

Problems in the Experimental Determination of Substrate-Specific H^+ /O Ratios During Respiration

Richard W. Hendler^{1,3} and Richard I. Shrager²

Received December 5, 1986; revised April 9, 1987

Abstract

Krab *et al.* (1984) have recently tried to resolve the long-standing controversy as to whether the mechanistic H^+ /O coupling ratio for electrons passing through sites II and III of the mammalian electron transport chain to O_2 is 6 or 8. Using a mathematical model they concluded that the higher number reported by Costa *et al.* (1984) was an overestimate because of the unaccounted for delayed response of the O_2 electrode. Responding to criticisms of Lehninger *et al.* (1985), they have recently used (Krab and Wikström, 1986) an improved mathematical model which shows that the higher number found by Costa *et al.* was probably due to an inadequate accounting for the effects of the proton leak process which accompanies the translocation process. The impression is left that the situation is now resolved in favor of the lower number. We agree that the procedures of Costa *et al.* do not properly account for the leak process, and provide further evidence in this paper of the magnitude of the problem. However, we disagree that the number 6.0, favored by Wikström *et al.*, rests on any more solid experimental support. We provide evidence here for this conclusion and raise the question as to whether or not any unique, fixed, integral number exists for the H^+ /O ratio accompanying the oxidation of a particular substrate.

Key Words: H^+ /O ratios; H^+ / e ratios; energy transduction; proton pumping; proton leaks.

Introduction

Since the introduction by Mitchell (1966) of the concept of proton translocating loops as a vehicle for moving protons across the membrane of a

¹Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892.

²Laboratory of Applied Studies, Division of Computer Research and Technology, National Institutes of Health, Bethesda, Maryland.

³To whom correspondence should be addressed.

respiring vesicle, a lively debate has ensued as to the precise number of protons translocated for each electron passing through each energy transducing site of the electron transport chain. For electrons originating in succinate and passing through sites II and III enroute to O_2 , different groups of investigators have strongly defended $H^+/2e$ ratios of 4, 6, or 8 (see Wikström and Saraste, 1984, for references). All of the groups have agreed that for electrons passing through site II, the $H^+/2e$ ratio is 4. Therefore, the controversy has been centered on site III, where the various groups have maintained that the $H^+/2e$ site ratio was 0, 2, or 4. Last year the major laboratory advocating 0 as the ratio for site III reassessed its position and now accepts 2 as the correct answer (West *et al.*, 1986). This has effectively narrowed the controversy to two contending groups, one maintaining that the H^+/O ratio for electrons passing through sites II and III is 6, and the other that it is 8.

In order finally to resolve the conflict, which has persisted for nearly twenty years, Krab *et al.* (1984) published a critique on the experimental procedures of Lehninger's group, which has been the strongest advocate for the higher number. The major thrust of this critique was a mathematical simulation which appeared to show that the Lehninger laboratory overestimated the H^+/O ratio (Costa *et al.*, 1984) because they failed to allow for the relaxation time delay of the O_2 electrode as it recovered from the rise in $[O_2]$ due to the initial injection, before it could adequately respond to the decrease in $[O_2]$ due to O_2 consumption.

Lehninger *et al.* (1985) pointed out serious flaws in the mathematical model of Krab *et al.* (1984). Krab and Wikström (1986) responded to this paper and admitted that their mathematical model was inadequate. In their latest paper, they have adopted the more complex model that was suggested, which includes exponential terms for electrode response, proton ejection, and proton leak. In their simulations based on the improved model, Krab and Wikström concluded that the technique used by Lehninger and co-workers does not deal adequately with the proton back-leak phenomenon and that this could cause an overestimate of H^+/O ratios. We entirely agree with this newer position, but we do not agree with the implied conclusion that having cast doubt on the ratio of 8, the alternative value of 6 is now more firmly established. The present communication is intended to amplify the experimental and theoretical considerations which, in our opinion, render both the values 6 and 8 equally uncertain, and which question the very existence of such an implied fixed, integral, mechanistic number.

In this analysis we have restricted the discussion to the direct approach of measuring pH and O_2 changes during or following a brief phase of respiration. Indirect approaches, some based on nonequilibrium thermodynamics

or the measurement of cation changes instead of pH changes, are not free from the problems we discuss here and they introduce other problems uniquely related to their indirectness. An attempt to deal with the many new questions involved in these other studies, however, would produce a cumbersome paper that would obscure the basic problems inherent to the system that we wish to illustrate here.

Experiments of Mitchell *et al.*

In 1967, Mitchell and Moyle introduced an oxygen pulse technique for obtaining H⁺/O stoichiometric ratios, characteristic of the oxidation of particular substrates. This is one of the techniques used by Wikström and co-workers (Krab and Wikström, 1979; Wikström and Pentillä, 1982). A small aliquot of aerated water or salt solution is injected into an anaerobic suspension of mitochondria, containing substrate and other essential components. Data are collected from a pH electrode during the phases of net proton translocation and resorption of extruded protons after the O₂ has been consumed. The object is to use the pH relaxation data to determine the total number of moles of protons extruded before the leak-back process diminished the amount. This number divided by the number of moles of O₂ injected is taken as the H⁺/O ratio. The pH data collected, which are on a decreasing curve, are extrapolated back to some earlier time. The number obtained is dependent on the method of back-extrapolation and the precise time selected. Mitchell and Moyle fixed this point by using two quantities, N_{O_2} , the amount of injected oxygen per unit weight of mitochondria, and Q_{O_2} , which is defined as the rate of state 3 respiration per unit weight of mitochondria:

$$\delta t = \frac{1}{2} N_{O_2} / Q_{O_2}$$

There are many serious problems with this technique.

Extrapolation of the resorption phase of the medium acidification time course back to any earlier time to determine the total amount of H⁺ that was translocated assumes either that all of the H⁺ was released in an instant of time or that the release process was completed in a time span that was very small in relation to the time for resorption. Since the release of H⁺ depends on mixing and O₂ uptake, it is obvious that the release is not instantaneous. In the back-extrapolation of an exponential function, the possibilities for significant error increase as the function approaches its origin. Therefore, uncertainties as to what time point represents the "effective" origin for this extrapolation procedure could affect the results. The respiratory pulse is initiated by injecting 0.1 or 0.05 ml of aerated salt solution into 6 ml of stirred

mitochondrial suspension. In this procedure there is no single zero time. Rather, different populations of mitochondria are exposed to O_2 in sequence during mixing. Protons continue to be released as long as O_2 is available to receive electrons. No O_2 -electrode measurements are made during the course of the experiment, but a "state 3 respiration rate" is assumed and it was stated (Mitchell and Moyle, 1967) that 1/2 of the O_2 was probably consumed in 0.75 s. If O_2 uptake is in steady state 3 all during the pulse, 1.5 s are required for its total utilization. If, however, dO/dt decreased in an exponential manner, as is usually seen (Costa *et al.*, 1984; Setty *et al.*, 1986; Reynafarje *et al.*, 1982), then 6% of the injected amount would still be present in 3 s. These considerations indicate a degree of uncertainty as to the assignment of a particular time as the effective zero time. In order to be able to represent this finite period of time for H^+ translocation as single time point for the purpose of back-extrapolating an exponential relaxation process, it is required that the time constant τ , for the resorption process [i.e., $\exp(-t/\tau)$] be much larger than the τ for the acid release process and that no data be taken from the decay curve near the inflection point, because it could be contaminated with the influence of the acid release process. Another important consideration stressed by Mitchell and Moyle is that knowledge of the relaxation time of the pH electrode must be available so that no data will be used from the resorption phase until the electrode has had time to adjust to the turnaround from the initial acidification of the medium to its subsequent alkalization. When all of the conditions necessary for the procedure to work have been met, the method is justified *only* if the resorption phase is truly a one-exponential process. That this requirement is not fulfilled is shown in Fig. 2 of Mitchell and Moyle (1967). A semilog plot of the H^+ data scaled to the size of the injected O_2 should be linear. Such data for respiratory pulses with different concentrations of succinate serving as electron donor are seen to be nonlinear and give the appearance of being exponential-like in the semilog coordinate system. Although the figure shows the back-extrapolation of all of these lines passing through a common "zero time" point, it is not obvious that the back-extrapolations along the apparent exponential trends of each curve would result in a common point of origin.

The only way that the data shown in Fig. 2 of Mitchell and Moyle (1967) could support the technique recommended by Mitchell and Moyle would be if they produced a series of straight lines with different slopes which all converged at the effective zero time. Therefore, we do not accept that this procedure can produce reliable measures of H^+/O stoichiometries such that it could distinguish between possible values of 4, 6, or 8 for electrons passing through sites II and III or even between values of 2 and 4 for electrons passing through site III.

Experiments of Lehninger *et al.*

In 1982, Lehninger and colleagues introduced new procedures designed to remove uncertainties concerning the O₂ uptake process and to obtain direct proton translocation data rather than try to estimate some bulk amount of released protons by a back-extrapolation of the decay curve (Costa *et al.*, 1984; Reynafarje *et al.*, 1982). This new approach was also intended to deal with the leak process by extrapolating the initial phase of pH acidification data back to zero time where the leak should be negligible or nonexistent. A further improvement was the use of the fast-responding Davies-type O₂ electrode in place of the slower Teflon-covered electrode used in previous studies. With this electrode, O₂ consumption rates could be measured much more quickly after the dead time of about 0.8 s which includes mixing and the recovery of the O₂ electrode from the sensing of the increase in [O₂] following the initial injection. Data from the O₂ and pH electrodes were expressed as rate increments over 0.2-s intervals and were then fitted to single-exponential expressions which could be extrapolated back to zero time. The rationale was to obtain the ratio of the two rates (i.e., H⁺/O ratio) at zero time when the $\Delta\tilde{\mu}_{\text{H}^+}$ should be zero and the leakage negligible. The data from both electrodes appeared to be well represented by straight lines on semilog plots, and the zero time H⁺/O ratios obtained for succinate oxidation were above 7 and close to 8.

In the absence of respiratory control, O₂ consumption would be expected to proceed at a steady rate until the concentration fell within the range of the K_m . However, the rate of O₂ consumption decreased all through the pulse, indicating the influence of respiratory control (Costa *et al.*, 1984; Setty *et al.*, 1986; Reynafarje *et al.*, 1982). Although the disappearance of O₂ appears to be fitted by a single-exponential function in the range where data were collected (i.e., from 0.8 to ~5 s), this does not prove that the decay is truly one-exponential. A plot of the residuals (i.e., difference between the actual and fitted time course of the [O₂]) shows nonrandom deviations, and the extrapolated curve shows a marked departure from the actual data as the time decreases from 0.8 s to zero. To illustrate these points, data from Setty *et al.* (1986) are presented (Fig. 1). These observations bring the actual extrapolated zero-time value of the dO/dt into question. The most serious problem, however, is not with the O₂ data but rather with the pH data. If it is assumed that the treatment of the O₂ data is error-free and that the zero time dO/dt is correct, then for each atom of O consumed, n ions of H⁺ will be translocated. The pH curve will reflect the O₂ curve in its rate of decay (i.e., the relaxation constants will be equal), but the coefficient for the pH exponential will be n times that of the oxygen exponential. Such a single-exponential curve, when fitted to a one-exponential expression, will yield the

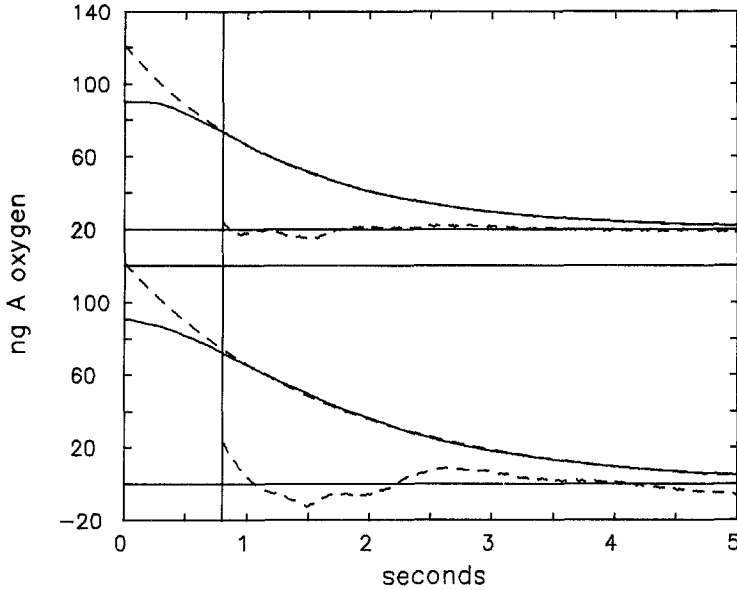


Fig. 1. The solid traces show standardized O_2 electrode signals obtained in two respiratory pulse experiments. Respiration was instantly initiated by photolysis of CO-inhibited cytochrome oxidase in a stirred suspension of rat liver mitochondria (18 mg protein/6 ml) containing 200 mM sucrose, 1.5 mM HEPES, 50 mM KCL, 2 μ M rotenone, 18 μ g oligomycin, 90 μ g NEM, 2.2 μ g valinomycin, 5 mM succinate, and 90 ng-atoms O under an atmosphere of 100% CO (Setty *et al.*, 1986). The O_2 electrode was a Davies fast-responding type (Costa *et al.*, 1984) with a relaxation constant of 0.2 s. The portion of the trace, in each case, from 0.8 to 5 s was fitted to the one-exponential function $A \cdot e^{-t/\tau} + A_{\text{baseline}}$ to produce the dashed theoretical line. The residuals $\times 10$ are shown for the fitted range in the bottom portion of each panel. These data were taken from experiments described in Setty *et al.* (1986).

same values for relaxation constant and coefficient regardless of the range of data used for fitting. Figure 2 shows pH data fit over four different time spans starting at 0.8 s and lasting to 1.5, 2, 3, and 4 s. The portion of the curve used for fitting is bracketed by vertical lines. It is apparent that the close fittings of the data in the specific range used are accompanied by wide deviations outside of the fitted range. Although the agreement between the experimental and fitted curves appears to be excellent in the regions used for fitting, the residuals show a tendency to deviate to the high side at early times and to the low side at later times in conformity with the obvious trends of the fitted curves shown in the figure. The extrapolated zero-time values of dH^+/dt are different in all cases. The relaxation constants, coefficients, and calculated zero time H^+/O ratios all increase with the increase in the fitted range (cf. Fig. 3). Over the range from 1.5 to 4 s shown in the figure, the average value was 8.0 ± 0.5 , but there was a steady trend from a value of 6.84 at the 1.5-s

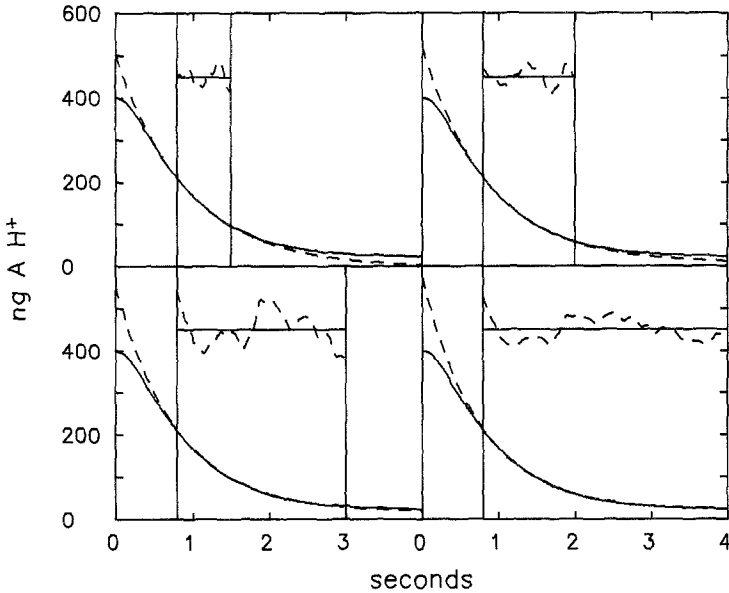


Fig. 2. The solid traces show the standardized pH electrode signals obtained for the experiment described in the upper panel of Fig. 1. A different portion of the data in the pH trace, as shown by the two vertical lines in each panel, was used for fitting to the one-exponential expression $A \cdot e^{-t/\tau} + A_{\text{baseline}}$ to produce the dashed theoretical line. The residuals $\times 100$ are shown for the fitted portion of the time course in the upper left panel, $\times 50$ for the fitted portions in the upper right and lower left panels, and $\times 25$ for the lower right panel. The ordinates have been shifted to show the magnitude of acidification of the external medium as obtained from the (uncorrected) pH readings (solid lines) and by extrapolation of the fitted one-exponential expression (dashed lines).

cut-off to 9.19 at the 4-s cut-off of data. This phenomenon is easily explained. The leak process acts to enhance the “apparent” relaxation constant for a single-exponential process, and the fitted constant for the pH curve becomes higher than that for the O_2 curve.

A different demonstration of this problem involves a combination of real and simulated data. The fitted relaxation constant from the O_2 data for the experiment shown in Fig. 2 was used as the exponential representing proton translocation, and the coefficient of this exponential was adjusted to represent a true H^+/O ratio of 6.0. Assuming an exponential for the leak process, the actual pH data were fitted over a 7-s range to a two-exponential function with the coefficient and relaxation constant of the exponential representing the translocation process held fixed as described above. This procedure was able to represent closely the actual pH data over the early range of 0.8 to 4 s (Fig. 4). Moreover, the fitted curve is a pure two-exponential curve having the quality of an outward-directed proton movement with a superimposed

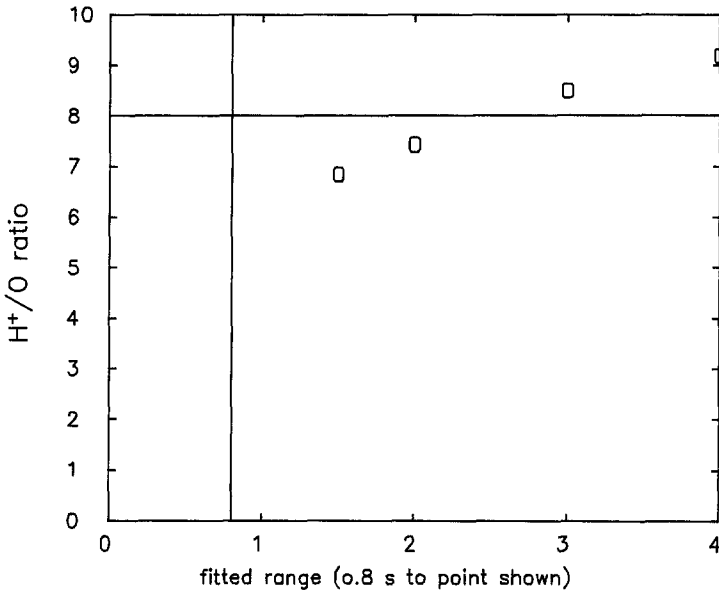


Fig. 3. The zero time H^+/O ratios for the experiment shown in Fig. 1 (upper panel) and Fig. 2 was obtained by dividing the extrapolated zero-time dH^+/dt obtained in each panel of Fig. 2 by the extrapolated zero time dO/dt obtained in Fig. 1 (upper panel).

inward flux. The two-exponential curve was fitted to a single-exponential expression using different ranges of the curve just as was done with the experimental data (Fig. 5 compared to Fig. 2). Once again, the fitted curve (one exponential) closely followed the simulated data (two exponentials) in the fitted range but deviated noticeably outside of the range. The coefficients, relaxation constants, and H^+/O ratios increased with the increase in range used in the fit just as was seen in fitting the experimental data to single exponentials. A summary of results obtained with real and simulated two-exponential data where the “true” H^+/O ratio was set at 6.0 for both of the experiments shown in Fig. 1 is shown in Fig. 6. The extrapolated zero-time ratio is seen to be a function of the ratio of the one-exponential constants obtained for the pH and oxygen curves. The ratio of the constants increases with the range of data used for the fit, but a particular ratio is not always obtained with the same time span in different experiments. Within the usable range of pH data obtained in experiments, values of H^+/O from about 6 to 10 are obtained and an average of such determinations will probably be close to 8.

In the above illustration, 6.0 was used as the “true” H^+/O ratio for fitting in order to obtain the best values for the coefficient and relaxation constant of the leak process. The computer-fitting algorithm was capable of

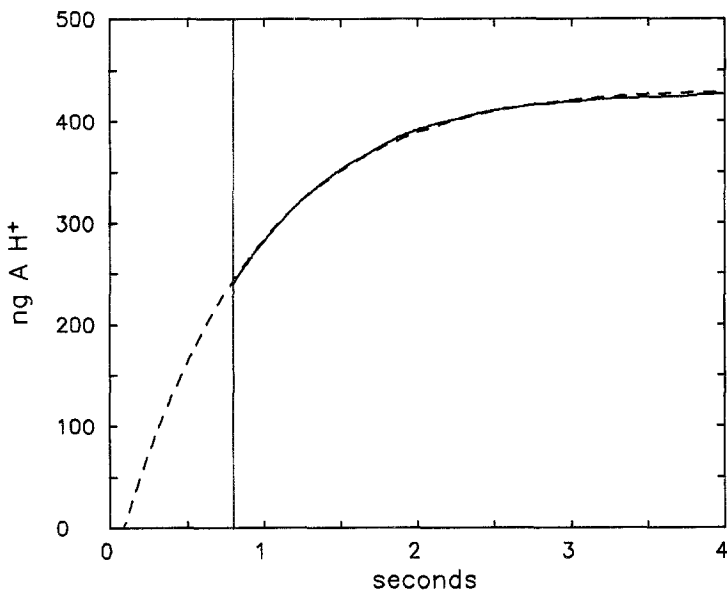


Fig. 4. The solid trace shows the standardized pH electrode signals obtained from 0.8 to 4 s for the experiment shown in Fig. 2 and Fig. 1 (upper panel). The one-exponential relaxation constant obtained for the fitting shown in Fig. 1 (upper panel) was used as the relaxation constant for the proton translocation relaxation constant in a two-exponential model for the pH time course shown in Fig. 2. Signal = $A_T e^{-t/\tau_{ox}} + A_L e^{-t/\tau_L} + A_{baseline}$. The coefficient for the translocation term (A_T) was fixed at 6 times the corresponding coefficient for the O₂ uptake process such that in the absence of a proton leak, the true H⁺/O would be seen as 6.0. The remaining parameters, A_L , τ_L , and $A_{baseline}$, were then fitted to yield the dashed curve shown in the figure. The two-exponential expression which represents a model for a process with proton translocation coupled to O₂ uptake with a true H⁺/O coupling ratio of 6.0 and the best-fit parameters A_L and τ_L for a concomitant leak process was then used for the analyses shown in Figs. 5 and 6.

simulating the experimental data quite closely in the two-exponential model (e.g., Fig. 4). Therefore, fitting restricted portions of the actual experimental curve and the simulated curve were bound to produce very nearly the same results (compare open and filled circles and squares, Fig. 6). Lest this illustration be taken as support for the ratio of 6.0 as the “true” ratio, we must extend the example. When the coefficient for the translocation exponential was adjusted to represent a “true” H⁺/O ratio of 8.0, the computer produced a third curve [in addition to the actual experimental curve and the one based on an H⁺/O ratio of 6.0 (not shown)], which was an extremely close representation of the first two. As to be expected, fitting the same restricted ranges of the new curve once again produced the same spectrum of extrapolated H⁺/O ratios and ratios of the fitted single-exponential kinetic constants. The solid stars in the figure show the results

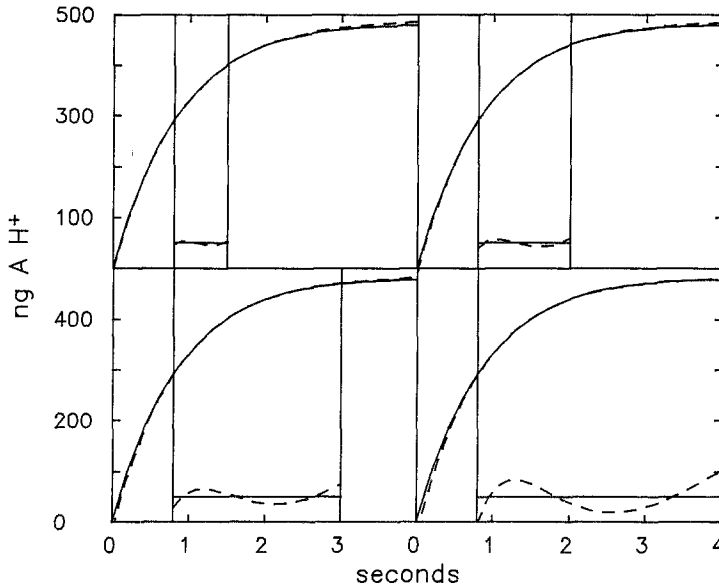


Fig. 5. The solid traces show two-exponential simulations for the appearance of H^+ in the external medium during a respiratory pulse experiment. The simulation employs an H^+/O translocation process with a ratio of 6.0 and an exponential leak process as described in the legend to Fig. 4 and the text. Different portions of the curve, as shown by the vertical lines in each panel, were fitted to the one-exponential expression $Ae^{-t/\tau} + A_{\text{baseline}}$ as described in the legend to Fig. 2. The residuals \times "factor" are shown in the fitted portions in each trace. The factors were 200, 100, 50, and 50, respectively, for the upper left and right panels and the lower left and right panels. The dashed traces show the fitted theoretical lines.

obtained with the fitted data from the same two experiments. The main purpose of the simulation is to show that two-exponential processes can be fitted to single-exponential functions, but that extrapolations based on these fittings produce unreliable H^+/O ratios covering the range that has been in contention in the literature.

The use of the one-exponential extrapolation procedure is based on the tacit assumption that the early time points will be virtually free of contamination by the leak process which should build during the continuing processes of proton ejection and oxygen uptake. The above analysis shows that this is not the case.

There is still another way to assess the magnitude of the leak during the process of the respiratory pulse. In a process of tightly coupled proton translocation with a concomitant leak, it would be expected that

$$J_H = nJ_O - J_L$$

where the J terms represent fluxes for the subscripted process which is either net proton translocation (H), O_2 consumption (O), or proton leak (L). The true stoichiometry is "n." The leak, J_L , then is simply $nJ_O - J_H$. We have

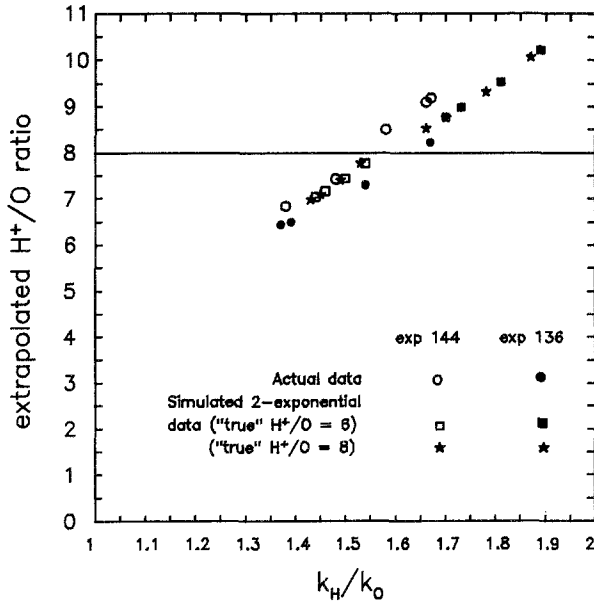


Fig. 6. The H⁺/O ratios were computed by dividing the extrapolated zero-time value for dH^+/dt by the extrapolated zero-time value of dO/dt . Two respiration pulse experiments are shown: exp 144 (Fig. 1, upper panel) and exp 136 (Fig. 1, lower panel). For each experiment three methods of analysis were used. In one case the actual pH data were fitted, using different portions of the time course as shown in Fig. 2. In the other two cases the exponential term for the O₂ process was used for a two-exponential fitting of the pH data in a model having a true H⁺/O stoichiometry of either 6.0 or 8.0 plus a leak process as described in Fig. 5. The first-order constants for k_H and k_O are the reciprocals of the relaxation constants τ_H and τ_O .

taken experimental J_H and J_O data which have been digitally filtered and corrected for the respective electrode relaxation processes (Setty *et al.*, 1986) and have computed J_L as a function of time for particular values of n . The J_L is viewed as strictly proportional to $\Delta\tilde{\mu}_{H^+}$,

$$J_L = L_H^1 \Delta\tilde{\mu}_{H^+}$$

where L_H^1 is a phenomenological constant. Therefore, J_L is an indication of $\Delta\tilde{\mu}_{H^+}$. The results of such an analysis are shown in Fig. 7. Only data after 0.5 s are considered in order to eliminate distortion due to a burst phenomenon, which should be completed by 0.3 s (Setty *et al.*, 1986). An n value of 2 can be eliminated because the leak would be in the wrong direction. This is also true for an n value of 4 up to the first second of the pulse. With n values of 6 and 8, the $\Delta\tilde{\mu}_{H^+}$ peaks at about 1.5 s although the peak in dO/dt was at about 0.4 s and the turnaround point for medium acidification where $nJ_O = J_L$ was beyond 4 s. Whether or not $\Delta\tilde{\mu}_{H^+}$ actually peaked so early in the experiment is not known, but according to this analysis and in opposition

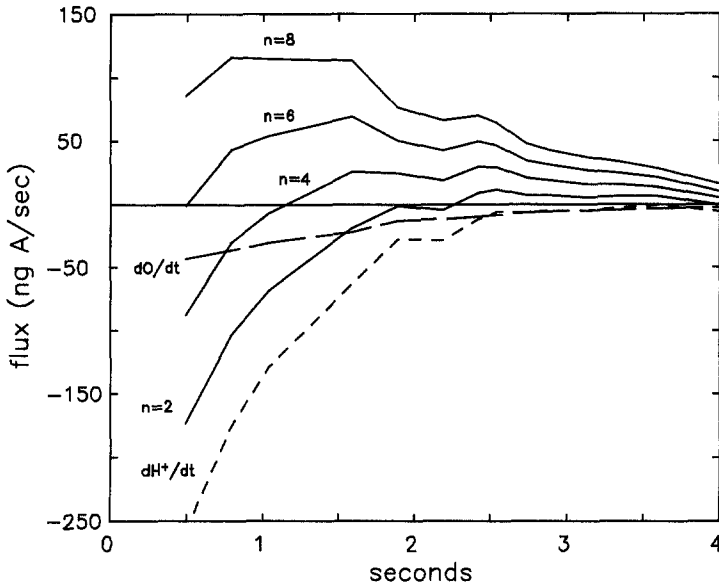


Fig. 7. The actual values for $dO/dt(J_O)$ and $dH^+/dt(J_H)$ vs t in the experiment shown in Fig. 1 (upper panel) and Fig. 2 were used to compute the leak flux (J_L) using the expression $J_L = nJ_O - J_H$, where n is the H^+/O stoichiometry. The curves with n set at 2, 4, 6, or 8 are shown, as well as the actual J_O and J_H vs t . J_L curves below the zero line are in the wrong direction (i.e., from “in” to “out”).

to the assumption on which zero-time extrapolation methods are based, data collected rather early in the pulse history would be the most affected by the kinetics of the leak process. In the absence of independent information on the actual magnitude of $\Delta\tilde{\mu}_{H^+}$ during the course of the experiment, another possibility is that $\Delta\tilde{\mu}_{H^+}$ builds as the integrals (i.e., total amounts) for the processes of proton translocation and oxygen uptake. The peak in magnitude of net proton translocation occurred at the “turnaround” point where the dO/dt was already quite low. If this represents the maximum for $\Delta\tilde{\mu}_{H^+}$, then the maximum leak is $J_L = nJ_O$, where J_O is only 10% of its maximum value. This picture says that J_L is quite insignificant during the major history of the burst, but it leaves unexplained why the H^+/O ratio drops most precipitously at the beginning of the pulse. A possible explanation is that respiratory control is mediated by something other than or in addition to $\Delta\tilde{\mu}_{H^+}$.

Experiments of Wikström *et al.*

The experiments of Wikström *et al.* which form the basis of their strong support for the H^+/O ratio of 6 for electrons moving through sites II and

III and of 2 for site III alone will now be examined. In one of the early papers, Wikström and Krab (1978) used potassium ferrocyanide as electron donor to site III of rat liver mitochondria. A Clark O₂ electrode and a pH electrode were used to monitor changes in medium O₂ and pH. The earliest part of the pH trace (~4 s) was lost because of an artifactual alkalinization which preceded the net acidification of the medium. The initial rates of O₂ consumption and acidification were obtained by drawing tangents to the early portions of the curves, and the H⁺/O stoichiometry was taken from the ratio of these two rates. In view of the above discussion we must question this approach and the value it produced. In particular, we would stress that:

- (1) The early part of the pH trace is contaminated with the influence of the leak process and the true tangent to this portion is not the rate of proton translocation.
- (2) The earliest portion of the pH curve, which should display the steepest slope, was not available.

In a subsequent paper (Krab and Wikström, 1979), two other procedures were employed. In one, a mathematical model was derived for the proton translocation and leak processes based on the assumptions that respiration and the internal volume remained constant during the experiment and that the leak process was determined only by ΔpH. A pH trace was shown for an experiment where electrons were introduced to site III of rat liver mitochondria by ascorbate/ferrocyanide. The equation used to analyze the data was

$$\Delta N_1 = (n_1 + n_2)rt + (n_2rV_2/k) [\exp(-kt/V_2) - 1]$$

where N_1 = total protons on the C (cytoplasmic) side

n_1 = H⁺/e stoichiometry on the C-side

n_2 = H⁺/e stoichiometry on the M (matrix) side

V_2 = internal volume with a particular buffer capacity such that

$[H^+]_2 = N_2/V_2$ on the M-side

r = rate of electron transfer

k = proton permeability of the membrane.

This equation predicts that the initial slope will be $n_1 \cdot r$, that a limiting asymptote with a slope of $(n_1 + n_2) \cdot r$ exists, that the intercept of the extended asymptote is $-n_2 \cdot r \cdot V_2/k$, and that a plot of the log of the distance from any part of the trace to the asymptote vs time will produce a slope equal to $-k/V_2$. When all of the slopes and distances are measured and the calculated value of n_1 corrected for the fact that 0.54 H⁺/e are liberated during ascorbate oxidation, n_1 is found to be 0.89 H⁺/e and n_2 to be -1.88 H⁺/e. In other words, the H⁺/e stoichiometry for site III is close to 1. The problems with this approach are:

- (1) It was never shown that the equation could adequately fit the experimental data.
- (2) Only one experiment is shown. There are no statistics showing reproducibility, range, variations, and goodness of fit.
- (3) It was not described how the asymptote to the pH trace at 9 min was obtained, nor was it shown that the rate of respiration remained constant after 6 min.
- (4) Controls on the possible autooxidation of ascorbic acid by O_2 were not presented.
- (5) The method depends on the assumption that neither the internal volume nor proton permeability changed during the 9 min of the experiment.

In the same paper an experiment of the Mitchell type is shown for electrons introduced by reduced cytochrome *c* to rat liver mitochondria. For a series of eight separate O_2 pulses of different magnitude, a H^+/e ratio of 0.78 ± 0.14 was obtained. In these experiments, the initial rapid decay of the pH trace was back-extrapolated to a point halfway between the addition of O_2 and anaerobiosis. The many problems with this technique were discussed above. In a later paper, Wikström and Penttilä (1982) again used the back-extrapolation method of Mitchell and Moyle to confirm that the H^+/e ratio for electrons introduced to site III of rat liver mitochondria by reduced cytochrome *c* was 0.6 to 0.7. However, in this paper the pH decay curve was back-extrapolated to zero time instead of the presumed time of $1/2 O_2$ utilization.

In discussing the method of Mitchell and Moyle we emphasized the precautions cited by the authors which required that the resorption kinetics be governed by a much slower time constant than the acidification kinetics and that no data should be used near the inflection point where the trace shows the beginning of the resorption phase. Furthermore, the original authors emphasized that the back-extrapolation be based on an exponential law. In their earlier paper, Krab and Wikström (1979) provided no details on the back-extrapolation, but in their more recent paper Wikström and Penttilä (1982) show in Fig. 3 a linear extrapolation from the inflection point back to the zero time of O_2 addition. As an example of the kind of error this procedure could produce in an idealized case where electrode delays are not present and for a process where the resorption phase is truly a single-exponential process, we present Fig. 8. In this figure we have simulated the H^+ translocation and resorption processes using a two-exponential model with time constants in the ratio of 1 to 3. The true external H^+/O stoichiometry was set at 4.0 and allowance was made for the net utilization of $2H^+$ per O for the scalar reaction. A linear extrapolation from the inflection

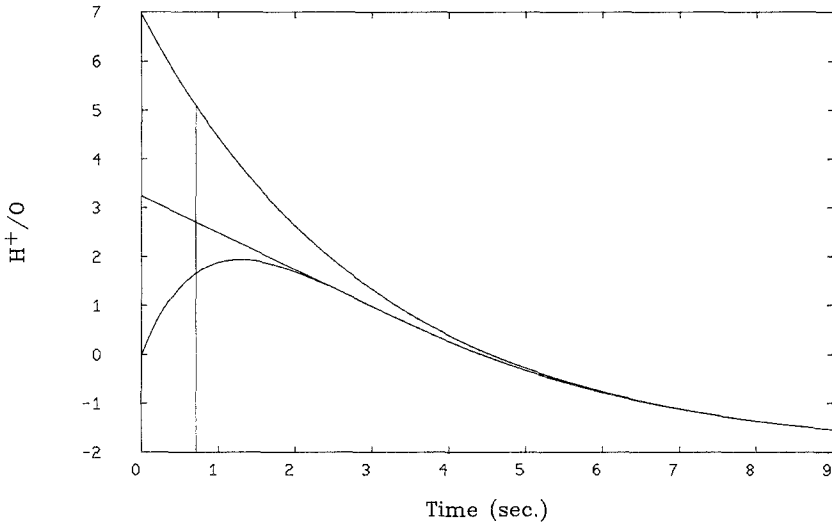


Fig. 8. A two-exponential simulation of an H^+ translocation process with a concomitant leak is shown in the bottom trace. [See Appendix, Eq. (A1).] Using the notation in the Appendix, the parameter values are $k = 1.0$, $D = 0.33$, $n = 4$, $\Delta = 2$, and $a = 0$. The middle trace shows a linear back-extrapolation from the inflection point to zero time, yielding $n = 3.24$. A similar extrapolation on a trace with $a = 1.08$ (not shown) yielded $n = 3.03$. The upper trace shows the extrapolation to zero time of the slow exponential associated with the leak process, yielding $n = 6.96$. A similar extrapolation with $a = 1.08$ yielded $n \approx 8.04$. The vertical line denotes the time of one-half O_2 utilization.

point to zero time resulted in an underestimation of n by approximately 20%. The true exponential back-extrapolation, which cannot be done visually, is also shown. It will be noted that the correctly extrapolated value of the slow exponential either to the time of O_2 addition or to the time of 1/2 utilization of O_2 does not produce the correct value of n . The true value of n could be obtained from the Y -intercept only by using formula (A₃) in the appendix. For back-extrapolation to the time of O_2 addition, $t = 0$; whereas for back-extrapolation to the time where 1/2 of the added O_2 was consumed, $t = \ln 2/k_2 = 0.7/k_2$. The value of $Y_{ex}(t)$ will approach n only as k_1/k_2 (c.f. Table I) approaches 0. It is important to emphasize that this treatment as well as the one which follows assumes that any complications due to the response characteristics of the pH electrode have been properly accounted for, otherwise the error in estimates of H^+/O stoichiometry will be greater. As a final comment, the theoretical single-exponential back-extrapolation shown in Fig. 8 is close to, but not exactly the same, as the method of Mitchell previously described, because his graphic method does not completely isolate the slow exponential. Therefore, Mitchell's values would be lower than Fig. 8, as indicated in Table I.

Table I. Extrapolation for $n(\text{H}^+/\text{O}$ Ratio) by the Semilog Method of Mitchell and Moyle (M) and by the Tangent Extrapolation of Wikström and Penttilä (W) for Several Values of k_2/k_1 ("True" $n = 4$)

k_2/k_1^c	M ^a	W ^b	W ^a
2	4.60	2.87	2.24
3	4.87	3.23	2.70
4	4.68	3.45	2.99
5	4.52	3.59	3.18
6	4.41	3.69	3.32
7	4.33	3.76	3.43
8	4.28	3.81	3.51
9	4.24	3.85	3.57
10	4.22	3.88	3.62

^aExtrapolation to the time of 1/2 utilization of O_2 (i.e., $t_{1/2} = \ln 2/k_2$).

^bExtrapolation to the time of O_2 addition (i.e., $t = 0$).

^c k_2 and k_1 are the exponential rate constants for H^+ translocation and backflow, respectively.

A further exploration of the theoretical basis of back-extrapolation of the resorption phase of the pH vs t curve in order to obtain the H^+/O stoichiometry follows. We have modified the mathematical model of Krab and Wikström (1979) to account for nonlinear O_2 consumption. Because in actual O_2 pulse experiments where $d\text{O}/dt$ has been measured it appears that $d\text{O}/dt$ declines in a nearly exponential manner (Costa *et al.*, 1984; Setty *et al.*, 1986), we have replaced the constant for $d\text{O}/dt$ by an exponential term. The resulting equation, which is analogous to Eq. (5) of Krab and Wikström (1979), is formula (A1) of the appendix. We have set the true external H^+/O stoichiometry at 4 and have allowed for the scalar uptake of H^+ in H_2O formation. In Table I we present the H^+/O stoichiometries which would be obtained upon back-extrapolation to the time at which 1/2 of the O_2 was consumed by the semilog method of Mitchell and Moyle (1967) and to either zero time or $t_{1/2}$ by a tangential procedure. The process is repeated for several situations where the ratio of the time constants for the two processes has been varied. If the resorption process were truly one-exponential, the table reveals the kind of uncertainty the method could produce. As we have shown above when we discussed the method originally, it appears that the resorption process cannot be treated as a single-exponential function and therefore no proper method of back-extrapolation has been presented.

More recently, Wikström and Casey (1985) have reported that intact rat liver mitochondria cannot oxidize reduced cytochrome *c*. The observed oxidation is due to 5–12% of mitochondria with damaged outer membranes. Because of this, they have concluded that the O_2 pulse normally used in this system is "oversized" by a factor of 9–20, probably resulting in rapid exhaustion of the matrix buffering capacity, subsequent fast back-leakage of

protons, and a dramatic lowering of the H^+/e ratio. This caveat must also apply to the H^+/O estimates of Wikström and Penttilä (1982) and Krab and Wikström (1979).

Should There be an Integral Mechanistic H^+/O Number?

In all of the methods discussed in this paper, it has been assumed that a single uniform translocation process is involved which proceeds in a monotonic manner from beginning to end. None of these methods has obtained reliable data in the first few tenths of a second that might substantiate this assumption. Using a new approach in which respiration is instantly initiated by photolysis of the CO-liganded cytochrome oxidase in a thoroughly mixed and equilibrated suspension of succinate, mitochondria, and O_2 , we have obtained data from zero time onward (Setty *et al.*, 1986). Using rapid-response pH and O_2 electrodes, we have corrected their earliest responses using experimentally determined relaxation constants. In this way, we have seen a rapid initial transient phenomenon in which there is an initial burst in proton translocation which peaks within the first 50 ms with H^+/O values about 30 and which phenomenon has totally relaxed by 300 ms. A similar phenomenon has since been observed using reduced cytochrome *c* as electron donor to rat liver mitoplasts (Hendler and Setty, 1987). Therefore, all methods which assume a single monotonic translocation process must be reconsidered.

We would like to emphasize one other potentially serious problem in the search for the true, mechanistic H^+/O ratio. There may not be such a number. The search for the true number presupposes that the mechanism of proton translocation employs a process linking each proton moved to an electron in the respiratory chain. If the chain is arranged in proton translocating loops, and if protons are necessarily transported through these loops, then there is a true value of n , which is the number of loops traveled by the electron and a coordinate proton. Although this is a very good basis on which to examine data, it has never been experimentally established as the mechanism involved. Thermodynamics permits a relationship between proton translocation and O_2 consumption in a less mechanistically coupled process. If energy liberated by a redox reaction is efficiently transferred to a proton pump, the number of protons that can be pumped will be $\Delta E_{ox}/\Delta\tilde{\mu}_{H^+}$. Because $\Delta\tilde{\mu}_{H^+}$ will rise more quickly with the number of protons pumped than will the ΔE_{ox} change, the operational n value in this process will fall in a continuous manner during the pulse. The magnitude of $\Delta\tilde{\mu}_{H^+}$ would be expected to rise at a more rapid rate in the beginning of the pulse than later, and therefore the H^+/O ratio would be expected to fall more rapidly in the beginning of the pulse than later. This is consistent with the observed course

for H^+/O ratios. We have not provided any evidence that a tightly coupled stoichiometric process is not occurring. Our finding of an initial high burst in the H^+/O ratio in the first 300 ms of a respiratory pulse says that if a basic locked process is functioning, then another process must also be considered to explain the burst. In the case of cytochrome oxidase, the same protein which accepts and donates the electrons may also perform the pumping function in a manner which utilizes a Bohr-type of redox/proton cooperativity. If such a mechanism is tightly coupled, a fixed H^+/O ratio might be expected provided that the ΔpH does not become too large. On the other hand, if the pH changes appreciably on either side of the membrane or if slippage occurs, as has been discussed by many others (Wikström and Penttilä, 1982; Pietrobon *et al.*, 1981; Wikström *et al.*, 1981; Brunori *et al.*, 1985; Westerhoff, 1983), the ratio may be nonintegral and variable. At the present state of our knowledge we cannot insist on tightly coupled processes as the only kind that can be operating. A dependable fixed H^+/e stoichiometry for any of the energy transduction sites in the respiratory chain has not been established.

Appendix: H^+ Time Course with Exponential O_2 Uptake

V_{out} = outside aqueous volume

V_{in} = inside aqueous volume, $V_{in} \ll V_{out}$

Y_{out} = ΔH^+ outside, $\dot{Y} = dY/dt$

Y_{in} = ΔH^+ inside

O_T = total oxygen in pulse.

Δ = $[-\dot{Y}_{out}(0) - \dot{Y}_{in}(0)]/O_T$ neglecting ascorbate,
i.e., Krab and Wikström's $2(n_1 - n_2)$

n = H^+/O ratio

a = ascorbate H^+/O contribution

d = diffusion constant

D = d/V_{in}

k = O_2 uptake rate constant

C_1 = $[k(n + a) + DC_3]/(k - D)$

C_2 = $C_1 + C_3$

C_3 = $\Delta - a$

Differential equations:

$$Y_{\text{out}}(0) = 0; \dot{Y}_{\text{out}} = (n + a)O_{\text{T}}k e^{-kt} - d(Y_{\text{out}}/V_{\text{out}} - Y_{\text{in}}/V_{\text{in}})$$

$$Y_{\text{in}}(0) = 0; \dot{Y}_{\text{in}} = -(n + \Delta)O_{\text{T}}k e^{-kt} + d(Y_{\text{out}}/V_{\text{out}} - Y_{\text{in}}/V_{\text{in}})$$

Solution:

$$Y_{\text{out}}/O_{\text{T}} = -C_1 e^{-kt} + C_2 e^{-Dt} - C_3 \quad (\text{A1})$$

Extrapolating the second exponential to any t yields

$$Y_{\text{ex}} = C_2 e^{-Dt} - C_3 \quad (\text{A2})$$

Solving for n :

$$n = (Y_{\text{ex}} + C_3)(k - D)e^{Dt} - a - kC_3 \quad (\text{A3})$$

References

- Brunori, M., Sarti, P., Colosimo, A., Antonini, G., Malatesta, F., Jones, M. G., and Wilson, M. T. (1985). *EMBO J.* **4**, 2365–2368.
- Costa, L. E., Reynafarje, B., and Lehninger, A. L. (1984). *J. Biol. Chem.* **259**, 4802–4811.
- Hendler, R. W., and Setty, O. H. (1987). Submitted for publication.
- Krab, K., and Wikström, M. (1979). *Biochim. Biophys. Acta* **548**, 1–15.
- Krab, K., and Wikström, M. (1986). *FEBS Lett.* **201**, 198–204.
- Krab, K., Soos, J., and Wikström, M. (1984). *FEBS Lett.* **178**, 187–192.
- Lehninger, A. L., Reynafarje, B., Hendler, R. W., and Shrager, R. I. (1985). *FEBS Lett.* **192**, 173–178.
- Mitchell, P. (1966). In *Chemiosmotic Coupling in Oxidation and Photosynthetic Phosphorylation*, Glynn Research Ltd., Bodwin.
- Mitchell, P., and Moyle, J. (1967). *Biochem. J.* **105**, 1147–1162.
- Pietrobon, D., Azzone, G. F., and Walz, D. (1981). *Eur. J. Biochem.* **117**, 389–394.
- Reynafarje, B., Alexandre, A., Davies, P., and Lehninger, A. L. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 7218–7222.
- Setty, O. H., Shrager, R. I., Bunow, B., Reynafarje, B., Lehninger, A. L., and Hendler, R. W. (1986). *Biophys. J.* **50**, 391–404.
- West, I. C., Mitchell, R., Moody, A. J., and Mitchell, P. (1986). *Biochem. J.* **236**, 15–21.
- Westerhoff, H. V. (1983). Thesis, Academisch Proefschrift, University of Amsterdam, The Netherlands.
- Wikström, M., and Krab, K. (1978). *FEBS Lett.* **91**, 8–14.
- Wikström, M., and Penttilä, T. (1982). *FEBS Lett.* **144**, 183–189.
- Wikström, M., and Saraste, M. (1984). In *Bioenergetics* (Ernster, L., ed.), Elsevier, Amsterdam, pp. 49–93.
- Wikström, M., and Casey, R. (1985). *FEBS Lett.* **183**, 293–298.
- Wikström, M., Krab, K., and Saraste, M. (1981). In *Cytochrome Oxidase, A Synthesis*. Academic Press, New York, p. 145.