# UNEQUAL CHARGE SEPARATION BY DIFFERENT COUPLING SPANS OF THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

M. D. BRAND, W. G. HARPER, D. G. NICHOLLS[+] and W. J. INGLEDEW*

*Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, England, [+]Department of Psychiatry, Ninewells Medical School, Dundee University, Dundee DD2 1UD* and **Department of Biochemistry, Dundee University, Dundee DD1 4HN, Scotland*

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## 1. Introduction

Although it is now widely accepted that $\Delta\tilde{\mu}H^+$ is the intermediate between electron transport and ATP synthesis in mitochondria [1], the quantitative relationships have yet to be resolved [2–8]. Two main lines of research have led to current ideas.

(1) If the phosphorylation potential attained by mitochondria in state 4 is compared with $\Delta\tilde{\mu}H^+$ the data suggest that ~3 $H^+$ must be translocated for each ATP synthesized [9–12]. This is consistent with the direct measurement of 2 $H^+$ ejected by the ATPase during ATP hydrolysis plus 1 $H^+$ transported with phosphate and adenine nucleotides [13].

(2) Observation of $H^+$ and other ion movements has been carried out. The results obtained by different methods and research groups disagree, and indicate that either 4 [3,6,7,14], 6 [2,15–17] or 8 [2,4,15,18–20] $H^+$ are transported out of the mitochondria per 2 $e^-$ passing from succinate to oxygen down the respiratory chain.

*Abbreviations:* $\Delta\tilde{\mu}H^+$, difference in electrochemical activity of $H^+$ across the mitochondrial inner membrane (consisting of an electrical component $\Delta\psi$ and a pH component $\Delta$pH); $H^+/O$, number of $H^+$ ejected from the matrix per 2 $e^-$ passing down the respiratory chain from specified substrate to oxygen; charge/O, number of positive charges (as $H^+$) crossing the inner membrane from the matrix side and/or negative charges (as $e^-$) crossing the inner membrane to the matrix side per 2 $e^-$ passing from substrate to oxygen

If a greater insight into the mechanism of the mitochondrial $H^+$-translocating systems is to be achieved it is important that these differences be resolved and the correct value of the $H^+/O$ ratio established.

We introduce here a new steady-state method of investigating $H^+/O$ or charge/O ratios. It involves varying the rate of $H^+$ ejection and measuring the steady-state $\Delta\tilde{\mu}H^+$ which results at constant membrane proton conductance. An importance advantage of this method is that it is free of many of the assumptions inherent in other procedures and it therefore gives an independent estimate of true $H^+/O$ and charge/O quotients. It allows comparison of $H^+/O$ or charge/O ratios for different substrates but does not give an absolute value for any one portion of the respiratory chain.

The results we have obtained in this study lead to two important conclusions.

(i) Classically, the respiratory chain is considered to contain three coupling sites at which 1 ATP is synthesized as 2 $e^-$ pass; however, our results show that the number of charges or $H^+$ translocated per 2 $e^-$ is not equal at each of these 'sites'. This means that more ATP may be synthesized per 2 $e^-$ at some 'sites' than at others and that P/O ratios are not integral with all substrates. We predict P/O ratios of 2.67 for NAD-linked substrates, 2.0 for succinate and 1.33 for ascorbate.

(ii) The charge/O and $H^+/O$ ratios for the spans isoascorbate → oxygen, succinate → oxygen, and 3-hydroxybutyrate → oxygen fall in the ratio

1.0 : 1.5 : 2.0. These values probably correspond to absolute $H^+/O$ quotients of 4, 6 and 8, respectively.

## 2. Experimental

### 2.1. *Theory*

The principle of the method is simple. Given a constant $H^+$ conductance of the coupling membrane, $\Delta\tilde{\mu}H^+$ is proportional to the rate of $H^+$ ejection by the respiratory chain. To maintain a given $\Delta\tilde{\mu}H^+$ will require a certain rate of $H^+$ ejection which will be independent of the nature of the substrate being oxidized. The rates of oxidation of different substrates needed to give the same rate of $H^+$ ejection will be inversely proportional to their $H^+/O$ ratios. It should thus be possible to relate $H^+/O$ ratios to steady-state oxidation rates. In practice greater accuracy is obtained by allowing $\Delta\tilde{\mu}H^+$ to vary and measuring the slope of a plot of $\Delta\tilde{\mu}H^+$ against rate of oxygen consumption to give the relative $H^+/O$ value, as shown below.

The rate of $H^+$ ejection from the matrix:

$$J_{H^+out} = H^+/O \times V \quad (1)$$

where $V$ is the rate of oxygen consumption. Under certain conditions the rate of $H^+$ re-entry:

$$J_{H^+in} = k\Delta\tilde{\mu}H^+ \quad (2)$$

where $k$ is a first order rate constant for natural plus artificially-induced $H^+$ re-entry. Equation (2) will be true up to $\Delta\tilde{\mu}H^+$ values of ~200 mV, when the leak rate becomes very great as state 4 is approached [10,11,21]. In the experiments described here, we kept $\Delta\tilde{\mu}H^+$ sub-maximal by restricting the rate of electron transport by using limiting substrate concentrations, and by deliberately increasing the maximum rate of $H^+$ re-entry by adding small amounts of uncoupler buffered with bovine serum albumin so that state 4 was never achieved.

Under these conditions in any steady-state:

$$J_{H^+out} = J_{H^+in}$$

thus from eq. (1) and eq. (2):

$$H^+/O \times V = k\Delta\tilde{\mu}H^+$$

thus

$$\Delta\tilde{\mu}H^+ = \frac{H^+/O}{k} \times V \quad (3)$$

Thus a graph of $\Delta\tilde{\mu}H^+$ against $V$ will have slope $(H^+/O)/k$. Substrates feeding into the respiratory chain at different points will give different slopes, and since $k$ will be the same in each case (it is due largely to the added uncoupler), the slopes will be in direct proportion to the $H^+/O$ quotients. The ratio of the slopes will be the ratio of the $H^+/O$ quotients.

$V$ is easily measured with the oxygen electrode and is varied by titration with the substrate being studied. It is not easy to determine $\Delta\tilde{\mu}H^+$ rapidly and accurately, but addition of nigericin and acetate will tend to minimize $\Delta pH$ and so make $\Delta\psi$ approximate $\Delta\tilde{\mu}H^+$. $\Delta\psi$ is readily measured using safranine [22]. It is important to note that under these conditions the method gives charge/O ratios. For the spans 3-hydroxybutyrate → oxygen, succinate → oxygen and isoascorbate → oxygen, charge/O is expected to equal app. $H^+/O$, but this would not be true for other spans such as cytochrome $c$ → oxygen, where charge/O is greater than $H^+/O$ by 2 units (see below).

### 2.2. *Materials and methods*

Experiments were carried out in an 8 ml cuvette stirred by an overhead motor and propeller. A Clark-type oxygen electrode was inserted through the cuvette lid, which protected the contents from contact with air. The electrode was periodically checked for linear response to oxygen concentration. The cuvette was placed in a thermostatted holder in a dual beam spectrophotometer. Additions were made with microsyringes through a special port. Rat liver mitochondria were prepared by conventional methods in 250 mM sucrose/1 mM EGTA (ethyleneglycolbis-(aminoethyl)tetraacetate)/5 mM Tris–HCl, pH 7.4. Fresh solutions of isoascorbate (which has very similar properties to ascorbate and was used as though the two were equivalent) and ferrocyanide were prepared each day.

## 3. Results

Figure 1 shows the results of a typical experiment.

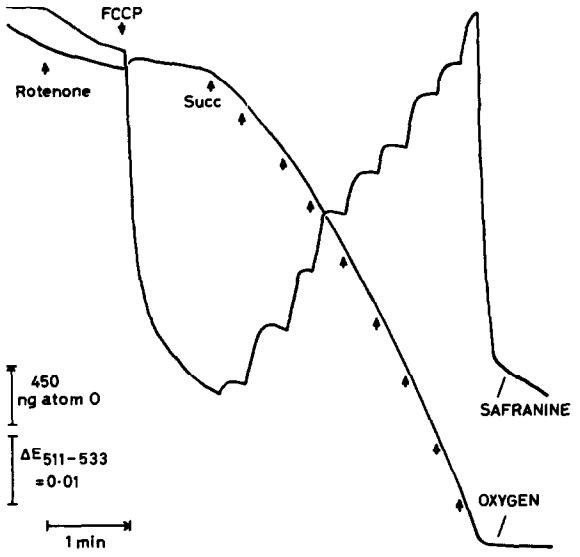


Fig.1. $\Delta\psi$ and oxygen consumption in mitochondria titrated with succinate. Mitochondria (18 mg protein) were suspended in medium containing 120 mM KCl, 10 mM Na acetate, 10 mM Na-TES (*N*-Tris(hydroxymethyl)-methyl-2-aminoethane sulphonate), 1 mM Na-EGTA (ethyleneglycolbis-(aminoethane)tetraacetate), 1 mg/ml bovine serum albumin, 20 $\mu$M safranine, 1.4 $\mu$g oligomycin/mg protein, 1 $\mu$g nigericin/mg protein (pH 7.1) 25°C, total vol. 8 ml. The safranine signal was measured at 511–533 nm; an upward deflection of the trace reflects increasing $\Delta\psi$. Where shown 3 $\mu$M rotenone and 188 nM FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) were added. At the times shown by arrows 1 $\mu$l aliquots of 0.5 M Na-succinate (succ) were injected; as $\Delta\psi$ increased larger aliquots were used. Final succinate was ~1 mM. At the end of the experiment (not shown) the oxygen trace was calibrated by injection of a small volume of $H_2O_2$, and the safranine signal was internally calibrated by addition of succinate to 3 mM to give a maximal signal. Anaerobiosis or addition of antimycin caused collapse of $\Delta\psi$, the signal difference was arbitrarily taken as the 100% control value. This varied by a few % from trace to trace.

As expected, treatment of mitochondria with rotenone and limiting amounts of uncoupler caused a rapid collapse of $\Delta\psi$ and a very low rate of oxygen consumption. Addition of a small amount of succinate allowed the oxidation rate to increase and $\Delta\psi$ to rise to a steady-state. Repeated additions of succinate gave successive steady-state rates of oxygen consumption and values of $\Delta\psi$.

Calibration of the safranine signal with $K^+$ diffusion gradients according to [22] showed that its maximum value was about 170–180 mV. The exact value is unimportant for the comparison of charge/O ratios.

Identical experiments were carried out with 3-hydroxybutyrate as substrate, titrating to maximum substrate 2 mM, and with isoascorbate, titrating ferrocyanide as mediator, to final ferrocyanide 5 mM. In the absence of isoascorbate, oxidation of 5 mM ferrocyanide generated only about 30% of max. $\Delta\psi$ owing to a slower rate of oxygen consumption, showing that ferrocyanide was acting catalytically and was not itself the major substrate under these conditions.

The amount of uncoupler added in these experiments varied slightly from day to day. The amount used was that which was just sufficient to give a maximum rate of oxygen consumption with 5 mM 3-hydroxybutyrate without lowering $\Delta\psi$ by $\lesssim$5%.

Figure 2 shows a graph of $\Delta\psi$ against rate of oxygen consumption for the three substrates under identical conditions. Although eq. (3) predicts that

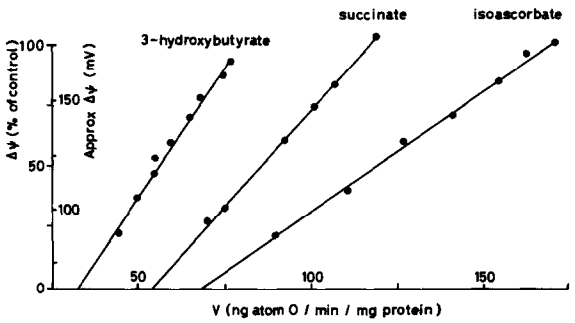


Fig.2. $\Delta\psi$ as a function of rate of oxygen consumption with different substrates. Experiments were carried out exactly as in fig.1. At each steady-state the rate of oxygen consumption, *V*, and $\Delta\psi$ (as % of the control value with succinate) were calculated. In the experiment with 3-hydroxybutyrate as substrate rotenone was omitted and 1 M Na-3-hydroxybutyrate was injected in aliquots from 1–5 $\mu$l up to a final concentration of ~ 2 mM. In the experiment with isoascorbate as substrate the medium contained 3 mM succinate (from the prior calibration of $\Delta\psi$ as in fig.1; omission of this succinate did not affect the results), 1 $\mu$g antimycin A/mg protein and 2.5 mM Na-D-isoascorbate. Aliquots, 10 $\mu$l, of 0.5 M Na-ferrocyanide were added up to a final concentration of ~5 mM. The approximate values for $\Delta\psi$ shown on the vertical axis were derived from control experiments using $K^+$ diffusion potentials as in [22].

the lines should have passed through the origin, they did not; this is probably an artifact due to the absence of safranine signal at $\Delta\psi$ values $\lesssim$60 mV. The scale labelled 'Approx $\Delta\psi$ (mV)' was derived from independent calibration experiments and is included to make this clearer. The extrapolated lines intersected at ~0 on this scale, with some scatter, which varied between experiments. The lines shown were fitted by the method of least squares. The slopes for isoascorbate, succinate, and 3-hydroxybutyrate were in the ratio 1 : 1.6 : 2.1, in this experiment; this is therefore the ratio of the charge/O quotients for these substrates. Analysis of many such experiments gave relative slopes ± SEM (no. independent pairs of determinations) of 1 : 1.47 ± 0.02 (18) : 1.99 ± 0.04 (18), very close to 1 : 1.5 : 2.0.

The fact that the lines in fig.2 were straight indicates that the charge/O quotient for each substrate did not vary over the range of $\Delta\psi$ studied.

## 4. Discussion

This new steady-state method shows that there are unequal numbers of charges translocated as electrons pass through different energy-conserving regions of the respiratory chain. If all coupling spans were equivalent we would expect the charge/O quotients to fall in the ratio 1 : 2 : 3 for isoascorbate, succinate and 3-hydroxybutyrate, which is very different from the ratio 1 : 1.5 : 2.0, which we found.

It is worth stressing that the method does not depend on accurate calibration of either $\Delta\psi$ or $V$ since any systematic errors are cancelled when ratios of slopes are taken. It does not rely on extrapolations to give maximum extents of $H^+$ ejection as in 'oxygen-pulse' experiments [14–16]. Transient ion movements are unimportant and thus transport inhibitors such as *N*-ethylmaleimide are not required for accurate results (cf. [2,6,13–16,18,19,23,24]) and since $Ca^{2+}$ is not involved no assumptions need be made about the mode of $Ca^{2+}$ transport (cf. [2–5, 15,17,19,25]). It is also free of criticisms that $\Delta\tilde{\mu}H^+$ may be underestimated, a potential hazard of using the thermodynamic approach to estimate stoichiometries [9–12,26], and that electrons may be arising from substrates other than that added [1,6,7] since (at least with 3-hydroxybutyrate and succinate) it is titration with substrate which causes the increase in $\Delta\psi$. For discussion of the possibility that ferrocyanide may allow oxidation of endogenous $H^+$ carriers see [7] and [8].

A disadvantage of the method is that it gives only relative values, not absolute ones, However, since we may ignore fractional charges, the only reasonably small absolute charge/O quotients consistent with our results for isoascorbate, succinate and 3-hydroxybutyrate are 2 : 3 : 4, or 4 : 6 : 8, or 6 : 9 : 12, or 8 : 12 : 16. Of these 4 : 6 : 8 best fits other experimental data.

We therefore conclude that the most probable charge/O and $H^+$/O ratios for the span 3-hydroxybutyrate → oxygen are 8.0; for the span succinate → oxygen they are 6.0; and for the span isoascorbate → oxygen they are 4.0. In the case of isoascorbate the pH equivalent of 2 $H^+$/O is generated by scalar reactions occurring in the medium and in the matrix, thus for the span cytochrome *c* → oxygen charge/O ratio is 4 but the true $H^+$/O ratio is 2.

These values are consistent with and support Wikström's contention that cytochrome oxidase is a proton pump with $H^+$/O ratio of 2 and charge/O ratio of 4 [23,24], and observations of $H^+$/O ratios of 6 for succinate oxidation using other methods [2,15–17]. They conflict with models requiring equal $H^+$ ejection in each energy-conserving region of the respiratory chain and with experiments suggesting that 2 $H^+$ [3,6,7,14] or 4 $H^+$ [2,4,15,18–20,25,26] are ejected in each region. Our results do not conflict with models which involve cytochrome oxidase as a proton pump [23,24], a protonmotive Q cycle [27], and NADH dehydrogenase as a proton pump (or Mitchellian loop) with stoichiometry of 2 $H^+$/2 $e^-$ [14], although other models of the mechanism of $H^+$ pumping (see, e.g., [28]) will also fit our data.

If $H^+$ ejection is indeed unequal in different energy-conserving regions of the respiratory chain then it follows that the P/2 $e^-$ ratios will not have simple integral values at all coupling sites. Specifically, we suggest that since the number of $H^+$ re-entering the mitochondria during synthesis of 1 ATP is most probably 3 ([2,9–13] but see [19]) (2 via the ATPase and 1 by the combined action of the phosphate and adenine nucleotide carriers [13,29]) then the P/O ratio for NAD-linked substrates is 8/3 = 2.67; for succinate it is 6/3 = 2.0; and for ascorbate (or

isoascorbate) it is 4/3 = 1.33. Literature values for P/O ratios do not appear to be inconsistent with these predictions (see, e.g., [30,31]).

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