Determination of the stoichiometry of redox-linked proton translocation from the kinetics of pulse experiments

A simulation study

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We have previously published a simple kinetic model to analyse possible pitfalls in kinetic measurements of H^+/O ratios in mitochondria [(1984) FEBS Lett. 178, 187–192]. While this model demonstrated how relative electrode response times may affect the results, it did not adequately describe the kinetics of proton back-diffusion across the membrane. Here this model is further developed and improved, and shown to give a good quantitative description of both oxygen-pulse type experiments as well as of experiments where the reaction is started by photolysis of the cytochrome *c* oxidase-CO complex. Simulations based on this model reveal that the extrapolation procedure used by Lehninger et al. [e.g. (1984) J. Biol. Chem. 259, 4802–4811] to estimate the H^+/O ratio will tend to yield overestimated values. This is mainly due to the back-diffusion of protons into the mitochondria, which is not correctly accounted for by this extrapolation.

Electron transport *Proton transport* *Mitochondria*

1. INTRODUCTION

To improve upon the interpretation of the results of oxygen-pulse experiments for the determination of stoichiometries of respiration-linked proton translocation [1], Lehninger's group [2,3] has introduced the use of fast-responding oxygen and pH electrodes to measure the early kinetics occurring after a pulse of oxygen. Using this technique, Costa et al. [3] determined the stoichiometry of mitochondrial proton translocation coupled to the oxidation of succinate, and reported an H^+/O ratio approaching 8.

In order to obtain the stoichiometry at 'level flow' conditions (where the force on back-leakage of ejected protons is zero), these workers extrapolated the observed rates of oxygen consumption and net proton ejection to 'zero time', using Guggenheim plots of the electrode signals [2,3]. As a basis for this procedure the authors stated that "the decrease in O_2 concentration... was found to follow an apparent first order course very closely, as indicated by the linearity of the semilog plot of O_2 disappearance *versus* time, fitted by regression analysis". They further stated that "Since the zero time O_2 level computed from this plot (48.2 ng atoms) agreed with the amount of O_2 injected (48.2 ng atoms), O_2 uptake evidently proceeded in this experiment with a monotonic rate constant from the very beginning of the reaction" (p.4805 in [3]).

On the basis of the above, it is surprising that Lehninger et al. [4] stated in their response to our earlier criticism [5] that the O_2 concentration to which the plot (fig.5 in [3]) extrapolated at zero time was, in fact, not 48.2 ng atoms, but was only incorrectly labelled as such in the figure. Therefore, as also pointed out by Lehninger et al.

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in a different context [4], the precise zero time at which mixing is complete is not known. This obviously means that the basis for the point in time to which extrapolation was carried out by Costa et al. [3] was incorrect.

Lehninger et al. [4] correctly criticised our previous simple simulation model [5] in that it did not adequately describe the effect of proton back-decay across the membrane. As pointed out in [4] a correct model requires at least three exponential terms to describe quantitatively net H^+ ejection (composed of proton ejection, proton backflow and electrode kinetics). Interestingly enough, the Guggenheim plot analysis by Lehninger et al. [2–4] does not take this important point into consideration, but analyses the kinetics as if they were composed of one exponential only.

Here we will present a model that adequately describes both proton and oxygen kinetics. The use of this model clearly demonstrates that the extrapolation procedure of Lehninger et al. [2–4] leads to overestimated H^+/O ratios. This is not due to the neglect of electrode kinetics as thought previously [5], but to the neglect of the effect of proton back-decay across the membrane. Therefore, the test of the extrapolation technique using known scalar reactions [2–4] does not validate this method of measuring vectorial events. Our model also shows that mixing effects are of little significance unless mixing becomes very slow. The model may be of more general use in future descriptions and analysis of redox-linked proton translocation.

2. THE SIMULATION MODEL

2.1. *The oxygen-pulse experiment*

The experimental principle is that first described by Mitchell and Moyle [1], where a suspension of anaerobic mitochondria is pulsed with a small amount of O_2 under appropriate conditions for measuring proton translocation. It differs from the original method in that both O_2 concentration and pH are monitored with rapidly responding electrodes allowing relatively early measurement of the kinetics (see [2–5]), rather than a measurement of the total amount of H^+ ejected as in [1].

The amounts of oxygen (x) and protons (y) are followed with an oxygen electrode (signal: s) and a pH electrode (signal: p), respectively, after a pulse of oxygen (size: x_0). We assume (see section 4) first-order kinetics of oxygen uptake from the beginning; and as a first approximation the mixing of oxygen is also described by a first-order process. The reaction may also be initiated by flash photolysis of CO bound to cytochrome *c* oxidase in the dark and presence of O_2, without a mixing problem [4]. Also this may be simulated as shown below.

2.2. *Set-up of the simulations*

Processes that occur after the pulse are:

(i) First-order mixing with rate constant k_m. When 'unmixed' oxygen is x^*,

$$dx^*/dt = -k_m x^* \tag{1}$$

and

$$(dx/dt)_{\text{mixing}} = -dx^*dt = k_m x^* \tag{2}$$

with the boundary conditions that $x^*(t=0) = x_0$ and $x(t=0) = 0$. These boundary conditions make it an oxygen-pulse experiment. Alternatively, an experiment where the reaction is initiated by flash photolysis of CO [4] may be simulated with the boundary conditions $x^*(t=0) = 0$ and $x(t=0) = x_0$.

(ii) First-order uptake of oxygen with rate constant k:

$$(dx/dt)_{\text{uptake}} = -kx \tag{3}$$

Linked to this there is proton ejection with a stoichiometric number n:

$$(dy/dt)_{\text{ejection}} = -n(dx/dt)_{\text{uptake}} = nkx \tag{4}$$

with the boundary condition that $y(t=0) = 0$.

(iii) First-order proton diffusion back into the mitochondria, with a rate constant k_d:

$$(dy/dt)_{\text{diffusion}} = -k_d y \tag{5}$$

(iv) The measurement of x and y with electrodes that have first-order responses with rate constants k_s and k_p, respectively:

$$ds/dt = k_s(x - s) \tag{6a}$$

and

$$dp/dt = k_p(y - p) \tag{6b}$$

with the boundary conditions that $s(t=0) = 0$ and

$p(t=0) = 0$. For experiments started by photodissociation of CO, $s(t=0) = x_0$. Note that x, y, s and p are expressed in the same electrode-derived units.

This yields the following set of differential equations:

$$
\begin{aligned}
dx^*/dt &= -k_m x^* && (7a)\\
dx/dt &= k_m x^* - kx && (7b)\\
dy/dt &= nkx - k_d y && (7c)\\
ds/dt &= k_s x - k_s s && (7d)\\
dp/dt &= k_p y - k_p p && (7e)
\end{aligned}
$$

The solutions to these equations (with the boundary conditions for the oxygen pulse experiment) are:

$$
\begin{aligned}
x^* &= x_0[\exp\{-k_m t\}] && (8a)\\
x &= x_0 L_1[\exp\{-k_m t\} - \exp\{-kt\}] && (8b)\\
y &= nx_0 L_1[L_4\exp\{-k_m t\} - L_5\exp\{-kt\} + (L_5 - L_4)\exp\{-k_d t\}] && (8c)\\
s &= x_0 L_1[L_2\exp\{-k_m t\} - L_3\exp\{-kt\} + \\
&\quad + (L_3 - L_2)\exp\{-k_s t\}] && (8d)\\
p &= nx_0 L_1[L_4 L_6\exp\{-k_m t\} - L_5 L_7\exp\{-kt\} + (L_5 - L_4)L_8\exp\{-k_d t\} + \\
&\quad + (L_5 L_7 - L_4 L_6 - (L_5 - L_4)L_8)\exp\{-k_p t\}] && (8e)
\end{aligned}
$$

wherein $L_1 = k_m/(k - k_m)$, $L_2 = k_s/(k_s - k_m)$, $L_3 = k_s/(k_s - k)$, $L_4 = k/(k_d - k_m)$, $L_5 = k/(k_d - k)$, $L_6 = k_p/(k_p - k_m)$, $L_7 = k_p/(k_p - k)$ and $L_8 = k_p/(k_p - k_d)$.

2.3. *The Guggenheim plot*

The electrode signals s and p can be plotted according to Guggenheim [6] by applying the transformations:

$$G(s) = \{s(t) - s(t + \Delta t)\}/\Delta t \quad (9)$$

and

$$G(p) = \{p(t) - p(t + \Delta t)\}/\Delta t \quad (10)$$

where Δt is the sampling time used in this method of velocity approximation.

The signs of $G(s)$ and $G(p)$ are such that in the phase of the experiment where s is decreasing (oxygen uptake) and p is increasing (proton ejection) $\ln\{G(s)\}$ and $\ln\{-G(p)\}$ can be calculated.

This procedure introduces a factor $(1 - \exp\{-k_i \Delta t\})/\Delta t$ in front of each exponential $\exp\{-k_i t\}$ in the equations given above.

3. RESULTS

3.1. *Simulation of an oxygen-pulse experiment*

Fig.1 shows the results of applying the simulation model with a parameter set appropriate to simulate the results of Costa et al. [3]. The values used for k_s and k_p were calculated from [4], and the value for k_m was estimated from a dead-time of 0.1 s [4]. k_d was 0.063 s^{-1} based on the $t_{1/2}$ for proton decay of 11 s reported in [3].

The shape of the pH-electrode trace (fig.1A) and the Guggenheim plot generated from it (fig.1B) differ from the results from our previous more simplified model [5], which did not adequately describe proton back-diffusion. The Guggenheim plot shows a large downward deviation from a straight line, which is specifically due to the H^+ back-diffusion term in the simulation equation.

The simulated data are plotted with $n = 1$ and $x_0 = 1$. Therefore, n is determined correctly from extrapolations to the ordinate when these extrapolations coincide for oxygen ($\ln\{G(s)\}$) and pH ($\ln\{-G(p)\}$). However, it is immediately clear from fig.1 that this is not the case. The nonlinearity inherent in the Guggenheim plot of the pH-electrode signal makes it incorrect to carry out such an extrapolation. But if this is done nevertheless (assuming as in [2–4] that this plot is linear), it is clear that n will be overestimated. The extent of such an overestimation is hard to assess because it depends on the extent of time over which the pH data are used, and on the proton back-diffusion constant k_d. Our simulations have shown, however, that with the parameters and reaction span covered by Costa et al. [3] the error could easily have led to an apparent enhancement of a true H^+/O ratio of 6 to one approaching 8.

3.2. *The effect of back-diffusion of protons*

Fig.2A demonstrates how proton back-diffusion

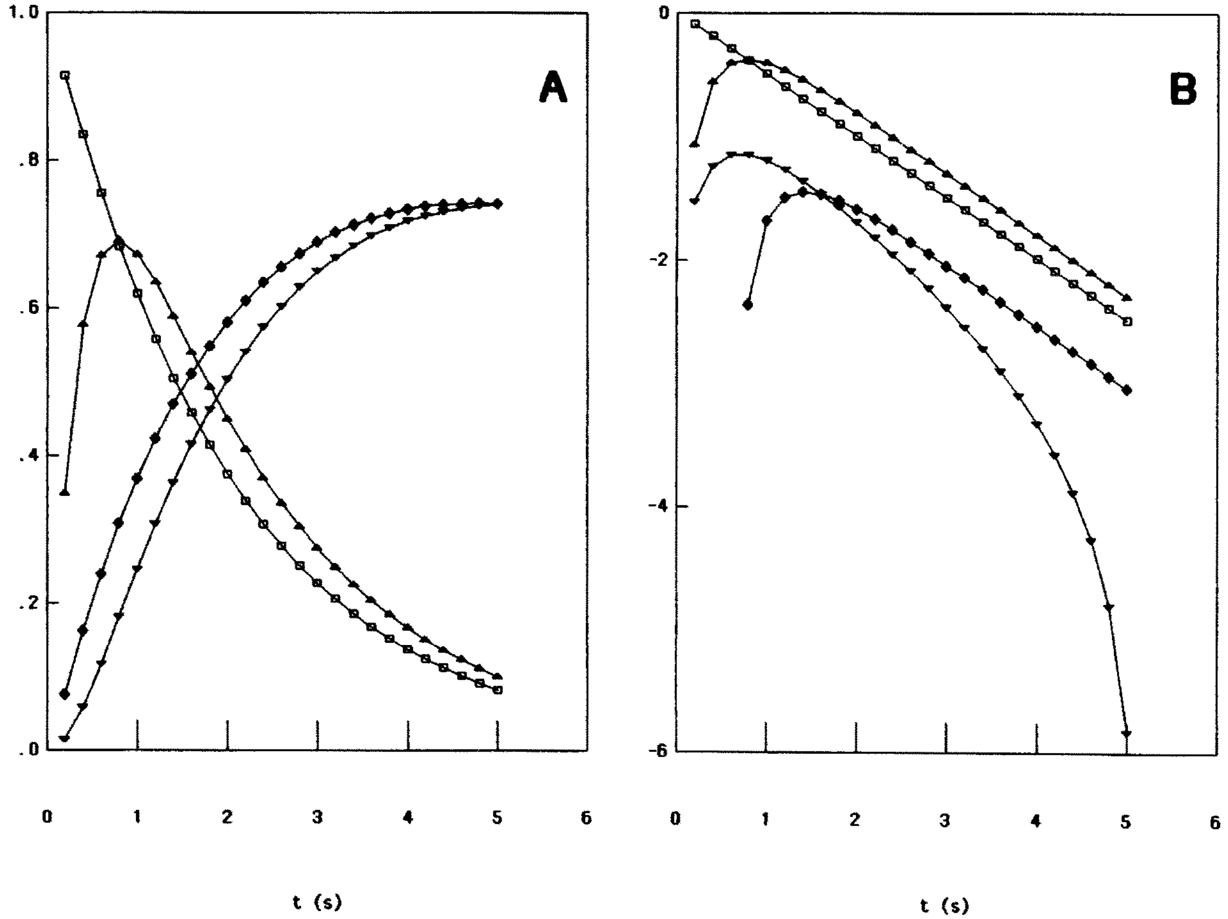

Fig.1. Simulation of an oxygen-pulse experiment. Conditions have been chosen to resemble those of fig.5 of Costa et al. [3]. $k = 0.5\ s^{-1}$, $k_m = 25\ s^{-1}$, $k_s = 2.88\ s^{-1}$, $k_d = 0.063\ s^{-1}$, $k_p = 2.56\ s^{-1}$ and $\Delta t = 0.2$ s. For convenience of plotting, $n = 1$ and $x_0 = 1$. Calculations were carried out on an Apple LISA 2/10, connected to an Apple Imagewriter. (A) Change of x (□), s (▲), y (◆) and p (▼) with time. (B) Change of $\ln\{x\}$ (□), $\ln\{s\}$ (▲), $\ln\{G(s)\}$ (◆) and $\ln\{-G(p)\}$ (▼) with time. For further explanation, see text.

affects the Guggenheim plot of the pH recordings, making it more nonlinear the higher the diffusion constant. Fig.2B shows comparable simulations of experiments where the reaction was started with CO photolysis rather than by O_2 addition (cf. [4]). The differential equations are solved for the different set of boundary conditions (solutions not shown). For the calculation, parameters applicable to fig.1 of [4] were used. The result is similar to that of figs 1 and 2A, showing the deviation of the plot from linearity due to proton back-diffusion, and leading inevitably to an overestimate of n when linear extrapolation is attempted.

3.3. *The effect of mixing*

Fig.3 shows the effect of mixing, as described in our model, on the Guggenheim plots of the electrode signals. Only relatively slow mixing events (e.g. $k_m = 2.5\ s^{-1}$, corresponding to a dead time of 1 s) have a significant effect on the curves.

4. DISCUSSION

The extrapolation of pH and oxygen traces to level-flow conditions is a way to account for proton back-diffusion. Our simulations show that Lehninger and co-workers [2–4] have done this in

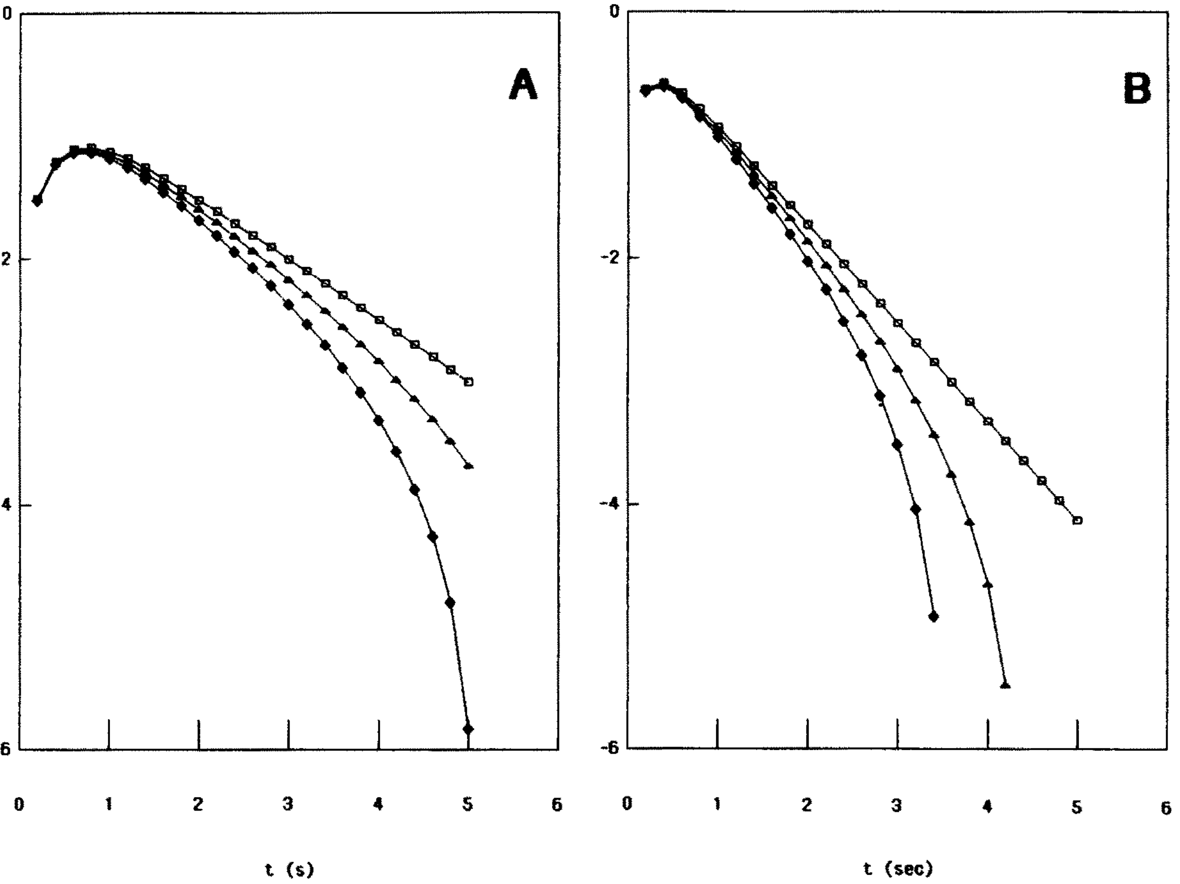

Fig.2. The effect of proton back-diffusion on $\ln\{-G(p)\}$. (A) Oxygen-pulse experiment. The set of constants was as in fig.1, but with $k_d = 0$ (□), 0.03 (▲), or 0.06 s^{-1} (◆). (B) Experiment started with CO photolysis. Conditions have been chosen to resemble those of fig.1 of Lehninger et al. [4]. $k = 0.8\ s^{-1}$, $k_s = 5\ s^{-1}$, $k_p = 5\ s^{-1}$ and $\Delta t = 0.2$ s. For convenience of plotting, $n = 1$ and $x_0 = 1$, $k_d = 0$ (□), 0.03 (▲) or 0.063 s^{-1} (◆).

an inadequate way by assuming linearity of the Guggenheim plots of the pH-electrode excursions. As shown by our model, it is precisely the back-diffusion of protons that makes these plots nonlinear. A correct account of the back-diffusion of protons requires more complicated curve-fitting procedures than a (linear) extrapolation of a Guggenheim plot. If such extrapolations are applied they will inevitably lead to an overestimation of the H^+/O ratio.

Our previous suggestion [5] that the H^+/O ratios of Costa et al. [3] were overestimated due to inaccurate accounting for the electrode response is probably incorrect. As pointed out by Lehninger et al. [4], the strongest evidence for a sufficient electrode response is the finding that a similar Guggenheim plot analysis of scalar reactions involving oxygen consumption and pH changes gave the theoretically expected result [3,4]. It is important to note that the error due to inadequate accounting for proton back-diffusion, identified in this paper, would obviously not show up when a scalar reaction is measured.

Precise knowledge of early events after an oxygen pulse (such as mixing and initially different kinetics of oxygen uptake) was stressed by Lehninger et al. [4] as necessary in order to carry out a proper simulation. This is not so because these factors only influence the extrapolated initial amount of oxygen (x_0). Since x_0 appears in the extrapolations of the Guggenheim plots of both oxygen and pH, errors in x_0 do not affect the resulting H^+/O

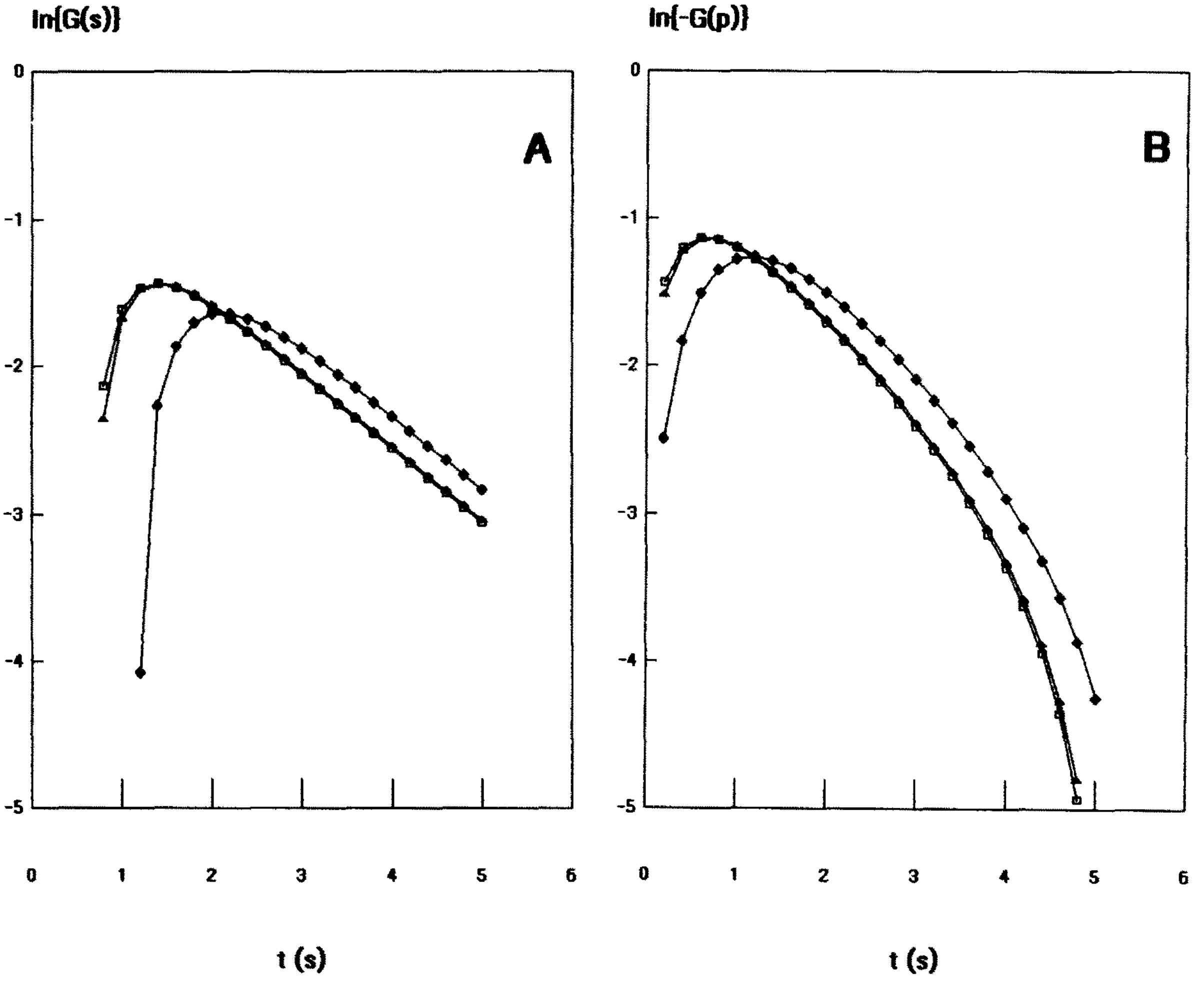

Fig.3. The effect of mixing. The set of constants was as in fig.1, but with k_m = 250 (□), 25 (▲) or 2.5 s^{-1} (◆). (A) ln{$G(s)$}, (B) ln{ $-G(p)$}.

ratio. Note also that in the treatment of the data by Lehninger and co-workers as well as in our simulation model early data points are either discarded [3,4] or do not affect determination of the asymptote intercepts ([5] and this paper).

The simulations in fig.3 further show that when mixing is accounted for by a first-order process (for mathematical convenience rather than for theoretical reasons), intercepts do not change significantly when mixing is relatively fast, and comparable to that in the actual experiments. Only very slow mixing adds a further error in the determination of *n*.

5. CONCLUSIONS

Determination of the 'level flow' H^+/O ratio of proton ejection in a pulse-type experiment in which oxygen consumption and proton ejection are monitored with relatively fast-responding electrodes has a number of pitfalls. Firstly, in analysis of the data the electrode characteristics and the effects of the Guggenheim transformation (when used) have to be taken explicitly into account [5]. In principle, this may be done by an extrapolation procedure. Secondly, as shown here, the back-decay of the ejected protons into the mitochondria has a strong effect on the shape of the Guggenheim plots of the pH data, making them nonlinear. Accounting for this effect requires more involved curve-fitting; a simple linear extrapolation procedure is inadequate and leads to significant overestimation of the H^+/O ratio.

As a consequence of this, the results of Lehninger et al. [2–4] overestimate the stoichiometric number *n*, and do not disagree with ratios of 6 and 2, respectively, for proton ejection per two electrons transferred from succinate or from cytochrome *c* to oxygen (see [7,8]).

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