Commentary

The H^+/O ratio of proton translocation linked to the oxidation of succinate by mitochondria

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In a recent communication Lehninger and co-workers (Costa, L.E., Reynaferje, B., and Lehninger, A.L. (1984) J. Biol. Chem. 259, 4802–4811) reported values approaching 8 for the H^+/O ratio of vectorial proton ejection from rat liver mitochondria respiring with succinate. Here we present a rigorous analysis of these measurements which reveals that they may significantly overestimate the true H^+/O stoicheiometry.

1. INTRODUCTION

The H^+/O stoicheiometry of proton translocation coupled to mitochondrial respiration is central for the understanding of both the thermodynamics and the mechanism of oxidative phosphorylation. Unfortunately, there has been a long-standing controversy in this area. Thus, for the oxidation of succinate (or ubiquinol) by O_2 it has been proposed in different laboratories that 4, 6 and 8 protons are transported across the inner mitochondrial membrane per pair of transferred electrons (for reviews, see [1–4]). As it is fully agreed that the $H^+/2e^-$ ratio of proton ejection is 4 for the span between succinate (or ubiquinol) and ferricytochrome *c* [1–6], the disagreement actually centers on the H^+/O ratio for oxidation of ferrocytochrome *c* by O_2, i.e., the function of cytochrome *c* oxidase. The different research groups referred to above suggest, accordingly, that cytochrome oxidase does not transport protons [7,8], or that either 2 [1–4,9,10], or 4 [11,12] H^+/O are ejected vectorially during cytochrome oxidase activity.

Recently, Lehninger et al. [13] published what they call the 'second generation' of measurements of the stoicheiometry of proton translocation linked to the oxidation of succinate by O_2 in rat liver mitochondria. It was reported that the data yielded H^+/O flow ratios above 7 and closely approaching 8, after extrapolation of the flows to zero time where no protonmotive force opposes proton transport (level flow conditions). They also reported that the H^+/O ratio had declined to about 6.0 at 3 s or earlier after the O_2 addition. It is therefore somewhat surprising that Lehninger et al. have reported similar very high H^+/O ratios in earlier accounts based on linear H^+ and O_2 excursions over much longer periods of time (see, e.g., [14,15]). In the second generation measurements of [13], both H^+ ejection and O_2 consumption were found to be highly non-linear with time from the earliest measurable point (about 0.8–0.9 s) onwards.

Here it will be shown that the conclusion in [13] with regard to the H^+/O ratio is due, in part, to an underestimation of the effect of the time constant of the oxygen-measuring system, relative to

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that of the pH-measuring system, and in part to the method of extrapolating the fluxes to zero time.

2. EXPERIMENTAL PROCEDURES

In the experiments of Lehninger et al. [13] known amounts of O_2 were injected at $t = 0$ to suspensions of rat liver mitochondria under appropriate conditions for measuring vectorial proton ejection coupled to oxidation of added succinate. The measuring chamber was equipped with O_2 and pH electrodes, the response times of which were reported to be matched. Although not reported in [13], the 90% response times of the electrodes may be judged to be within 0.5–1 s. Rates of H^+ ejection and O_2 consumption were extrapolated to $t = 0$, based on the assumption of first-order kinetics, and using Guggenheim plots (see [16]), i.e., essentially plotting the logarithm of rate *vs* time. The assumption of first-order kinetics of oxygen consumption was based on the finding that a semilogarithmic plot of oxygen concentration *vs* time extrapolated to the concentration of oxygen added at $t = 0$ [13]. However, it is noteworthy here that in [13] the rates of oxygen consumption at $t = 0$ were nevertheless obtained from extrapolated Guggenheim plots rather than from the semilogarithmic plots of oxygen *vs* time. Division of the (extrapolated) rates at $t = 0$ of H^+ ejection and oxygen consumption yielded the reported H^+/O ratios at level flow conditions. Extrapolation to $t = 0$ is motivated by the fact that only at level flow (where no protonmotive force opposes transport of H^+) will the flow ratio approach the stoicheiometry of proton translocation.

3. SIMULATIONS

3.1. *Oxygen flux*

When O_2 is injected into an anaerobic suspension of mitochondria supplemented with substrate, the resulting oxygen electrode response will be governed by the response characteristics of the oxygen-measuring system as well as by the oxygen consumption by the mitochondria. It is assumed that the electrode signal S (converted to units of oxygen concentration) reaches the true oxygen concentration C, after a step change in the latter, by a first-order process that is characterised by the time constant k_e of the oxygen-measuring system. Then S will be related to C by

$$dS/dt = k_e(C - S) \tag{1}$$

Consumption of oxygen by a first-order reaction (see [13] and above), after a step change at $t = 0$ from $C = 0$ to $C = C_o$ (where C_o is the concentration of added oxygen), with a rate constant k will be governed by

$$dC/dt = -kC \tag{2}$$

which yields after integration

$$C = C_o e^{-kt} \tag{3}$$

Combination of eqns 1 and 3 yields

$$dS/dt = k_e C_o e^{-kt} - k_e S \tag{4}$$

The differential equation, eqn 4, can be solved to yield

$$S = \frac{k_e C_o}{(k_e - k)} \cdot (e^{-kt} - e^{-k_e t}) \tag{5}$$

for the case where $k \neq k_e$.

Fig.1A shows a simulated oxygen electrode trace using eqn 5 in conditions comparable to those described in section 2, with a 90% response time of 0.6 s ($k_e = 3.84\ s^{-1}$) for the electrode and $k = 0.5\ s^{-1}$ (cf. below). Fig.1B shows the corresponding semilogarithmic plot of oxygen *vs* time. From the latter a deviation from the 'true' first-order reaction is apparent at times before approx. 1.5 s. This underestimation of the rate of O_2 consumption due to the electrode response is borne out more clearly in the Guggenheim plot of the same function (time interval 0.2 s, as in [13]), given in Fig.1C. This plot also clearly reveals the relative slowness of the electrode, the signal of which becomes confluent with the true reaction rate only at times later than approx. 2.5 s.

From this analysis it is clear that even though the 90% response time of the oxygen-measuring system is 0.6 s, it will nevertheless not measure rates of oxygen consumption with 90% accuracy until much later in time. This is obviously due to the fact that the oxygen-measuring system must first respond to

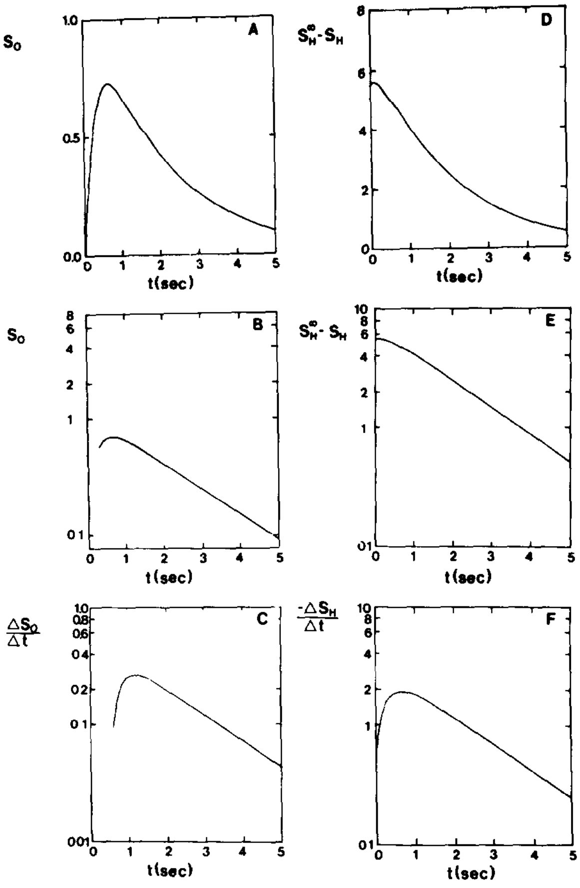

Fig.1. Simulations of oxygen electrode (A–C) and pH electrode (D–F) excursions during an oxygen pulse experiment. The traces were simulated with the parameters $k = 0.5\ s^{-1}$ (oxygen consumption), $k' = 0.53\ s^{-1}$ (proton ejection) ($k_d = 0.03\ s^{-1}$), $k_{eO} = 3.84\ s^{-1}$ and $k_{eH} = 2.5\ s^{-1}$. Electrode excursions are given relative to the total amount of oxygen atoms injected, and with an assumed H^+/O ratio of 6.0. Shown are linear (A,D), logarithmic (B,E) and (logarithmic) Guggenheim (C,F) plots. The sampling time in the latter was taken to be 0.2 s. S_O and S_H refer to the signals of the oxygen and pH electrodes, respectively. S_H^∞ is the pH signal at infinite time. The simulations were carried out using the equations given in the text and a Sinclair Spectrum microcomputer interfaced with a Servogor 210 strip chart Y-t recorder.

the large increase in O_2 concentration before relaxing towards the kinetics of oxygen consumption.

3.2. *Proton flux*

Simulation of the proton ejection can be done by analogy to the case of oxygen above. Thus if k_e and k are the rate constants of the electrode and the H^+ ejection, respectively, and C^∞ is the concentration of ejected protons at infinite time, then the electrode signal S is

$$S = \frac{C^\infty}{(k_e - k)} \cdot (k_e e^{-kt} - k e^{-k_e t}) \qquad (6)$$

This equation neglects the back-flux of protons across the membrane, driven by the ΔpH continuously generated during the reaction. However, the proton leak can be accounted for as well by substituting k' for k and C'^∞ for C^∞ in eqn 6, where

$$k' = k + k_d \qquad (7)$$

and

$$C'^\infty = C^\infty k/(k + k_d) \qquad (8)$$

in which k_d is the rate constant for the proton leak.

In contrast to the above apparent discrepancy between the response time of the oxygen electrode and the time taken to measure oxygen consumption accurately (fig.1A–C), no such discrepancy occurs in the measurements of H^+ ejection. Obviously, this is because the latter start with H^+ (ejected) = 0 at $t = 0$. As shown by the corresponding simulations of H^+ ejection in fig.1D–F (plotted as the difference from the final level, C'^∞, with $k_e = 2.5\ s^{-1}$ and $k' = 0.53\ s^{-1}$), the pH electrode will approach the true reaction at a much earlier point in time than the O_2 electrode, even though the rate constant of the former has been chosen to be somewhat slower than that of the latter.

This kinetic discrepancy between the two electrode measurements is borne out most clearly in fig.2, which shows the simulated H^+/O ratio *vs* time taken from the individual curves of fig.1F and C. Fig.2 shows that, perhaps surprisingly, there is a significant overestimation of the H^+/O flow ratio not only at times before about 2.5 s, but at all times.

To evaluate the reasons for the overestimation at times >2.5 s we note that the equation simulating the H^+/O flow ratio by the Guggenheim method, derived from eqns 5 and 6, reduces to the following expression when time approaches infinity

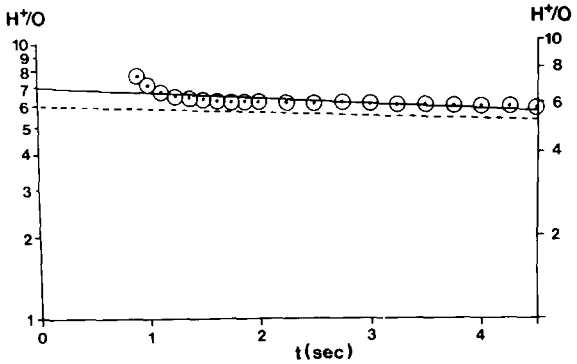

Fig.2. Logarithmic plot of the rate of H^+ ejection divided by the rate of oxygen uptake (rates as obtained from Guggenheim plots, see figs.1C and F). The encircled points were obtained by simulation using the parameters of fig.1 for the time period between 0.9 and 4.5 s, and assuming a true H^+/O flow ratio of 6.0. The dashed line was obtained in the same way, but assuming very fast electrode responses (k_e for both electrodes = $1000\,s^{-1}$). The full line was obtained by linear regression analysis of the circled points, yielding after extrapolation an H^+/O ratio of 6.98 at $t = 0$, and a correlation coefficient of 0.81. The scaling of ordinate and abscissa is similar to that in [13] to facilitate comparison.

$$(H^+/O)_{app} = NABe^{-k_d t} \tag{9}$$

where N is the correct H^+/O ratio, and

$$A = \frac{k_{eH}k(k_{eO} - k)}{k_{eO}k'(k_{eH} - k')} \tag{10}$$

in which k_{eH} and k_{eO} refer to the pH and oxygen electrodes, respectively, k and k' are the rate constants of oxygen consumption and proton ejection (see above), and

$$B = \frac{1 - e2^{-k'\Delta t}}{1 - e^{-k\Delta t}} \tag{11}$$

The factor B is due to the Guggenheim method of rate approximation, Δt being the time interval (0.2 s in [13]), and drops out when Δt approaches zero.

Looking now for the factor by which this apparent H^+/O ratio is erroneous we make the electrodes infinitely fast-responding, from which we obtain

$$(H^+/O)_{app} = N\frac{k}{k'}Be^{-k_d t} \tag{12}$$

The error factor (F) at infinite time due to electrode slowness is then eqn 9 divided by eqn 12, which yields

$$F = k_{eH}/k_{eO} \cdot (k_{eO} - k)/(k_{eH} - k') \tag{13}$$

With the rate constants used in our simulations $F = 1.104$, which is a considerable overestimation. Eqn 13 shows, however, that F is very sensitive to even small changes in the rate constants of the oxygen and pH electrodes. It is unity when they are kinetically perfectly matched ($k_{eH}/k' = k_{eO}/k$), and less than unity when the pH electrode is relatively faster than the oxygen electrode ($k_{eH}/k' > k_{eO}/k$). However, from [13] we have the impression that the oxygen electrode was indeed slightly faster than the pH electrode, though no mention is given of actual electrode response times. Note, incidentally, that if the pH electrode were faster than the oxygen electrode the error at times before 3 s would be enhanced.

From this section we may now conclude, and referring to fig.2, that there are two reasons for the overestimation of the flow ratio borne out by these simulations. Unless both electrodes are very fast-responding and/or have identical relative response times, the ratio is overestimated at all times when the oxygen electrode is faster than the pH electrode. Secondly, even if the electrode responses are exactly matched there will be an overestimation at times before about 2–3 s due to the different nature of the oxygen and pH transients (see above). In this connection it is of interest to note that it was reported in [13] that the directly measured H^+/O flow ratio already fell to 6.5 at 2 s and to 6.0 at 3 s (figs.4A and 5 of [13]). In this experiment the ratio was reported to be 7.25 after extrapolation to $t = 0$. However, also the *extrapolated* flow ratio in [13] is subject to error, which is in part related to the above analysis (see below).

4. DATA ANALYSIS

Fig.3 shows data points derived from the experiment of fig.4A of [13]. The semilogarithmic plot of oxygen *vs* time apparently extrapolates to the point of added oxygen at $t = 0$ (cf. [13]). From the slope we obtain a first-order rate constant of $0.503\,s^{-1}$. From this, and the amount of added oxygen (48.2 nmol O; see [13]) an initial rate of

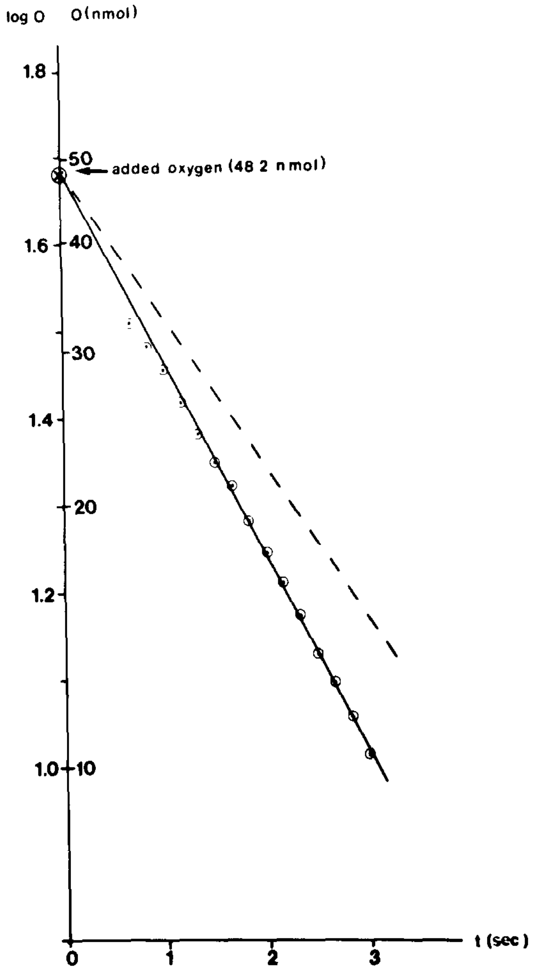

Fig.3. A plot of the logarithm of oxygen *vs* time from the data of fig.4A in [13]. A straight line was drawn through the experimental points and the point of oxygen added at $t = 0$. The slope yields an apparent first-order rate constant of $0.503\ s^{-1}$, from which an initial rate of oxygen consumption of 24.2 nmol O/s is obtained. The slope of the dashed line was obtained by using the initial rate given in [13] (18.9 nmol O/s) and the amount of oxygen added (48.2 nmol), and was drawn through the point of added oxygen on the ordinate.

24.2 nmol O/s is obtained. At first sight it is curious that this initial rate disagrees with that of 18.9 nmol O/s reported by Lehninger et al. [13] for this experiment. However, the latter authors obtained their value, not from the semilogarithmic plot of oxygen *vs* time, but from a Guggenheim plot extrapolated linearly to $t = 0$. This procedure introduces much scatter of the experimental points (see [13]). Hence, it is less reliable and in fact not even theoretically preferable over the conventional semilogarithmic plot of oxygen *vs* time, since the concentration of oxygen at infinite time is known (zero; cf. [14]). But the major factor that contributes to the discrepancy is the use in [13] of the data of the Guggenheim plot between about 0.9 and 4.5 s (see fig.5 in [13]) as the basis for *linear* regression and extrapolation to $t = 0$. As shown in fig.2, such data should not fall on a straight line in the Guggenheim plot when the 90% electrode response times exceed about 0.5 s. While the curvatures in this plot were not obvious in [13], probably due to scatter of the data points, a linear regression and extrapolation to $t = 0$ in the mentioned time interval is bound to yield an overestimated flow ratio. This conclusion is supported by the fact that fig.2 simulates the experiment of fig.5 of [13] rather well. The solid line (fig.2) is the result of forcing the 'data' points to a straight line by linear regression. This line yields an apparent H^+/O ratio of 6.98 at $t = 0$ (correlation coefficient −0.81), which may be compared with the value of 7.25 given in [13]. Yet, no effort was made here to optimise the simulation with respect to the data in [13]. Hence, the results in [13] are in accordance with the proposal that the true H^+/O ratio is near 6.0 for the oxidation of succinate (or ubiquinol) by O_2 in mitochondria.

5. CONCLUSIONS

From simulations of pH and oxygen electrode excursions following initiation of electron flow and proton translocation in mitochondria by injection of O_2 into an anaerobic suspension, the following may be concluded:

(i) Due to the different forms of the oxygen and pH transients the oxygen electrode signal approaches the true reaction at much later times than does the pH electrode, at equal electrode response times. When the electrode response times for 90% excursion are of the order of 0.5–1 s and the apparent first-order rate constant of the biochemical reaction about $0.5\ s^{-1}$, the measured H^+/O flow ratio is significantly overestimated at reaction times below about 3 s.

(ii) The phenomenon in (i) not only leads to an overestimation of directly measured flow ratios at early times, but also causes an error in extrapolations of flow ratios between 1 and 4.5 s to $t = 0$, as done in [13]. This error leads to an overestimated (extrapolated) flow ratio.

(iii) Unless the oxygen and pH electrodes are exactly matched with respect to their response times relative to the kinetic parameters of the biochemical reactions, and the former responds relatively even slightly faster than the latter, H^+/O ratios will be overestimated also much beyond the above approximate limit of 3 s.

(iv) Initial rates of oxygen consumption calculated from extrapolated plots of log oxygen concentration *vs* time and log oxygen rate *vs* time disagree for experiments in [13]. This is likely to be due to the errors of (i) and (ii) which become more apparent in the latter than in the former plot. Moreover, the conversion of original data to the latter plot introduces much scatter of the data points which further obscures the extrapolation procedure.

When the above is taken into account it seems very probable that the data in [13] conform to the observations by others [1–4,17–25] that the true H^+/O ratio is near 6.0 for the oxidation of succinate (or ubiquinol) by O_2. This is supported by the finding that the data in [13] can be simulated with a true H^+/O ratio of 6.0 and reasonable values for electrode and reaction rate constants.

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