K.), except for choline base, obtained from Fluka AG (Buchs, Switzerland); myxothiazol, obtained from BCl Ltd. (East Sussex, U.K.); and hexammineruthenium(III) chloride ($[Ru(NH_3)_6]Cl_3$), which was a gift from Dr. P. Hinkle. Before use the choline base was purified using activated charcoal, and the rotenone was recrystallized twice in ethanol.

Treatment of data

Calibration and removal of drift. The pH_O scale in each experiment was calibrated by quantitative additions of anaerobic H^+ solution (about 100 nmol H^+ each). The equivalents of ΔpH_O and $\Delta(\Delta pH_O)$, ΔH_O^+ and $\Delta(\Delta H_O^+)$, respectively, were produced by reference to this calibration. Time-courses of ΔpH_O were produced from the primary data (time-courses of pH_O) by subtracting the background drift. This was obtained by linear extrapolation of the drift rate of change of pH_O measured over the 25 s before the addition of the oxidant.

Calculation of time-courses of $\Delta(\Delta pH_O)$. To correct for H^+ back flow, as described in the next section, the time-course of the baseline ΔpH_O, to which the observed ΔpH_O in each experiment was tending to decay, was required, i.e., the time-course ΔpH_O would have followed if the H^+ conductance of the inner membrane had been infinite. This baseline could not be measured directly using FCCP because the H^+ conductance of the inner membrane was not sufficiently increased even at saturating concentrations of this uncoupler. However, an approximate time-course could be computed on the basis of the following assumptions (see labels in Figs. 1A, 3A, 4 and 5).

(1) The final ΔpH_O that would have been reached after completion of the back flow of the translocated H^+ should be the same as that observed in parallel experiments with FCCP present.

(2) This final ΔpH_O, which corresponds to the net scalar reaction for the section of the respiratory chain under examination, should be generated linearly with the oxidant consumption, i.e., the 'steady-state' rate of change of the baseline ΔpH_O should be equal to the final ΔpH_O divided

by the time taken to add the oxidant.

(3) The time-course of the baseline ΔpH_o should exhibit lag phases at the start of the oxidant addition and just after the end of the oxidant addition. These lag phases should have the same kinetics as the equivalent phases in the time-course of the observed ΔpH_o after correction of the latter for H^+ back flow.

The last assumption creates difficulties because we need the time-course of the baseline ΔpH_o before we can correct the time-course of the observed ΔpH_o for H^+ back flow. However, a compromise is possible. The kinetics of both lag phases in the baseline ΔpH_o can be derived from the initial lag phase in the observed ΔpH_o on the assumption that negligible H^+ back flow occurred during this phase. For a first approximation this was achieved by scaling the initial lag phase in the observed ΔpH_o by the ratio of the final ΔpH_o (see Ref. 1) to the peak value of the observed ΔpH_o ('OP' in Figs. 1, 3, 4 and 5). In subsequent refinements the initial lag phase in the observed ΔpH_o was scaled by the ratio of the final ΔpH_o to the 'final' value of the observed ΔpH_o after the previous correction for H^+ back flow ('PK' in Figs. 1, 3, 4 and 5). The refinement was continued until successive ΔpH_o values at 'PK' differed by less than 0.1%. In all cases two or less iterations were required.

Having computed the time-course of the baseline ΔpH_o, values of $\Delta(\Delta pH_o)$ (see definition in abbreviations) were generated as the difference between the computed baseline ΔpH_o values and the observed ΔpH_o values.

Correction for H^+ back flow. When correcting for the back flow of H^+ that occurred during the consumption of the oxidant, the rate of back decay of $\Delta(\Delta pH_o)$ was assumed to be proportional to the magnitude of $\Delta(\Delta pH_o)$.

$$\frac{d(\Delta(\Delta pH_o))}{dt} \simeq -k \cdot \Delta(\Delta pH_o) \quad (1)$$

Justification for this assumption comes from the observation that the back decay of $\Delta(\Delta pH_o)$ after exhaustion of the oxidant approximates to such behaviour, as originally reported by Mitchell and Moyle [28] for rat liver mitochondria (pH_o in the range 7.0–7.1) and as observed by Krishnamoorthy and Hinkle [40] for liposomes. A more fundamental question, however, is whether a transient $\Delta\psi$, developed during the consumption of the oxidant, could contribute significantly to the rate of back flow of H^+. It has long been recognised that such a $\Delta\psi$ develops during conventional oxidant-pulse experiments [41] and it has been argued that this could lead to underestimation of protonmotive stoichiometries if an efficient charge compensation system were not present [41,42]. We consider that the system used in the present experiments, i.e., K^+ in the presence of valinomycin, is more than sufficient in this respect. In support of this contention we cite the work of Brand et al. [8], who, in oxygen-pulse experiments, observed the same stoichiometry for the ubiquinol to O_2 span of the respiratory chain of rat liver mitochondria regardless of whether the charge compensation system consisted of K^+ plus valinomycin or endogenous Ca^{2+} or endogenous Ca^{2+} plus exogenous Ca^{2+}, or all three together. We also cite the observation of Mitchell and Moyle [28] that neither FCCP, gramicidin nor valinomycin significantly affected the observed stoichiometry for the NADH to O_2 span of the respiratory chain of rat liver mitochondria (endogenous Ca^{2+} was always present as charge compensating ion) even when the catalysed rate of decay of $\Delta(\Delta pH_o)$ in the presence of FCCP of gramicidin approached ten times the uncatalysed decay rate.

The first-order rate constant for the back decay of $\Delta(\Delta pH_o)$, k, was estimated from the slope of a plot of $\log(\Delta(\Delta pH_o))$ versus time for a portion of the decay of $\Delta(\Delta pH_o)$ (e.g., see 'plot region' in Fig. 1A (a)).

To correct time-courses of $\Delta(\Delta pH_o)$ for back decay caused by H^+ back flow each $\Delta(\Delta pH_o)$ value was first multiplied by $k \cdot \Delta t$ to determine the change in $\Delta(\Delta pH_o)$ due to the H^+ back flow during the interval Δt, i.e.,

$$\Delta[\Delta(\Delta pH_o)] = \Delta(\Delta pH_o) \cdot k \cdot \Delta t \quad (2)$$

where Δt is the length of time between each $\Delta(\Delta pH_o)$ value. The $\Delta(\Delta pH_o)$ values at given times were then corrected by addition of the cumulative changes due to H^+ back flow over all the Δt increments up to those times. Note that the relationship in Eqn. 2 is valid, provided $k \cdot \Delta t$ is

small. The values of $k \cdot \Delta t$ encountered in this work were in the range 0.001–0.005 (k in the range 0.007–0.023 s^{-1}, Δt either 0.23 or 0.15 s), giving errors in the range 0.06–0.26%.

Having corrected the time-courses of $\Delta(\Delta pH_O)$ for back decay of $\Delta(\Delta pH_O)$ due to H^+ back flow, corrected time-courses of ΔpH_O were produced by simply adding back the appropriate baseline time-course of ΔpH_O.

The accuracy of this correction procedure, including the baseline ΔpH_O computation, was tested by stimulation (based on that described in the final part of Results). The results of this study indicated that the errors introduced by the correction procedure would be less than 1%.

Results

Protonmotive stoichiometry of the ubiquinol to O_2 span of the respiratory chain of rat liver mitochondria

The protonmotive stoichiometry of the ubiquinol to O_2 span (cytochrome *c* reductase and cytochrome *c* oxidase combined) of the rat liver mitochondrial respiratory chain was investigated by adding air-saturated 150 mM KCl at a known rate to suspensions of anaerobic mitochondria. The mitochondria had been treated with oligomycin, to block protonmotive events connected with ATP hydrolysis, with rotenone, to prevent electron flow through NADH dehydrogenase, and with *N*-ethylmaleimide, to prevent phosphate transport on the phosphate/OH^- antiporter. A charge compensation system consisting of valinomycin and K^+ was present. Charge compensation by movement of endogenous Ca^{2+} was prevented by the addition of EGTA. Endogenous substrate was supplemented with choline. Because of the possibility of diffusion of O_2 from the tip of the syringe needle, a 1-μl pre-addition of air-saturated KCl was made 5 s before the main addition (40 μl, approx. 1 nmol O/mg of mitochondrial protein) to ensure that the KCl in the latter addition contained a uniform concentration of dissolved O_2.

Fig. 1A (a) shows the average time-course of ΔH_O^+ obtained with the highest oxygen addition rate used, which was close to the maximum rate possible with the remote-controlled syringe. H^+ ejection was observed after a lag of about 0.5 s, reaching a maximum about 2 s after the end of the

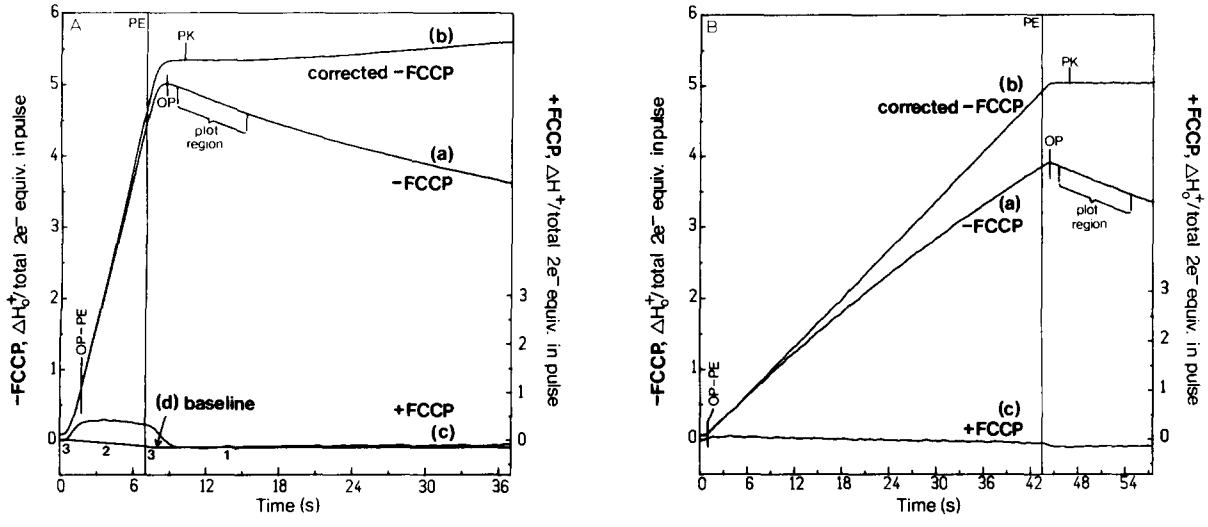

Fig. 1. Time-courses of changes in pH_O induced by pumping air-saturated 150 mM KCl solution into anaerobic suspensions of rat liver mitochondria. Experimental details are described in the legend to Table I. (A) (a) Addition rate = 2.84 nmol O/s, sampling rate = 110.0 s^{-1}, average data from 20 experiments on 6 days; (b) same as (a) but corrected for H^+ back flow; (c) same as (a) but + FCCP, average data from six experiments on the same 6 days as above, weighted using the number of − FCCP experiments done on the same day; and (d) the computed baseline. (B) (a) Addition rate = 0.46 nmol O/s, sampling rate = 70.7 s^{-1}, average data from four experiments on 3 days; (b) same as (a) but corrected for H^+ back flow; and (c) same as (a) but + FCCP, average data from three experiments on the same 3 days as above, weighted using the number of − FCCP experiments done on the same day. The vertical lines on the time axes mark the end of the oxygen addition. The labels are referred to in the text and in the legend to Table I.

TABLE I

$\Delta H_O^+/2e^-$ RATIOS, ESTIMATED FOR THE UBIQUINOL TO OXYGEN SPAN OF THE RAT LIVER MITOCHONDRIAL RESPIRATORY CHAIN

Mitochondria (6 mg of mitochondrial protein/ml) were incubated anaerobically at 25 °C in 3.3 ml of 150 mM KCl containing 1 mM EGTA, 1 mM glycylglycine, 2 mM choline chloride and 20 μg/ml carbonic anhydrase (pH_O 7.0–7.15). FCCP (1 μM, added as indicated) and oligomycin (0.17 μg/mg of mitochondrial protein) were added as aerobic solutions in ethanol at 1 and 1.5 min, respectively, after the start of the incubation. Valinomycin (0.1 μg/mg of mitochondrial protein), rotenone (0.6 μM) and *N*-ethylmaleimide (33 nmol/mg of mitochondrial protein) were added as anaerobic solutions in ethanol at 2, 13.5 and 14 min, respectively. The remote-controlled syringe, freshly filled with air-saturated 150 mM KCl (preincubated at 25 °C), was then placed in position (by about 16 min). Between 19 and 21 min into the incubation data sampling was started and after 25 s a 1 μl pre-addition of air-saturated KCl was made to the reaction cell. The main oxygen addition (40 μl ≡ 20 nmol O) began 5 s later. ΔpH_O was calibrated in terms of ΔH_O^+ with alternate additions of 2 μl of 50 mM HCl and KOH (between six and nine of each), with only the H^+ additions being taken as standards. The H^+ ejection observed in the experiments where FCCP was not present was corrected for H^+ back flow as described in Materials and Methods, using the rate constant obtained from a plot of $\log(\Delta(\Delta pH_O))$ versus t (between $t = 9.6$ s and $t = 15.4$ s, see Fig. 1A (a)). The meanings of 'total quantity ratio' and 'rate ratio' measurements are apparent from the text. 'n' is the number of experiments; 'p' is the number of mitochondrial preparations used. The data sampling rate was 110.0 s^{-1} and the oxygen addition rate was 2.84 nmol O/s, i.e., total addition time 7.0 s.

Addition	Type of measurement	$\Delta H_O^+/2e^-$	S.E.	n	p
None	Total quantity ratio	5.24	0.03	20	6
	rate ratio	5.21	0.03	20	6
+ FCCP	total quantity ratio	−0.18	0.07	8	7

oxygen addition. This maximum, on average, corresponded to a $\Delta H_O^+/2e^-$ ratio of just less than +5 (uncorrected for H^+ back flow).

Fig. 1A (b) shows the same time-course, but after correction for H^+ back flow as described in the section on the treatment of data. Note that the assumption used in this correction, that the rate of back decay of $\Delta(\Delta pH_O)$ due to H^+ back flow was proportional to $\Delta(\Delta pH_O)$, is not completely justified since a log plot over a larger portion of the decay of $\Delta(\Delta pH_O)$ than that indicated in Fig. 1A (a) is non-linear. This shows up in Fig. 1A (b) as an apparent slow increase in ΔH_O^+ about 9 s after the end of the oxygen addition. However, in practice, the deviations from exponential behaviour were small and, further, the estimates of the rate constant for $\Delta(\Delta pH_O)$ back decay were made over a region of each time-course, after the addition of the oxygen, where the rate of back flow of the ejected H^+ was almost maximal. This corresponds to the region, during the addition of the oxygen, where there was greatest deviation between the observed and the corrected time-courses of ΔH_O^+. Fig. 1B shows the equivalent results to those in Fig. 1A, except that a much lower rate of oxygen addition was used.

The salient point to be made from these results is that, after allowance for H^+ back flow and after the initial lag, the rate of H^+ ejection is constant. Therefore, there are two ways in which to calculate the $\Delta H_O^+/2e^-$ ratio. The first is by dividing the total quantity of H^+ appearing in the suspension medium (taken to be the value at time 'PK' on Fig. 1A and B) by the total quantity of oxygen added. The second is by dividing the constant rate of change of ΔH_O^+ (measured between times 'OP–PE' and 'PE' as indicated on Fig. 1A and B) by the rate of addition of oxygen. These two ways are somewhat analogous to the pulse and rate methods, respectively, for determining $\Delta H_O^+/2e^-$ ratios from conventional oxygen-pulse experiments as described in the Introduction, and are termed the total quantity ratio and rate ratio measurements, respectively.

As can be seen in Table I, the results of the two methods of calculation agree closely *. This is an

* These $\Delta H_O^+/2e^-$ values have been corrected for the small ΔH_O^+ observed on the same day, when air-saturated KCl was added to medium only (termed the 'medium only' correction; average of seven measurements on 7 days equivalent to +0.016 $\Delta H_O^+/2e^-$ (S.E. of the mean = 0.002)).

important observation since it allows us to conclude, with reasonable certainty, that the $\Delta H_O^+/2e^-$ ratio was the same during the initial lag and during the final tail off as it was during the constant rate of change of ΔH_O^+. In other words, the overall $\Delta H_O^+/2e^-$ ratio for cytochrome *c* reductase and cytochrome *c* oxidase is independent of ΔpH over the range encountered in these experiments.

So far we have been concerned with the $\Delta H_O^+/2e^-$ ratio. The question now is, how does this ratio relate to the $\leftarrow H_o^+/2e^-$ ratio. This question may seem trivial since, from the net scalar reaction

$$2QH_2 + O_2 \rightarrow 2Q + 2H_2O \qquad (3)$$

we might expect there to be no ΔH_O^+ other than that due to translocated H^+. However, as can be seen in Fig. 1 and in Table I, a small but reproducible final ΔH_O^+ (about $-0.2/2e^-$) was observable when uncoupler (FCCP) was present. It seems that this uptake must be associated with the metabolism, in the matrix, of the substrates that ultimately donate electrons to the Q pool. If it is assumed, as seems reasonable, that this uptake in the matrix was only communicated with the suspension medium by non-carrier-mediated diffusion of H^+ through the inner membrane, then the $\Delta H_O^+/2e^-$ ratio and the $\leftarrow H_o^+/2e^-$ ratio are the same in these particular experiments. This is because this diffusion will be included in the correction for back decay of $\Delta(\Delta pH_O)$ caused by H^+ back flow and, hence, the uptake will not be observed after correction. However, if the H^+ uptake in the matrix were rapidly communicated with the medium by some means the $\leftarrow H_o^+/2e^-$ ratio would be equal to the $\Delta H_O^+/2e^-$ ratio minus the final $\Delta H_O^+/2e^-$ ratio.

Fig. 2 shows the effects on the rate-ratio $\leftarrow H_o^+/2e^-$ measurements of the age of the mitochondrial preparation, and the time taken to add the oxygen (A and B, respectively). In both cases the $\leftarrow H_o^+/2e^-$ ratio decreased slightly with

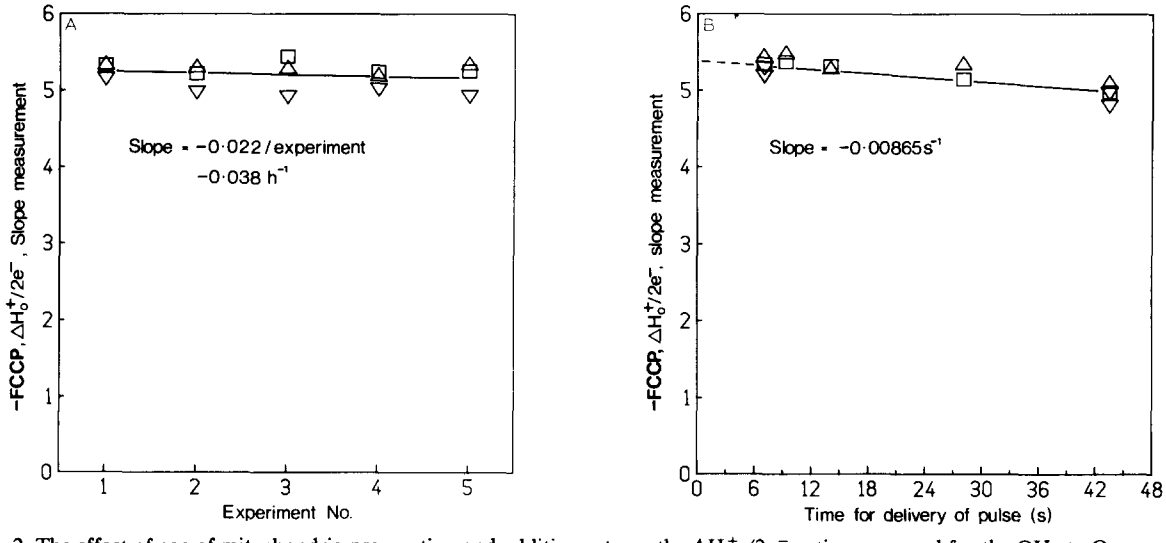

Fig. 2. The effect of age of mitochondria preparation and addition rate on the $\Delta H_O^+/2e^-$ ratios measured for the QH_2 to O_2 span of the rat liver mitochondrial respiratory chain. Experimental details were as described in the legend to Table I. (A) $\Delta H_O^+/2e^-$ ratios (rate ratio measurements, total addition time = 7 s) versus experiment number for 3 day's experiments (identified by the different symbols). Experiment 1 in each case was started about 1.5 h after the end of the mitochondria preparation and subsequent experiments (numbered 2–5) were at about 35-min intervals. One or two +FCCP experiments were also done on each day. (B) $\Delta H_O^+/2e^-$ ratios (rate ratio measurements) versus total time taken to add oxygen for 3 day's experiments. For the longest time the data sampling rate was 70.7 s^{-1}; for the rest it was 110.0 s^{-1}. The experiments were balanced with respect to time after mitochondria preparation so that the average result at each pump rate was equivalent to experiment No. 3 in (A). The lines in each case were fitted by linear least-squares analysis. In all cases the $\Delta H_O^+/2e^-$ ratios were obtained after correction for H^+ back flow using rate constants from log plots between OP + 1.1 s and OP + 10.2 s for the longest addition time, OP + 0.7 s and OP + 6.5 s for the rest.

increasing time. From these results, on the assumption of linear extrapolation, for an oxygen addition taking 1.5 s, i.e., about as fast as the respiratory chain could consume the added oxygen, and with the measurement carried out as soon as possible after the mitochondria had been prepared, we would expect a rate ratio $\leftarrow H_o^+/2e^-$ measurement of just under 5.4 on average.

Protonmotive stoichiometry of the cytochrome c to O_2 span of the respiratory chain of rat liver mitochondria

High ionic strength medium. The protonmotive stoichiometry of the cytochrome *c* to O_2 span (cytochrome *c* oxidase) of the rat liver mitochondrial respiratory chain was investigated in essentially the same way as the stoichiometry of the ubiquinol to O_2 span except that the mitochondria were treated with antimycin and myxothiazol, to prevent electron flow through cytochrome *c* reductase, and hexammineruthenium(II) ($[Ru(NH_3)_6]^{2+}$) replaced choline as exogenous reductant. The main addition of oxygen was 100 μl of air-saturated 150 mM KCl (equivalent to 2.5 nmol of O/mg of mitochondrial protein) instead of 40 μl as before. The results of this investigation are summarized in Table II. Fig. 3 shows data from one day's experiments. A correction was made for H^+ back flow as described in the section on the treatment of data. After doing this correction, the rate of ejection of H^+ was again found to be constant (Fig. 3A–D, (b) in each case). Therefore, the same methods of calculation of the $\Delta H_O^+/2e^-$ ratio can be used as de-

TABLE II

$\Delta H_O^+/2e^-$ RATIOS, ESTIMATED FOR THE CYTOCHROME *c* TO OXYGEN SPAN OF THE RAT LIVER MITOCHONDRIAL RESPIRATORY CHAIN

Experimental details were as described in the legend to Table I, with the following differences/additions. The medium contained 120 μM hexammineruthenium(III) (added as 20 μl of 20 mM stock solution just before each incubation) instead of choline chloride. Antimycin (0.1 μg/mg of mitochondrial protein) and myxothiazol (0.15 μg/mg of mitochondrial protein) were added as anaerobic solutions in ethanol at 15 and 15.5 min into the incubation, respectively. The additions of air-saturated 150 mM KCl were 100 μl ($\cong$ 50 nmol O). The H^+ ejection observed in the experiments where FCCP was not present was corrected for H^+ back flow as described in Materials and Methods, using the rate constant obtained from a plot of $\log(\Delta(\Delta pH_O))$ versus *t* (between $t = OP + 1.1$ s and $t = OP + 6.5$ s). The $\Delta H_O^+/2e^-$ have also been corrected for $\Delta H_O^+/2e^-_{medium\text{-}only}$ (average of five measurements on 5 days = +0.015 (S.E. of the mean = 0.002)). The data sampling rate was 70.7 s^{-1}. The term 'average experiment number' is defined in the legend to Fig. 2.

Comment	Addition time (s)	Average expt. No.	Type of measurement	$\Delta H_O^+/2e^-$	S.E.	*n*	*p*
	15.5	2.00	total quantity ratio	1.62		4	
	15.5	2.00	rate ratio	1.64		4	
	18.0	3.00	total quantity ratio	1.58		3	
	18.0	3.00	rate ratio	1.59		3	
	21.7	3.00	total quantity ratio	1.62		2	
	21.7	3.00	rate ratio	1.64		2	
	27.2	1.75	total quantity ratio	1.61		4	
	27.2	1.75	rate ratio	1.62		4	
Average	20.6	2.31	total quantity ratio	1.61	0.01	13	4
Average	20.6	2.31	rate ratio	1.62	0.01	13	4
+FCCP	15.5		total quantity ratio	−1.95		4	
+FCCP	15.5		rate ratio	−1.95		4	
+FCCP	27.2		total quantity ratio	−1.97		4	
+FCCP	27.2		rate ratio	−1.98		4	
Average +FCCP	21.4		total quantity ratio	−1.96	0.01	8	4
+FCCP	21.4		rate ratio	−1.96	0.01	8	4

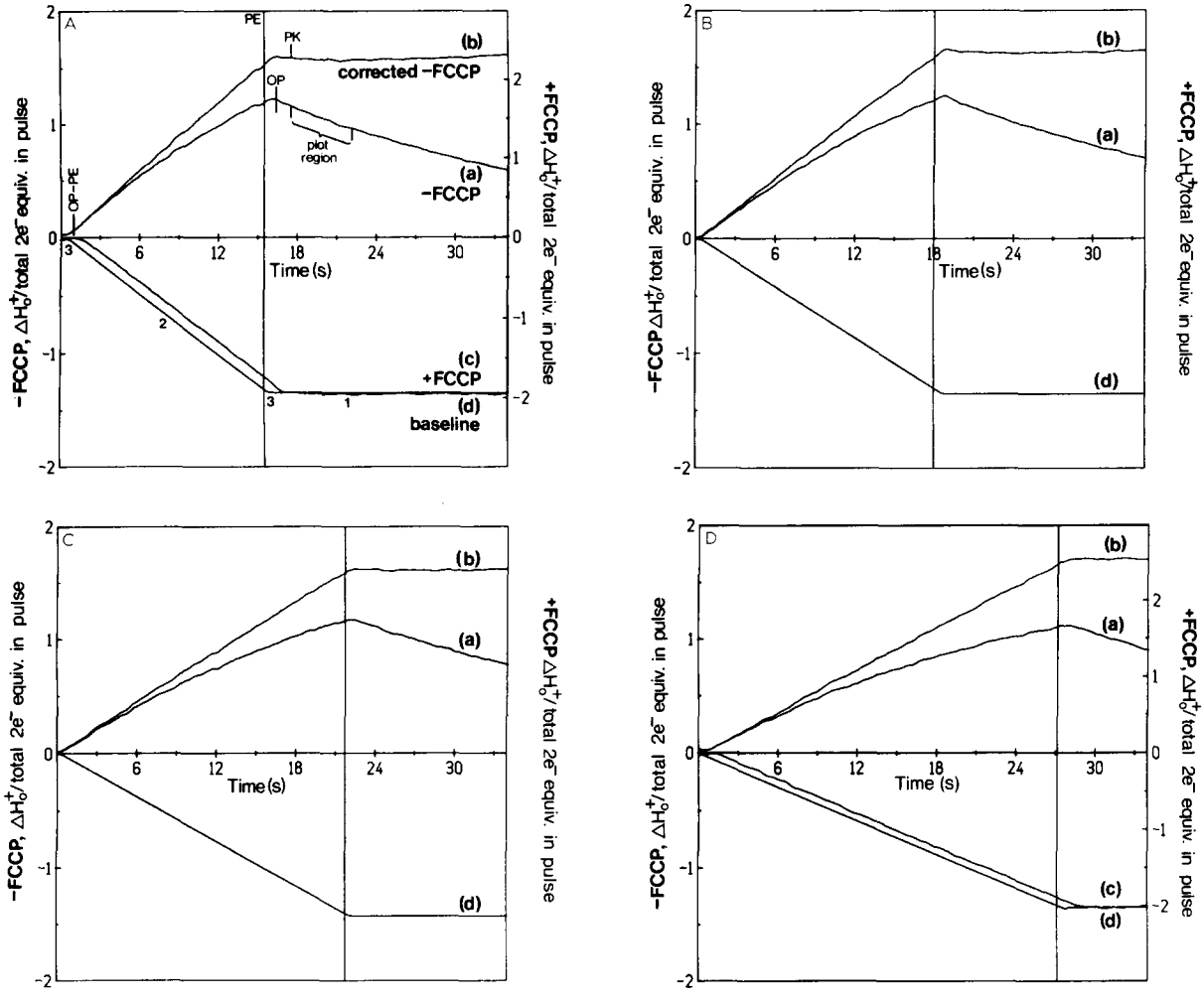

Fig. 3. Time-courses of changes in pH_O induced by pumping air-saturated 150 mM KCl solution into anaerobic suspensions of rat liver mitochondria. Experimental details were as described in the legend to Table II. This figure represents data from six experiments done on 1 day, in the order D(a), B(a), A(a), C(a), D(c), A(c). The data sampling rate was 70.7 s^{-1}. In each case, (a) Addition rates were 3.11, 2.68, 2.22 and 1.77 nmol O s^{-1} for A to D, respectively; (b) same as (a) but corrected for H^+ back flow; (c) same as (a) but + FCCP; and (d) the computed baseline. The other labels are as in Fig. 1.

scribed in the last section. The results of these calculations are shown in Table II.

As can be seen in Table II, the final $\Delta H_O^+/2e^-$ value reached after complete H^+ back flow (observed in the presence of uncoupler) was a few percent lower than that expected for the net scalar reaction catalysed by cytochrome *c* oxidase,

$$4\text{Cyt } c^{2+} + O_2 + 4H^+ \rightarrow 4\text{Cyt } c^{3+} + 2H_2O \quad (4)$$

where the uptake of H^+ occurs at the matrix side of the inner mitochondrial membrane [43]. The same $\Delta H_O^+/2e^-$ value was also obtained by dividing the 'steady-state' rate of change of ΔH_O^+ observed in the presence of uncoupler by the rate of addition of oxygen. This 'shortfall', though small here, has been observed before in the conventional oxygen pulse type of experiment, and its possible causes have been discussed by West et al. [14]. Under the conditions of the experiments described here, i.e., high ionic strength medium and valinomycin present together with FCCP, the likely reason for the 'shortfall' is that there was a side-reaction of a fraction of the added oxygen with

hydrogenated reductant(s), as shown in Eqns. 5 and 6.

$$2RH_2 + O_2 \rightarrow 2R + 2H_2O \quad (5)$$

or

$$2RH + O_2 + 2H^+ \rightarrow 2R + 2H_2O \quad (6)$$

where $\Delta H^+_O/2e^-_{\text{side-reaction}} = 0$ and -1 for Eqns. 5 and 6, respectively.

This side-reaction might have occurred in either aqueous phase, or in both. There is, therefore, some uncertainty as to how to interpret the observed $\Delta H^+_O/2e^-$ ratio values in terms of the $\leftarrow H^+_o/2e^-$ ratio. The following are possible interpretations assuming the side-reaction of oxygen to be non-protonmotive.

1. If the side-reaction were in the inner aqueous phase then the $\leftarrow H^+_o/2e^-$ ratio can be obtained from the $\leftarrow H^+_o/2e^-$ ratio values in Table II by allowing for the quantity of $2e^-$ accepted (i.e., O consumed) in the side-reaction, $2e^-_{\text{side-reaction}}$, as given by

$$2e^-_{\text{side-reaction}} = \frac{\Delta H^+_{O,\text{shortfall}}}{\Delta H^+_O/2e^-_{\text{side-reaction}} - \text{final } \Delta H^+_O/2e^-_{\text{main-reaction}}} \quad (7)$$

Hence, $\leftarrow H^+_o/2e^- = \Delta H^+_O/2e^- \cdot f$, where $f = 1.02$ and 1.04, if the side-reaction is as in Eqns. 5 and 6, respectively.

2. If the side-reaction were in the outer aqueous phase then the $\leftarrow H^+_o/2e^-$ ratio would be given by

$$\leftarrow H^+_o/2e^- = \{(\Delta H^+_O/2e^-_{\text{observed}} \cdot 2e^-_{\text{total}}) - (\Delta H^+_O/2e^-_{\text{side-reaction}} \cdot 2e^-_{\text{side-reaction}})\} \times \{2e^-_{\text{main-reaction}}\}^{-1} \quad (8)$$

where $2e^-_{\text{side-reaction}}$ and $2e^-_{\text{main-reaction}}$, the quantities of $2e^-$ accepted in the side-reaction and main-reaction, respectively, can be obtained using Eqn. 7. Hence, $\leftarrow H^+_o/2e^- = \Delta H^+_O/2e^- \cdot f$, for the reaction in Eqn. 5, and $\leftarrow H^+_o/2e^- = (\Delta H^+_O/2e^- \cdot f) + 0.04$, for the reaction in Eqn. 6, where f in both cases is as above.

Of course, if the side-reaction were protonmotive with a stoichiometry greater than that of the oxidase the $\Delta H^+_O/2e^-$ ratio might be an overestimate of the $\leftarrow H^+_o/2e^-$ ratio. This seems unlikely because rotenone, antimycin and myxothiazol were present. The possibility of significant re-reduction of $[Ru(NH_3)_6]^{3+}$ by hydrogenated reductant(s) also seems unlikely, for the same reason.

As can be seen in Table II, no significant effect of the oxygen addition rate on the $\Delta H^+_O/2e^-$ measurements was observed, although an effect of the same relative magnitude as that observed in the $\Delta H^+_O/2e^-$ measurements of the ubiquinol to O_2 span might be lost in the experimental variation because a much smaller range of addition rates was used.

Low ionic strength medium. Fig. 4 shows the average time-courses obtained for one day's experiments carried out in exactly the same way as those in Table II and Fig. 3, except that a low

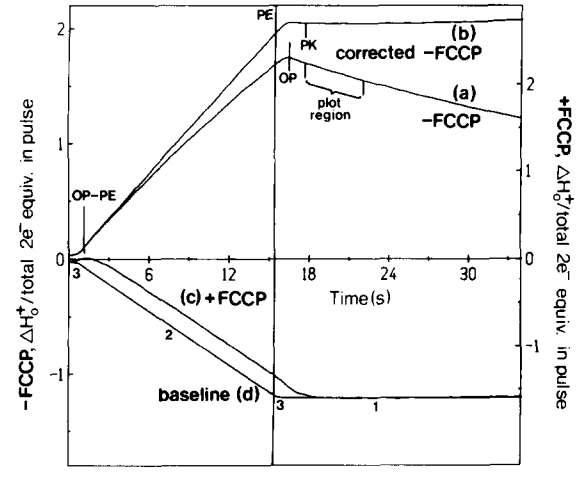

Fig. 4. Time-courses of changes in pH_O induced by pumping air-saturated 240 mM sucrose/10 mM KCl solution into anaerobic suspensions of rat liver mitochondria. Experimental details were as described in the legend to Table II except that the medium was 240 mM sucrose containing 10 mM KCl, 1 mM glycylglylcine, 1 mM EGTA, 121 μM hexamminеruthenium(III) and 20 μg/ml carbonic anhydrase, and air-saturated 240 mM sucrose/10 mM KCl was added. The addition rate was 3.19 nmol O/s (total addition time = 15.5 s) and the data sampling rate was 70.7 s^{-1}. (a) Average data from four experiments done on 1 day; (b) same as (a) but corrected for H^+ back flow; (c) same as (a) but +FCCP, average data from two experiments on the same day as above; and (d) the computed baseline. The other labels are as in Fig. 1.

ionic strength sucrose-based medium was used. The results are qualitatively similar to those in high ionic strength medium. However, the $\Delta H_O^+/2e^-$ ratio, after correction for H^+ back flow, is now much higher at 2.01 (S.E. of the mean = 0.03, $n = 4$) and 2.02 (S.E. of the mean = 0.03, $n = 4$) for the total quantity ratio and rate ratio measurements, respectively. Also, there is a considerable 'shortfall' in the final $\Delta H_O^+/2e^-$ ratio, observed in the presence of uncoupler, amounting to 0.43 and 0.41 for total quantity ratio and rate ratio measuremens, respectively. As discussed in Ref. 14, most of this 'shortfall' can be attributed to ion-exchange interactions between the hexammineruthenium cations and the mitochondria, leading to the generation of H^+ in the outer aqueous phase. When this is allowed for, by subtracting 0.39 from the $\Delta H_O^+/2e^-$ values, i.e., the difference between the 'shortfall' in the present medium and the 'shortfall' in the high ionic strength medium, one can see that the results obtained are almost identical to those obtained in the high ionic strength medium.

Protonmotive stoichiometry of the ubiquinol to cytochrome c span of the respiratory chain of rat liver mitochondria

The protonmotive stoichiometry of the ubiquinol to cytochrome *c* span (cytochrome *c* reductase) of the rat liver mitochondrial respiratory chain was investigated in essentially the same way as the stoichiometry of the ubiquinol to O_2 span, except that ferricyanide dissolved in nearly anaerobic 150 mM KCl was used instead of oxygen (air-saturated 150 mM KCl) as oxidant. Also, previous experience in this laboratory using ferricyanide in conventional oxidant-pulse experiments had shown that, when using some

TABLE III

$\Delta H_O^-/2e^-$ RATIOS, ESTIMATED FOR THE UBIQUINOL TO CYTOCHROME *c* SPAN OF THE RAT LIVER MITOCHONDRIAL RESPIRATORY CHAIN

Experimental details were as described in the legend to Table I, with the following differences/additions. The remote-controlled syringe was filled at the beginning of the day's experiments with nearly anaerobic 150 mM KCl containing 4 mM potassium ferricyanide. The syringe was placed in position at about 13 min into each incubation and a 10-μl addition of ferricyanide solution made to the reaction cell at about 15 min (data was not sampled during this addition). Between 19 and 21 min into the incubation data sampling was started and after 25 s 1 μl of ferricyanide solution was added. The main addition (40 μl, $\equiv$ 39 nmol of ferricyanide) began 5 s later. The H^+ ejection observed in the experiments where FCCP was not present was corrected for H^+ back flow as described in Materials and Methods, using the rate constant obtained from a plot of $\log(\Delta(\Delta pH_O))$ versus t (between $t = 11.1$ s and $t = 14.0$ s). The $\Delta H_O^+/2e^-$ ratio values have also been corrected for $\Delta H_O^+/2e^-_{\text{medium-only}}$ (average of 5 measurements on 5 days was +0.063 (S.E. of the mean = 0.01)). The terms 'O_2 correction 1' and 'O_2 correction 2' are explained in the text. 'n' is the number of experiments; 'p' is the number of mitochondrial preparations used. The data sampling rate was 110.0 s^{-1} and the pump rate was 5.62 nmol ferricyanide/s (plus 0.13 nmol O/s; total addition time 7.0 s). The amount of contaminating O_2 dissolved in the ferricyanide solution was estimated from the change in the oxygen electrode signal observed on pumping 40 μl of the ferricyanide solution into medium only, compared with the change observed when 40 μl of air-saturated 150 mM KCl (preincubated at 25 °C) was pumped into the same batch of medium. This measurement was performed at the beginning and end of a day's experiments and the average value used. The ferricyanide solution was standardized by its A_{420} using a millimolar extinction coefficient of 1.05 [44].

Addition	Type of measurement	$\Delta H_O^+/2e^-$	S.E.	n	p
None	Total quantity ratio	3.68	0.03	15	5
	O_2 correction 1	3.61	0.03	15	5
	O_2 correction 2	3.61	0.03	15	5
	Rate ratio	3.72	0.02	15	5
	O_2 correction 1	3.65	0.02	15	5
	O_2 correction 2	3.65	0.02	15	25
+FCCP	Total quantity ratio	1.62	0.01	8	5
	O_2 correction 1	1.71			
	O_2 correction 2	1.70			

mitochondrial preparations, some of the added ferricyanide was reduced by an unknown reductant via a pathway that was either non-protonmotive or protonmotive with a stoichiometry less than that of cytochrome *c* reductase. This unknown reductant could be removed, at least for the duration of an oxidant-pulse experiment, by adding a small amount of ferricyanide (10 nmol was more than sufficient) about 10 min before the pulse (Mitchell, R., unpublished results). Therefore, in the present rate/pulse experiments a pre-addition of 10 nmol of ferricyanide was made 5 min before the main addition. The results of this investigation are summarized in Table III. Fig. 5 shows the average time-courses obtained. A correction for H^+ back flow was made as described in the section on the treatment of the data. Once again, after this correction was made, the rate of H^+ ejection was found to be constant.

A major problem in these experiments was caused by the presence of a measurable residue of oxygen in the anaerobic ferricyanide solution. Therefore, there must also have been some protonmotive electron flow through cytochrome oxidase. However, by using the $\Delta H^+_O/2e^-$ ratios for the ubiquinol to O_2 span or the cytochrome *c* to O_2 span ($\Delta H^+_O/2e^-_{(QH2\text{-}O_2)}$ and $\Delta H^+_O/2e^-_{(Cyt\ c\text{-}O_2)}$, respectively), which we have already determined, we can allow for this protonmotive electron flow and, hence, estimate the $\Delta H^+_O/2e^-$ ratio for the ubiquinol to cytochrome *c* span ($\Delta H^+_O/2e^-_{(QH2\text{-}Cyt\ c)}$). The following equations were derived for this purpose.

$$\Delta H^+_O/2e^-_{QH_2 \rightarrow cyt.c} = \frac{(\Delta H^+_O/2e^-_{observed} \cdot 2e^-_{total}) - (\Delta H^+_O/2e^-_{cyt.c \rightarrow O_2} \cdot 2e^-_{(O)})}{2e^-_{total}} \quad (9)$$

$$\Delta H^+_O/2e^-_{QH_2 \rightarrow cyt.c} = \frac{(\Delta H^+_O/2e^-_{observed} \cdot 2e^-_{total}) - (\Delta H^+_O/2e^-_{QH_2 \rightarrow O_2} \cdot 2e^-_{(O)})}{2e^-_{ferricyanide}} \quad (10)$$

where $2e^-_{(O)}$ and $2e^-_{(ferricyanide)}$ are the quantities of $2e^-$ accepted by oxygen and by ferricyanide, respectively, and $2e^-_{(total)}$ is the total quantity of $2e^-$ accepted.

The results of using these equations are shown in Table III: 'O_2 correction 1' using Eqn. 9, and 'O_2 correction 2' using Eqn. 10. Values of 1.7 and 5.2 were used for $\Delta H^+_O/2e^-_{(Cyt\ c\text{-}O_2)}$ and $\Delta H^+_O/2e^-_{(QH2\text{-}O_2)}$, respectively, and values of -2 and -0.2 were used for the final $\Delta H^+_O/2e^-_{(Cyt\ c\text{-}O_2)}$ and final $\Delta H^+_O/2e^-_{(QH2\text{-}O_2)}$, respectively (see Tables I and II). The results obtained using these equations agree closely.

However, it is apparent, even allowing for the oxygen contamination, that there is a 'shortfall', amounting to about 0.3, on the theoretical final $\Delta H^+_O/2e^-$ ratio of 2 for the net scalar reaction

$$QH_2 + 2(Fe(CN)_6)^{4-} \rightarrow Q + 2(Fe(CN)_6)^{3-} + 2H^+ \quad (11)$$

This 'shortfall' can be accounted for in two ways. First, we should recall that a small final

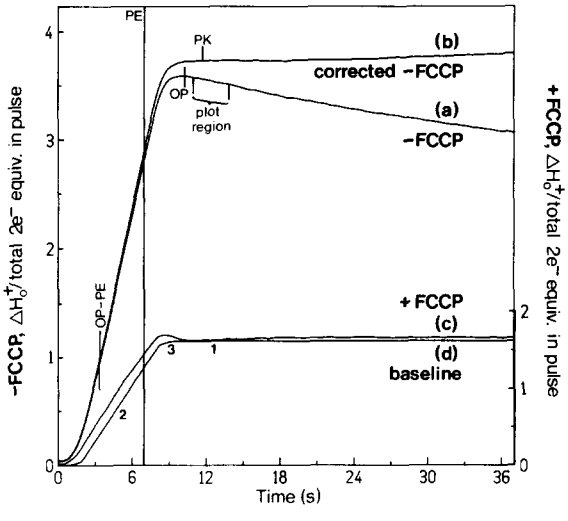

Fig. 5. Time-courses of changes in pH_O induced by pumping 150 mM KCl containing 4 mM ferricyanide (and a small, known amount of O_2) into anaerobic suspensions of rat liver mitochondria. Experimental details are described in the legend to Table III. (a) Addition rate = 5.62 nmol ferricyanide/s (plus 0.13 nmol O/s), sampling rate = 110.0 s^{-1}, average data from 15 experiments on 5 days; (b) same as (a) but corrected for H^+ back flow; (c) same as (a) but +FCCP, average data from eight experiments on the same 5 days as above, weighted using the number of −FCCP experiments done on the same day; and (d) the computed baseline. The labels are the same as in previous figures.

$\Delta H^+_O/2e^-$ ratio was observed for the ubiquinol to O_2 span. We should also recall that this was attributed to the uptake of H^+ occurring in the mitochondrial matrix associated with the metabolism of the respiratory substrate(s), and, as such, it should also be present in the current experiments. Therefore, the observed final $\Delta H^+_o/2e^-$ ratio should, on this basis, be corrected upwards by 0.18 ± 0.07 (mean $\pm$ S.E.). Second, on examination of the time-course of ΔH^+_O observed in the presence of uncoupler (Fig. 5(c)), it is apparent that the reaction of the ferricyanide was not complete at the time ('PK') that the final $\Delta H^+_O/2e^-$ measurement was made. The maximum final $\Delta H^+_O/2e^-$ value was reached about 6 s after this time and was about 0.07 greater.

The reasons for the latter observation and its effect on the validity of these particular experiments will be discussed in the next section. For the moment, we can obtain an estimate of the $\leftarrow H^+_o/2e^-$ ratio of the ubiquinol to cytochrome *c* span by using the equation

$$\leftarrow H^+_o/2e^- (\text{total-quantity}\,\Delta H^+_O/2e^-_{\text{observed}}) \times \left(\frac{\text{final}\,\Delta H^+_O/2e^-_{\text{expected}}}{\text{final}\,\Delta H^+_O/2e^-_{\text{observed}}}\right) \quad (12)$$

where the expected final $\Delta H^+_O/2e^-$ is taken to be 1.82, i.e., the theoretical value of 2 plus the correction of -0.18 already described, and the difference between this and the observed final $\Delta H^+_O/2e^-$ (as in Table III) is assumed to be a consequence of there still being some ferricyanide present at the time when the measurements were made. When this correction is made we find an average $\leftarrow H^+_o/2e^-$ ratio of 3.86 for the ubiquinol to cytochrome *c* span. One should note, however, that there is an inevitable accumulation of errors in these calculations such that if we take the limits of one standard error above and below the average values and calculate the $\leftarrow H^+_o/2e^-$ ratios possible within these limits we arrive at a range of 3.64–4.07.

A simple simulation study

In order to facilitate the understanding of the experimental results presented in this paper the following simulation study has been carried out. A similar simulation of the results of conventional oxidant-pulse experiments (in particular, those in Ref. 18) can be found in Ref. 33. The processes occurring during and after the addition of the oxidant are represented in the following sets of differential equations.

A. During the addition of oxidant

$$dO^*/dt = b - k_m O^* \quad (13)$$

$$dO/dt = k_m O^* - k_r O \quad (14)$$

$$dH/dt = nk_r O - k_b H \quad (15)$$

$$dS/dt = k_e H - k_e S \quad (16)$$

B. After the addition of oxidant

$$dO^*/dt = -k_m O^* \quad (17)$$

dO/dt, dH/dt and dS/dt are as in *A*.

where O^*, O, H and S are the amount of unmixed oxidant, the amount of mixed oxidant, the amount of ejected H^+ and the signal from the H^+ electrode, respectively; b is the constant rate of oxidant addition; k_m, k_r, k_b and k_e are first-order rate constants for mixing, reaction, H^+ back flow and H^+ electrode response, respectively; and n is the protonmotive stoichiometry.

After rearrangement into linear differential equation form these equations can be solved by standard methods using the following boundary conditions. A. During the addition of the oxidant: When $t = 0$ then O^*, O, H and $S = 0$. B. After the addition of the oxidant: When $t = 0$, then O^*, O, H and S = the respective values these variables reached at the end of the addition of the oxidant and are termed O_0^*, O_0, H_0 and S_0, respectively.

The equations obtained (not shown), describing the relationships between t and S, the H^+ electrode signal, and $O + O^*$, the total amount of unreacted oxidant present, were used to generate the plots shown in Fig. 6. Values of k_m and k_e were chosen using information on the mixing and electrode kinetics for a reaction cell similar to that used in the experiments described in this paper (Mitchell, R., unpublished results). The value of k_b chosen, equivalent to a half time for H^+ back flow of 60 s, was representative of that observed with the mitochondrial preparations used in these

experiments. In the simulations oxidant was added at 3.33 nmol of $2e^-$ acceptor/s for 6 s (total = 20 nmol; i.e., slightly faster than the fastest rate used in the actual experiments) and an arbitrary protonmotive stoichiometry of 6 was chosen. Note that the use of first-order kinetics to describe the reaction of the oxidant for a system approximating to Michaelis-Menten kinetics is only justified if the concentration of the mixed oxidant in the system is always well below the apparent K_m of the system for the oxidant. On this assumption the relationship

$$k_r = \frac{V_{max}}{K_m} \tag{18}$$

was used in choosing values of k_r appropriate for the oxidants, oxygen and ferricyanide. The apparent K_m of rat liver mitochondria for oxygen was taken to be about 0.1 μM [45,46] and the K_m for ferricyanide was taken to be about 10 μM [47]. These are equivalent to k_r values of about 100 and 1 s^{-1}, respectively. Fig. 6 shows simulations produced using k_r values of 100, 10 and 1 s^{-1}. On the scale used in this figure a simulation produced using a k_r value of 1000 s^{-1} would be indistinguishable from that produced using a k_r value of 100 s^{-1}.

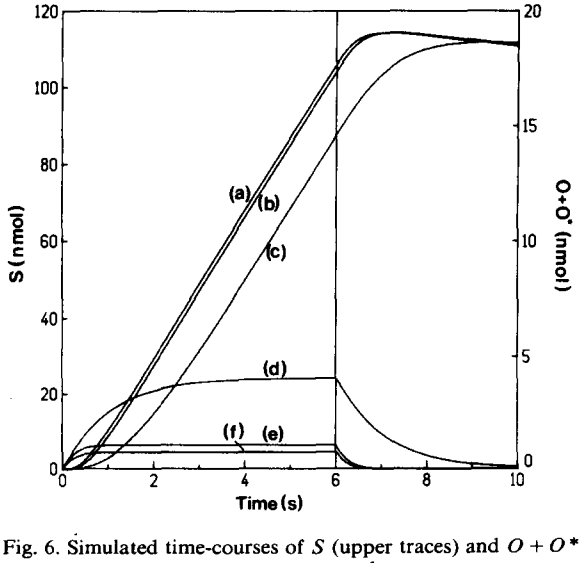

Fig. 6. Simulated time-courses of S (upper traces) and $O + O^*$ (lower traces). (a) and (f), $k_r = 100$ s^{-1}; (b) and (e), $k_r = 10$ s^{-1}; (c) and (d), $k_r = 1$ s^{-1}. In all cases $k_d = 0.012$ s^{-1}, $k_m = 4.6$ s^{-1}, and $k_e = 3.5$ s^{-1}. Other details are given in the text.

On the basis of the simulation where k_r was set at 100 s^{-1} oxygen is shown to be ideal for this type of experiment since the amount of mixed/unreacted oxidant in the simulation rapidly reaches a steady-state (where the concentration of mixed/unreacted oxidant is about 10% of the K_m for the oxidant), the time taken to reach the steady-state being controlled mainly by k_m, the rate constant for the mixing of the oxidant. Hence, as required, the rate of oxidation in the steady-state is defined by the rate of oxidant addition. Furthermore, the steady-state amount of unreacted/mixed oxidant quickly becomes negligible when compared with the total amount added. As a consequence of this, we would predict that addition of oxygen at a constant rate to suspensions of anaerobic mitochondria would produce time-courses of ΔH_O^+ (corrected for H^+ back flow) with kinetics indistinguishable from the time-courses of ΔH_O^+ obtained on addition of H^+ at a constant rate to suitably buffered medium in our reaction cell. This has indeed been found to be the case *.

In contrast to oxygen, the simulation where k_r was set to 1 s^{-1} indicates that the concentration of mixed/unreacted ferricyanide would not quite reach a steady-state by the end of the addition. Further problems with ferricyanide become apparent when we increase the complexity of our simple model by qualitatively considering the ef-

* Since the original drafting of this paper it has proved possible to detect the steady-state level of oxygen during rate/pulses using a modified Clark-type oxygen electrode set-up. A level of about 10 nM O (≡0.033 nmol total) was observed shortly after the addition of 100 μl rate/pulses (taking 15.5 s to add) to anaerobic mitochondria treated with valinomycin, rotenone, antimycin, myxothiazol and *N*-ethylmaleimide, in the presence of hexammineruthenium(II) as reductant. A level of about 4 nM O (≡0.013 nmol total) was observed after the addition of 40 μl rate/pulses (taking 7 s to add) to mitochondria treated with valinomycin, rotenone and *N*-ethylmaleimide. However, the 90% response time of the oxygen electrode under these circumstances was around 15 s and so these levels must be underestimated. Nevertheless, they are certainly consistent with the results of the simulation.

fect of variation in k_r during the reaction of the oxidant.

For oxygen the variation in k_r is considered to be small: first, because the pH changes involved are relatively small ($\Delta pH_O < 0.1$ and $\Delta pH_I < 0.4$ [48]); and second, because the oxidation is essentially irreversible. In any case, the simulations indicate that a variation in k_r between 10 and 1000 s^{-1} would have little effect on the results obtained.

For ferricyanide the situation is different. The simulation indicates that even a small change in k_r might have a considerable effect on the results. In addition, the electron transfer from cytochrome *c* to ferricyanide is inhibited at high ferrocyanide/ferricyanide ratios (Moyle, J., unpublished results). Therefore, one would expect that the net rate of electron transfer to the ferricyanide would decrease at high ferrocyanide/ferricyanide ratios. Such a situation would occur after the end of the ferricyanide addition as the remaining unreacted ferricyanide is converted to ferrocyanide. Hence, in a more realistic simulation the reaction of the last few percent of the ferricyanide would be retarded. This effect seems to have been observed experimentally, as noted in the previous section.

Discussion

In the new rate/pulse method of measuring $\leftarrow H_o^+/2e^-$ ratios, described in this paper, proton ejection was estimated from the time-course of the pH of the medium (pH_O), while electron flow through cytochrome *c* oxidase and/or cytochrome *c* reductase was initiated and maintained by addition of oxidant (oxygen or ferricyanide) at a constant rate to suspensions of initially anaerobic rat liver mitochondria. In all cases, it was found that, after a short initial lag, the rate of H^+ ejection (corrected for H^+ back flow into the mitochondria) reached a constant level. Furthermore, the $\leftarrow H_o^+/2e^-$ ratio was found to be the same whether it was calculated from the total amount of H^+ output divided by the total amount of $2e^-$ accepted, or whether it was calculated from the constant rate of H^+ output divided by the rate of addition of $2e^-$ acceptor.

We conclude that the protonmotive stoichiometries of rat liver cytochrome oxidase and cytochrome reductase are independent of ΔpH under the conditions of these experiments (i.e., $0 < \Delta pH < 0.5$ [48]). This conclusion is important because it validates the conventional pulse method for these two enzymes. In the conventional pulse method all that is measured is the total H^+ ejection associated with the consumption of a known amount of oxidant. If the protonmotive stoichiometry of the enzyme or enzymes in question were to vary, i.e., decrease in response to the increasing protonmotive force [35,36], then this method could only give the mean stoichiometry for that particular amount of oxidant, which would of course be an underestimate of the mechanistic stoichiometry. Since we observe no dependence of these stoichiometries on the protonmotive force, there should be no underestimation of the stoichiometry. One should note here that the present results do not eliminate the possibility of variable protonmotive stoichiometry as the result of protonmotive force-dependent 'slippage' at higher values of protonmotive force, since only the ΔpH component is significant in these experiments and the range encountered is small. Furthermore, it could still be argued that the stoichiometry measured in these experiments is an underestimate because the starting protonmotive force is not strictly zero. That this is so arises from the use of a small pre-addition of oxidant to ensure a uniform concentration of oxidant in the main addition, and also from the presence of small leaks of oxygen into the reaction vessel (see Ref. 14). However, identical results have recently been obtained for the ubiquinol to O_2 span without the use of the pre-addition and with the oxygen leaks reduced effectively to zero by performing the experiments in an anaerobic glove box where the concentration of oxygen in the atmosphere surrounding the reaction cell was maintained at less than 0.025% of the ambient concentration (Moody, A.J., unpublished results).

The observed protonmotive stoichiometry of the ubiquinol to O_2 span of the mitochondrial respiratory chain was found to decrease slightly as the rate of addition of oxygen was decreased (Fig. 2B). One possible reason for this decrease is that there was some re-equilibration of phosphate via residual phosphate/OH^- antiport or its equivalent (despite the presence of *N*-ethylmaleimide),

occurring at a slow and essentially constant rate during the addition of the oxygen. Loss of protons in such a way would be 'invisible' in that it would not be included in the correction for H^+ back flow. On the basis of this explanation the best estimate of the protonmotive stoichiometry should be obtained by extrapolating to zero addition time. The stoichiometry obtained on doing this is about 0.06 greater than that obtained with the shortest addition time (7 s) possible with the motorized syringe. It would also be desirable to do such an extrapolation for the observed stoichiometries of the ubiquinol to cytochrome *c* and cytochrome *c* to O_2 spans. However, the variation of these stoichiometries over a wide range of oxidant addition rates is not yet known.

It is evident given the previous considerations that the results obtained by the conventional pulse method should be the same as those obtained by the present rate/pulse method (for the fastest addition rate used or, where possible, by extrapolation to zero addition time) using an otherwise identical experimental design. We have obtained essentially identical results for the ubiquinol to O_2 span using the conventional oxidant pulse method under the same conditions (Moody, A.J. and Mitchell, R., unpublished results), and similar results have been obtained previously in this laboratory for ubiquinol to O_2 and ubiquinol to cytochrome *c* spans, but under slightly different conditions [49]. Similar results have also been obtained for the cytochrome *c* to O_2 span [14], under identical conditions, except that the total amount of oxygen added in each experiment was about 2.5-fold greater in the present experiments (It should be noted that the 'shortfall' in the observed net scalar H^+ uptake associated with this span was found to be somewhat smaller in the present experiments.).

As indicated above, there has been a fundamental objection to the conventional pulse method in that the protonmotive stoichiometries of the oxidase and the reductase might decrease as the protonmotive force increases during the consumption of the oxidant. This was one reason for introducing the rate/pulse method described in this paper. However, there is another method already in use that ostensibly provides the same information as the rate/pulse method. This is the rate method, which has been developed by Lehninger and colleagues over the past 10 years or so [30,18–21]. In the rate method the simultaneous rates of H^+ ejection and oxygen consumption are measured on addition of a 'pulse' of oxygen to anaerobic mitochondria (or mitoplasts). The conclusions reached by Lehninger and colleagues from the results of their rate measurements are in complete disagreement with the conclusions reached in this paper, i.e., they have concluded that the stoichiometries of the ubiquinol to O_2 and cytochrome *c* to O_2 spans are variable and that the values obtained on extrapolation to 'zero' protonmotive force are consistent with a protonmotive stoichiometry of 4 for cytochrome oxidase. However, for several reasons, we think that the results obtained by the rate/pulse method are more reliable than those obtained by the rate method.

The rate/pulse type of experiment has the advantage that there is a known and constant rate of consumption of oxidant (at least in the case where oxygen is used). Therefore, no oxidant measurements need be made. The fact that simultaneous measurements of the rates of H^+ ejection and oxidant consumption are required in the rate method introduces two problems that are completely avoided in the rate/pulse method. First, the response times of the methods of measurement must be closely matched, otherwise significant errors will occur [2]. Second, even if the response times are matched a time-dependent error will be introduced because the concentration of oxygen effectively undergoes a step change at the start of the measurements while the concentration of H^+ does not [50].

In addition, the method of analysis used by Lehninger et al. incidentally introduced errors that are not intrinsic to the rate method. Krab and Wikström [33] have shown clearly, in a simulation study, that the method of extrapolation of Guggenheim plots to infer the rate of H^+ ejection at zero time, as used by Lehninger et al., is mistaken, even if these plots appear to be linear.

We can see, therefore, that the rate/pulse method combines the virtues of both the pulse method and the rate method, while eliminating their shortcomings. The pulse method requires no oxidant measurements but gives no information as to the relationship of the protonmotive stoichiom-

etry to the protonmotive force *. Conversely, the rate method could in principle give information on the latter relationship but in practice it is complicated by the need for oxidant measurements.

It is apparent that the protonmotive stoichiometries obtained by the present rate/pulse method are non-integral, even if the age of the mitochondria and the slight effect of the rate of oxidant addition on the stoichiometry (Fig. 2) are taken into account. The next highest integers are 2 for cytochrome oxidase, 4 for the cytochrome reductase and 6 for the oxidase and reductase in combination. Since the protonmotive stoichiometry of cytochrome reductase is generally agreed to be 4 on mechanistic grounds, it seems sensible to address the question as to why non-integral stoichiometries were observed.

An obvious reason for the failure to observe integral stoichiometries is that some of the mitochondria have proton-leaky inner membranes; the simplest possibility here is that the inner membranes of some of the mitochondria are actually broken. We can derive the following equation to predict the effect that such mitochondria would have on the observed protonmotive stoichiometry for each measurement,

$$OS = MS \cdot PI + NS \cdot (1 - PI) \quad (19)$$

or rearranged,

$$PI = (OS - NS)/(MS - NS) \quad (20)$$

where OS is the observed stoichiometry, MS is the mechanistic stoichiometry, PI is the proportion of intact (i.e., not proton-leaky) mitochondria and NS is the net scalar $\Delta H^+_O/2e^-$ stoichiometry for the reaction. Note that the effect of the protons taken up or released in the net scalar reaction is taken to be 'hidden' in the matrix if the mitochondria are intact but apparent in the medium if the inner membranes of the mitochondria are proton-leaky.

From Eqn. 19 it can be seen that the observed stoichiometry is only decreased from the mechanistic stoichiometry in proportion to the fraction of intact mitochondria for the special case where the net scalar $\Delta H^+_O/2e^-$ stoichiometry (i.e., net acid production or consumption) of the reaction in question is zero, as is approximately the case for the combined reaction of cytochrome *c* reductase and cytochrome *c* oxidase. If the net scalar stoichiometry is positive, as is the case for the reductase reaction, then the decrease in observed protonmotive stoichiometry will be less than proportional to the amount of intact mitochondria. Conversely, if the net stoichiometry is negative, as is the case for the oxidase reaction, then the decrease in observed stoichiometry will be more than proportional.

Eqn. 20 has been applied to the results presented in this paper, together with the cumulative results of conventional oxidant-pulse experiments carried out under similar conditions in this laboratory for many years (see e.g. Ref. 49). When this is done, we find that, if the mechanistic stoichiometries of the oxidase and the reductase are taken to be 2 and 4, respectively, the proportion of intact mitochondria calculated for the measurement of the protonmotive stoichiometry of cytochrome oxidase, cytochrome reductase and both together, agrees well at between 85 and 95%, decreasing with the age of the mitochondria. The agreement is much less satisfactory if the stoichiometry of the oxidase is taken to be greater than 2. On this basis, therefore, the presence, in the mitochondrial suspensions, of mitochondria that are proton-leaky (perhaps because their inner membranes are broken) seems a reasonable explanation for the observation of non-integral stoichiometries.

The obvious test of this explanation would be to try to obtain a direct estimate of the proportion of intact mitochondria by measuring enzyme activities, e.g., pyridine nucleotide transhydrogenations [51], that indicate if the matrix is accessible to water-soluble substrates that are normally

* This is only true for an individual experiment. If pulses of different sizes are used over a series of experiments, that are otherwise identical, the relationship between protonmotive stoichiometry and protonmotive force can be inferred from the dependence of the stoichiometry on pulse size. Such series of experiments can be found in Refs. 28 and 8. In the presence of *N*-ethylmaleimide the stoichiometry of the ubiquinol to O_2 span was found to be independent of pulse size over the range 0.4 (the lowest size used) to 1.7 nmol O/mg of mitochondrial protein (A total of about 1 nmol O/mg was used in the rate/pulse experiments reported here.).

excluded if the inner membrane is intact. However, it should be noted that such measurements might not be totally reliable indicators of some types of breakage, e.g., where the structure of the mitochondria remains largely intact and the matrix protein is still associated with the inner membrane. Given the relative bulk of the substrates and the well-known high mobility of the H^+ (strictly, H_3O^+) and OH^- ions compared to other small monovalent ions [52], it would not be surprising if enzyme activity studies failed to be diagnostic of the true level of 'breakage' with respect to protons.

In summary, we conclude that a good case can be made for the presence of mitochondria with proton-leaky inner membranes being the major, but as yet still untested, reason for our failure to observe integral protonmotive stoichiometries in the rate/pulse experiments described in this paper, and that the results are, therefore, consistent with a mechanistic $\leftarrow H_o^+/2e^-$ ratio of 2 for cytochrome *c* oxidase.

Acknowledgements

We thank the Medical Research Council and the Commission of the European Communities for grants in support of this work. We are also indebted to other benefactors, named in the brochure of the U.K. Registered Charity called the Glynn Research Foundation Ltd, for general financial support of the research facilities at the Glynn Research Institute. We are grateful to Alan Jeal and Sally Madgwick for skilled technical assistance, and to Robert Harper for help in preparing the manuscript. I.C.W. is on leave of absence form the University of Newcastle-upon-Tyne. We thank Dr. Peter Rich for helpful suggestions during the revision of the manuscript. We are also indebted to Dr. J.M. Moyle for access to her wealth of unpublished material.

References

1 Lorusso, M., Capuano, F., Boffoli, D., Stefanelli, R. and Papa, S. (1979) Biochem. J. 182, 133–147
2 Papa, S., Capuano, F., Markert, M. and Altamura, N. (1980) FEBS Lett. 111, 243–248
3 Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., Boffoli, D., Capuano, F., Capitanio, N. and Altamura, N. (1980) Biochem. J. 192, 203–218
4 Papa, S. (1982) in Membranes and Transport, Vol. 1 (Martonosi, A.N., ed.), pp. 363–368, Plenum Press, New York
5 Papa, S., Guerrieri, F. and Izzo, G. (1983) Biochem. J. 216, 259–272
6 Papa, S., Lorusso, M., Capitanio, N. and De Nitto, E. (1983) FEBS Lett. 157, 7–14
7 Al-Shawi, M.K. and Brand, M. (1981) Biochem. J. 200, 539–546
8 Brand, M.D., Reynafarje, B. and Lehninger, A.L. (1976) Proc. Natl. Acad. Sci. USA 73, 437–441
9 Brand, M.D., Harper, W.G., Nicholls, D.G. and Inglewdew, W.J. (1978) FEBS Lett. 95, 125–129
10 Wikström, M.K.F. and Krab, K. (1979) Biochim. Biophys. Acta 549, 177–222
11 Wikström, M. and Pentillä, T. (1982) FEBS Lett. 144, 183–189
12 Sigel, E. and Carafoli, E. (1980) Eur. J. Biochem. 111, 299–306
13 Casey, R.P., Chappell, J.B. and Azzi, A. (1979) Biochem. J. 183, 149–156
14 West, I.C., Mitchell, R., Moody, A.J. and Mitchell, P. (1986) Biochem. J. 236, 15–21
15 Beavis, A.D. and Lehninger, A.L. (1986) Eur. J. Biochem. 158, 315–322
16 Azzone, G.F., Pozzan, T. and Di Virgilio, F. (1979) J. Biol. Chem. 254, 10206–10212
17 Di Virgilio, F., Pozzan, M. and Azzone, G.F. (1981) Eur. J. Biochem. 117, 225–231
18 Costa, L.E., Reynafarje, B. and Lehninger, A.L. (1984) J. Biol. Chem. 259, 4802–4811
19 Reynafarje, B., Alexandre, A., Davies, P. and Lehninger, A.L. (1982) Proc. Natl. Acad. Sci. USA 79, 7218–7222
20 Reynafarje, B., Costa, L.E. and Lehninger, A.L. (1986) J. Biol. Chem. 261, 8254–8262
21 Setty, O.H., Shrager, R.I., Bunow, B., Reynafarje, B., Lehninger, A.L. and Hendler, R.W. (1986) Biophys. J. 50, 391–404
22 Papa, S., Capitanio, N. and De Nitto, E. (1987) Eur. J. Biochem. 164, 507–516
23 Mitchell, R., West, I.C., Moody, A.J. and Mitchell, P. (1986) Biochim. Biophys. Acta 849, 229–235
24 Moyle, J. and Mitchell, P. (1978) FEBS Lett. 88, 268–272
25 Moyle, J. and Mitchell, P. (1978) FEBS Lett. 90, 361–365
26 Mitchell, P. (1980) Ann. NY Acad. Sci. 341, 564–584
27 Mitchell, P. (1982) in Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M., eds.), pp. 1225–1246, Pergamon Press, Oxford
28 Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162
29 Mitchell, P., Moyle, J. and Mitchell, R. (1979) Methods Enzymol. 55, 627–640
30 Reynafarje, B., Brand, M., Alexandre, A. and Lehninger, A.L. (1979) Methods Enzymol. 55, 640–656
31 Lehninger, A.L., Reynafarje, B., Alexandre, A. and Villalobo, A. (1979) in Membrane Bioenergetics (Lee, C.P., Shatz, G. and Ernster, L., eds.), pp. 393–404, Addison-Wesley, Reading, MA
32 Lehninger, A.L. (1984) Biochem. Soc. Trans. 12, 386–388

33 Krab, K. and Wikström, M. (1986) FEBS Lett. 201, 198–204
34 Mitchell, P. (1982) in Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M., eds.), pp. 1247–1268, Pergamon Press, Oxford
35 Pietrobon, D., Azzone, G.F. and Walz, D. (1981) Eur. J. Biochem. 117, 389–394
36 Pietrobon, D., Zoratti, M. and Azzone, G.F. (1983) Biochim. Biophys. Acta 723, 317–321
37 Mitchell, P., Mitchell, R., Moody, A.J., West, I.C., Baum, H. and Wrigglesworth, J.M. (1985) FEBS Lett. 188, 1–7
38 Wikström, M. (1977) Nature 266, 271–273
39 Wikström, M. and Saari, H. (1977) Biochim. Biophys. Acta 462, 347–361
40 Krishnamoorthy, G. and Hinkle, P.C. (1984) Biochemistry 23, 1640–1645
41 Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, pp. 70–82, Glynn Research Ltd, Bodmin
42 Heinz, E., Westerfoff, H.V. and Van Dam, K. (1981) Eur. J. Biochem. 115, 107–113
43 Mitchell, P. and Moyle, J. (1979) in Cytochrome Oxidase (King, T.E., Orii, Y., Chance, B. and Okunuki, K., eds.), pp. 361–372, Elsevier/North-Holland, Amsterdam
44 Turnbull, D.M., Sherratt, H.S.A., Davies, D.M. and Sykes, A.G. (1982) Biochem. J. 206, 511–516
45 Degn, H. and Wohlrab, H. (1971) Biochim. Biophys. Acta 245, 347–355
46 Petersen, L.C., Nicholls, P. and Degn, H. (1974) Biochem. J. 142, 247–252
47 Estabrook, R.W. (1961) J. Biol. Chem. 236, 3051–3057
48 Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162
49 Mitchell, P. and Moyle, J. (1979) Biochem. Soc. Trans. 7, 887–894
50 Krab, K., Soos, J. and Wikström, M. (1984) FEBS Lett. 178, 187–192
51 Rydström, J. (1977) Biochim. Biophys. Acta 463, 155–184
52 Mitchell, P. (1967) Nature 214, 1327–1328