DIRECT MEASUREMENT OF THE INITIAL AND EARLY RATIOS OF PROTON EXTRUSION TO OXYGEN UPTAKE ACCOMPANYING CYTOCHROME *c* OXIDATION BY RAT LIVER MITOPLASTS

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ABSTRACT We have recently described new methods that enable the sharp initiation of a respiratory pulse by photolysis of the CO complex of cytochrome oxidase in a stirred suspension of mitochondria, succinate, O_2, and CO (Setty, O. H., R. I. Shrager, B. Bunow, B. Reynafarje, A. L. Lehninger, and R. W. Hendler. 1986. *Biophys. J.* 50:391–404). Data are collected directly into a microcomputer at 10-ms intervals from fast responding O_2 and pH electrodes. These procedures eliminate delays and uncertainties due to mixing times, recorder response, and recovery of the O_2 electrode from responding to the injection of O_2. Correction procedures were also described for the inherent electrode delays. These procedures revealed an initial burst in medium acidification and a lag in O_2 uptake that led to H^+/O rates of 20–30 during the first 50 ms and relaxed to "normal" levels by 300 ms. Subsequent changes in $[H^+]$ and $[O_2]$ followed time courses that appeared to be, but were not strictly, first order. We describe here similar studies in which cytochrome *c* served as electron donor to site III of rat liver mitoplasts. A qualitatively similar but quantitatively smaller burst in medium acidification and H^+/O ratio was seen in these studies. Implications of the previous (Setty et al., 1986) and current studies on defining "mechanistic" H^+/O ratios are discussed.

INTRODUCTION

We have recently described new methods for instantly initiating a respiratory pulse in a thoroughly mixed suspension of mitochondria-containing succinate and O_2, and collecting data from zero time at 10-ms intervals from fast responding O_2 and pH electrodes (1). At the same time we described new methods for characterizing the relaxation of each electrode and correcting the early data for delays in electrode response. The purpose of these measures was to remove uncertainties present in the usual methods for defining H^+/O ratios in which data from 0.8 s onwards are extrapolated back to some arbitrary earlier point in time over a period that includes mixing and electrode delays, and in which the data used for the extrapolation are not free from the influence of the proton leak kinetics. In our recent paper, we described an initial burst in acidification of the external medium that reached a peak within the first 50 ms and was completed by 300 ms. During this early phase, there was an initial delay in the rate of O_2 uptake such that early transient H^+/O ratios of 30 were seen. In confirmation of earlier studies, we found that both the pH and O_2 data obtained after 0.8 s could be fit to single exponential expressions which, when extrapolated back to zero time, produced "initial" H^+/O ratios of 8.0. On the other hand, we have pointed out why we question the validity of the number and the methods that produced it (references 1, 2 and in this paper). Evidence for a similar initial burst in H^+ extrusion has been published previously by two other laboratories (3–5).

In the present paper, we describe the results obtained from an application of the new techniques to a study of the H^+/O ratios which occur during the oxidation of reduced cytochrome *c* via site III of rat liver mitoplasts. These results confirm the transient initial phase seen with succinate as electron donor (1), which includes a rapid proton burst and initial lag in O_2 uptake leading to high values of H^+/O ratio, which were from ⅓ to ½ of that seen in the succinate incubations.

EXPERIMENTAL PROCEDURES

The experimental system and protocol have already been described (1). In brief, a solution (~5.5 ml) containing 200 mM sucrose, 50 mM KCl, and 1.5 mM Hepes adjusted to pH 7.05 was placed in a water-jacketed reaction vessel maintained at 10°C and made anaerobic by bubbling with a stream of N_2. The gas was then stopped and 100% carbon monoxide was passed over the surface at a rate of 80 ml/min. Next rotenone (2.0 μM) and succinate (5.0 mM) were added followed by rat liver mitoplasts (18 mg of protein) in 0.3 ml to adjust the final volume to 6.0 ml. The pH, if

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changed, was adjusted back to 7.05. Any O_2 admitted during the additions was removed by respiration which proceeded upon illumination of the suspension. After 5 min of complete anaerobiosis, 18 μg of oligomycin and 90 μg of *N*-ethyl-maleimide were added, followed in 3–5 min by 2.0 μg of valinomycin, all additions being in microliter amounts. After 5–10 min, 100 μl of 5 mM cytochrome *c* (equine heart, Sigma Chemical Co., St. Louis, MO) was added and 10 min allowed for the reduction of the cytochrome. Antimycin (1.5 μl of 1 mM) was then added, followed in ~1 min by 0.2 ml of air-saturated buffer in the dark. The calibration for the O_2 electrode was taken from this injection. When chemically reduced cytochrome *c* was used, succinate and antimycin were omitted. After 30 s, a photographic shutter was opened to photolyze the CO complex of cytochrome oxidase and thereby initiate respiration. Concentrations of O_2 and H^+ in the medium were continuously monitored by fast responding O_2 and pH electrodes with a sampling rate of 10 ms per reading per electrode. After the O_2 uptake was complete, the pH electrode was calibrated by injection of 200 or 400 nmol HCl. The rat liver mitoplasts were prepared in the laboratory of Dr. A. L. Lehninger according to the procedure described in reference 6. The respiratory control index of each preparation was measured by the difference in the rate of succinate oxidation before and after the addition of ADP or carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) using a standard Clark O_2 electrode. These ratios were 4–5 with ADP and close to 6 with FCCP for all preparations of mitoplasts. The relaxation constants for each electrode were determined in each set of experiments and electrode responses were corrected for intrinsic delays as previously described (1).

RESULTS

Raw and filtered data obtained from O_2 and pH electrodes in two pulse experiments where cytochrome *c* reduced in situ served as electron donor are shown in Fig. 1. As was the case for O_2 pulse experiments in mitochondria with succinate as the electron source, the filtered data are a virtually noiseless representation of the original data with little or no distortion. In Fig. 2, corresponding data are shown for two experiments where chemically reduced cytochrome *c* served as electron donor. Although a little more noise is evident in the O_2 traces in this figure, the filtered data appear to be a dependable representation of the original data. With the smoothed data from the O_2 and pH electrodes, it was possible to obtain the first and second derivatives of concentration with respect to time. This information and the experimentally determined relaxation constants for the two electrodes were then used as previously described (1) to obtain a record of the true rates of change of $[O_2]$ and $[H^+]$ updated every 10 ms from the start of the pulse. The ratios of dH^+/dt to dO/dt were used as a dynamic record of H^+/O ratio during the entire incubation. Fig. 3 shows results obtained in two experiments where cytochrome *c* was reduced in situ by succinate followed by antimycin, and in two experiments where chemically reduced cytochrome *c* was used in the absence of succinate and antimycin. As was the case for succinate oxidation by mitochondria, there is an initial burst in H^+/O ratios which decays within 0.3 s when corrections for electrode responses are made but whose effects are clearly seen from 0.5 to 1.0 s if raw electrode data are used. The traces in the figure also show a noise pattern that is more pronounced in the corrected curves and becomes

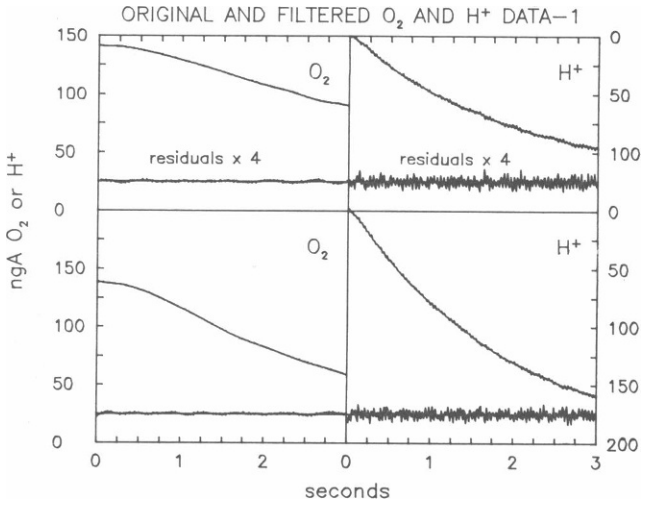

FIGURE 1 Two experiments in which cytochrome *c* reduced in situ served as electron donor to site III of rat liver mitoplasts. The top two panels show raw electrode signals plus the superimposed digitally filtered traces during the first 3 s of an O_2 pulse initiated by photolysis of the CO-complexed cytochrome oxidase. The bottom two panels show comparable data from another experiment. Electrode data were collected every 10 ms from each electrode. The residuals X4 from the filtered and original data are shown in each panel. The incubation medium contained mitoplasts (18 mg protein in 6 ml), 200 mM sucrose, 1.5 mM Hepes, 50 mM KCl, 2 μM rotenone, 18 μg oligomycin, 2.0 μg valinomycin, 90 μg of *N*-ethyl maleimide, and 5 mM succinate under an atmosphere of 100% CO. After 10 min of complete anaerobiosis, 100 μl of 5 mM cytochrome *c* was added and 10 min were allowed for the reduction of cytochrome *c*. Antimycin (1.5 μl of 1 mM) was then added followed by ~1 min by 0.2 ml of air saturated buffer in the dark. After several seconds the pulse was initiated by illumination of the suspension.

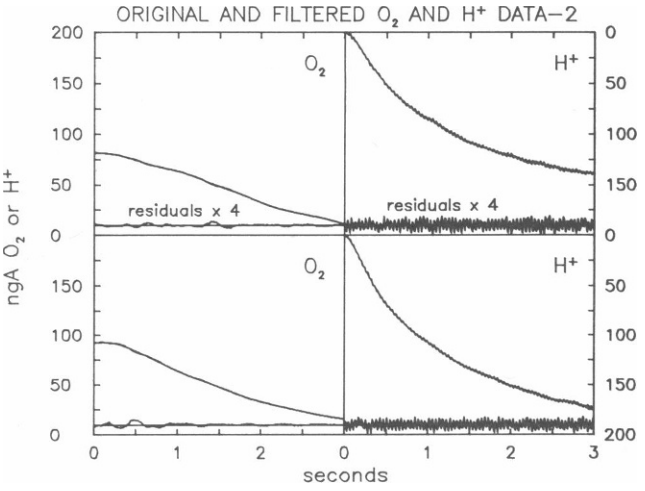

FIGURE 2 Two experiments in which chemically reduced cytochrome *c* served as electron donor to site III of rat liver mitoplasts. The legend to Fig. 1 is appropriate for this figure with the following changes. Succinate and antimycin were omitted and replaced by 100 μl of 5 mM reduced cytochrome *c*.

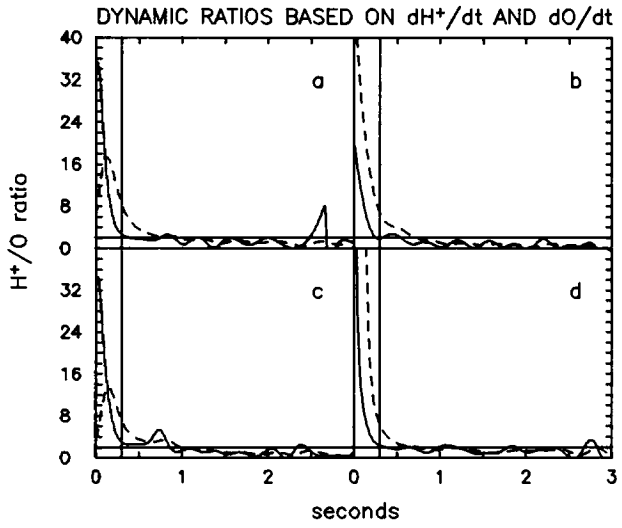

FIGURE 3 In *a* and *b*, the data obtained in the two experiments shown in Fig. 1 were corrected for electrode response delays as previously described (1) to obtain values for dH^+/dt and dO/dt updated every 10 ms. The H^+/O ratio was calculated for each 10-ms interval from the H^+ and O derivatives. These data are represented by the solid lines. The dashed lines show the H^+/O ratios from each 10-ms interval obtained from the raw (uncorrected) electrode data. *c* and *d* show the results of the same treatment for the data obtained from the two experiments shown in Fig. 2.

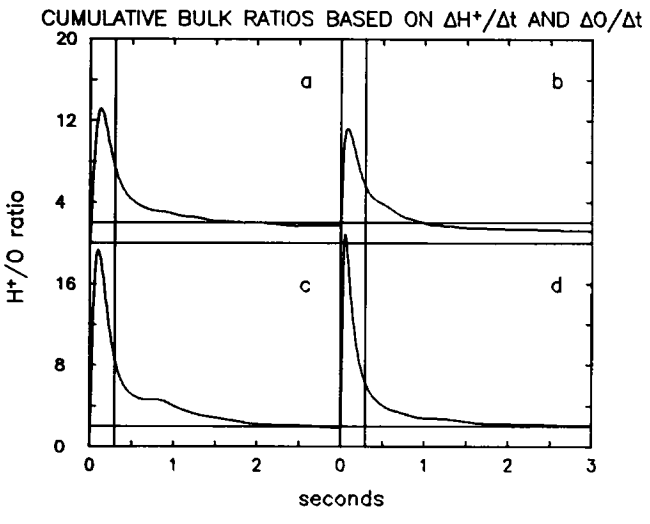

FIGURE 4 In *a* and *b*, the corrected electrode data obtained in the two experiments shown in Fig. 1 were used to compute ΔH^+ and ΔO which occurred during the experiment. The cumulative bulk H^+/O rate shown at each time point was calculated by $\Delta H^+_i/(\Delta O_i + O_i)$ where the increments represent the total change at each time point and O_i represents the quantity of O inside the mitoplast at zero time. *c* and *d* show the results of the same treatment for the data obtained from the two experiments shown in Fig. 2.

more evident after the early burst. This is understandable because although the filter is very effective, a small amount of noise does pass. This noise is greatly magnified during the process of correcting for response times as discussed previously (1). As both dH^+/dt and dO/dt decrease because of respiratory control, the ratio of the two becomes more sensitive to noise especially as dO/dt approaches zero. The true curve is easily seen as the average running through the regular noise pattern. In addition to the dynamic record of H^+/O ratios where a new H^+/O ratio is computed every 10 ms, the data can be used to compute an accumulative bulk ratio. In this approach the total amount of H^+ appearing in the external bulk phase from zero time is divided by the total amount of O_2 disappearing, plus the total amount of O_2 that was contained in the mitoplast matrix at the start of the pulse. The cummulative H^+/O ratios for the same four experiments shown in Fig. 3 are presented in Fig. 4. The individual changes in $[O_2]$ and $[H^+]$ normalized to 100% of their changes during the first 3 s of the pulse for two experiments using cytochrome *c* reduced in situ are shown in Fig. 5 (*a* and *b*). It is seen that the early burst in H^+/O ratio is based on an initial rapid rate of proton ejection and an initial slow phase of O_2 uptake. In *c* and *d*, $[H^+]$ data from 0.8 to 3 s were fit to a single exponential expression and extrapolated to zero time (*dashed curves*). The difference between the actual (corrected) data and the fitted one exponential curve indicates the magnitude and duration of the burst phenomenon. It was shown in the studies with succinate oxidation, where a similar burst phenomenon

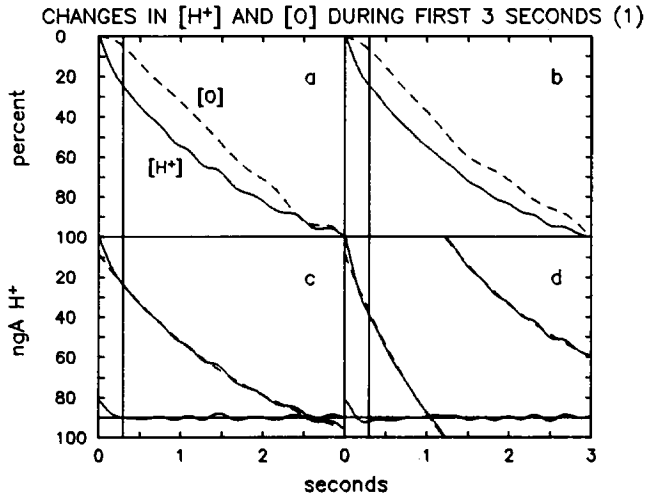

FIGURE 5 The corrected electrode readings for the two experiments shown in Fig. 1 were normalized over the first 3 s of the pulse and are shown in *a* (from Fig. 1, *top*) and in *b* (from Fig. 1, *bottom*). In *c*, the corrected data (from *a*) for $[H^+]$ vs. time from 0.8–3 s were fit to a one exponential function and are shown extrapolated back to zero (*dashed line*). The actual $[H^+]$ data are shown as a solid line and a vertical line is drawn at 0.3 s. The difference between the actual $[H^+]$ data and the one-exponential line is shown at the bottom of the panel. This indicates the extent and time course of the extra $[H^+]$ in the proton burst. *d* shows a similar treatment for the $[H^+]$ date in *b*. In this case more protons were released, and the continuation of the curve going out of the panel at 1.2 s is shown at the top of the panel with a displacement of the *y*-axis.

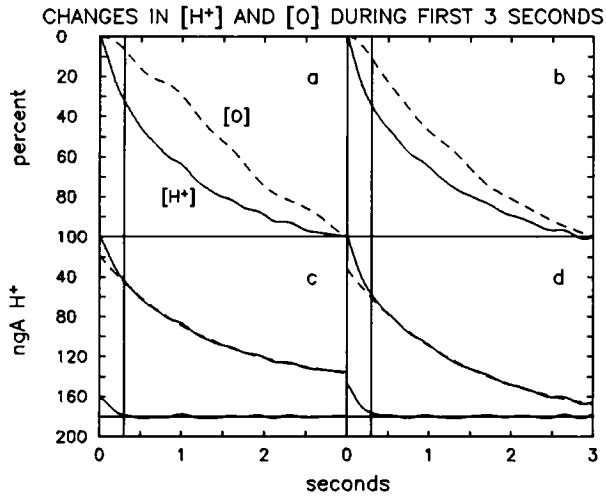

FIGURE 6 The legend to Fig. 5 applies here except for the fact that *a* and *c* relate to the date shown in the upper panel of Fig. 2 and *b* and *d* relate to the data shown in the lower panel of Fig. 2.

was evident, that the correction procedure used for electrode response delay, could not cause an artifactual burst in a truly one exponential process (1). Similar data for two experiments using exogenously chemically reduced cytochrome *c* are shown in Fig. 6.

Controls

The preparation of mitoplasts from mitochondria involves the use of detergents and centrifugations. In comparing the H^+/O stoichiometries for site III in mitoplasts to those for sites II and III in rat liver mitochondria, it is necessary to establish that both preparations are similar with respect to proton fluxes accompanying O_2 uptake. For this purpose, we have observed proton translocation accompanying succinate oxidation at 25°C in a series of experiments using mitoplasts and mitochondria. Table I shows that the two preparations are similar both for the dynamic studies where ratios are computed and updated every 10 ms, and for the cumulative bulk ratios.

Other important controls are shown in Fig. 7. *a* and *b* show the rapid initiation of O_2 uptake and proton translocation that occur upon illumination of the stirred mitoplast suspension containing reduced cytochrome *c*, O_2, and CO. In *a*, cytochrome *c* was reduced in situ by succinate, after which antimycin was added to block electron flow from succinate through site II. In *b*, exogenously reduced cytochrome *c* was added in the absence of succinate and antimycin. Both panels show the respiration driven acidification of the external medium which quickly reaches a peak followed by the slow return of the extruded protons to the mitoplast interior. In *c*, a respiratory pulse with succinate at 25°C in the absence of antimycin is shown. *d* shows that when 0.25 μM antimycin was present in an incubation at 10°C, there was no succinate oxidation or medium acidification initiated by illumination of the sample. The presence of antimycin prevents both the CO-resistant and light-stimulated changes in O_2 uptake and proton translocation. In the absence of added substrate,

TABLE I
COMPARISON OF MITOPLASTS AND MITOCHONDRIA FOR H^+/O STOICHIOMETRIES DURING SUCCINATE OXIDATION

I Dynamic ratios

	Burst H^+/O				H^+/O Ratio			
					Uncorrected		Corrected	
	O_2	Peak value	1/2 Peak	End	0.5 s	1.0 s	0.5 s	1.0 s
	μM		*s*	*s*				
Mitoplasts	7.3 ± 0.8 (8)	23 ± 5.4 (8)	0.21 ± 0.5 (7)	0.29 ± 0.01 (6)	7.0 ± 0.4 (8)	4.5 ± 0.08 (8)	4.6 ± 0.18 (8)	4.0 ± 0.11 (8)
Mitochondria	7.8 ± 0.4 (14)	23 ± 3.2 (14)	0.24 ± 0.03 (14)	0.33 ± 0.02 (13)	7.8 ± 0.3 (14)	4.9 ± 0.2 (14)	5.9 ± 0.2 (14)	4.2 ± 0.2 (14)

II Bulk ratios and amounts

	Burst H^+/O		H^+/O Ratio at			Total changes during burst	
	Peak value	Time	1 s	2 s	3 s	H^+	0
		s				*ngA/mg protein*	
Mitoplasts	13.9 ± 1.8 (8)	0.14 ± 0.01 (8)	5.4 ± 0.10 (8)	4.8 ± 0.07 (8)	4.6 ± 0.09 (7)	4.1 ± 0.3 (6)	0.44 ± 0.06 (6)
Mitochondria	17.7 ± 1.4 (14)	0.14 ± 0.01 (14)	7.0 ± 0.1 (14)	5.6 ± 0.02 (14)	5.1 ± 0.02 (14)	7.3 ± 0.5 (13)	0.61 ± 0.05 (13)

The incubation medium and procedure are as described in the legends to Figs. 1 and 2 and in the Experimental Procedures section with succinate as substrate (−antimycin and −cytochrome *c*). The incubations were at 25°C and either mitoplasts or mitochondria were used. The dynamic ratios were computed from corrected values of dH^+/dt and dO/dt updated every 10 ms. The bulk ratios were computed as described in the legend to Fig. 4. Data are presented as the average ± SEM for the numbers of experiments in parentheses.

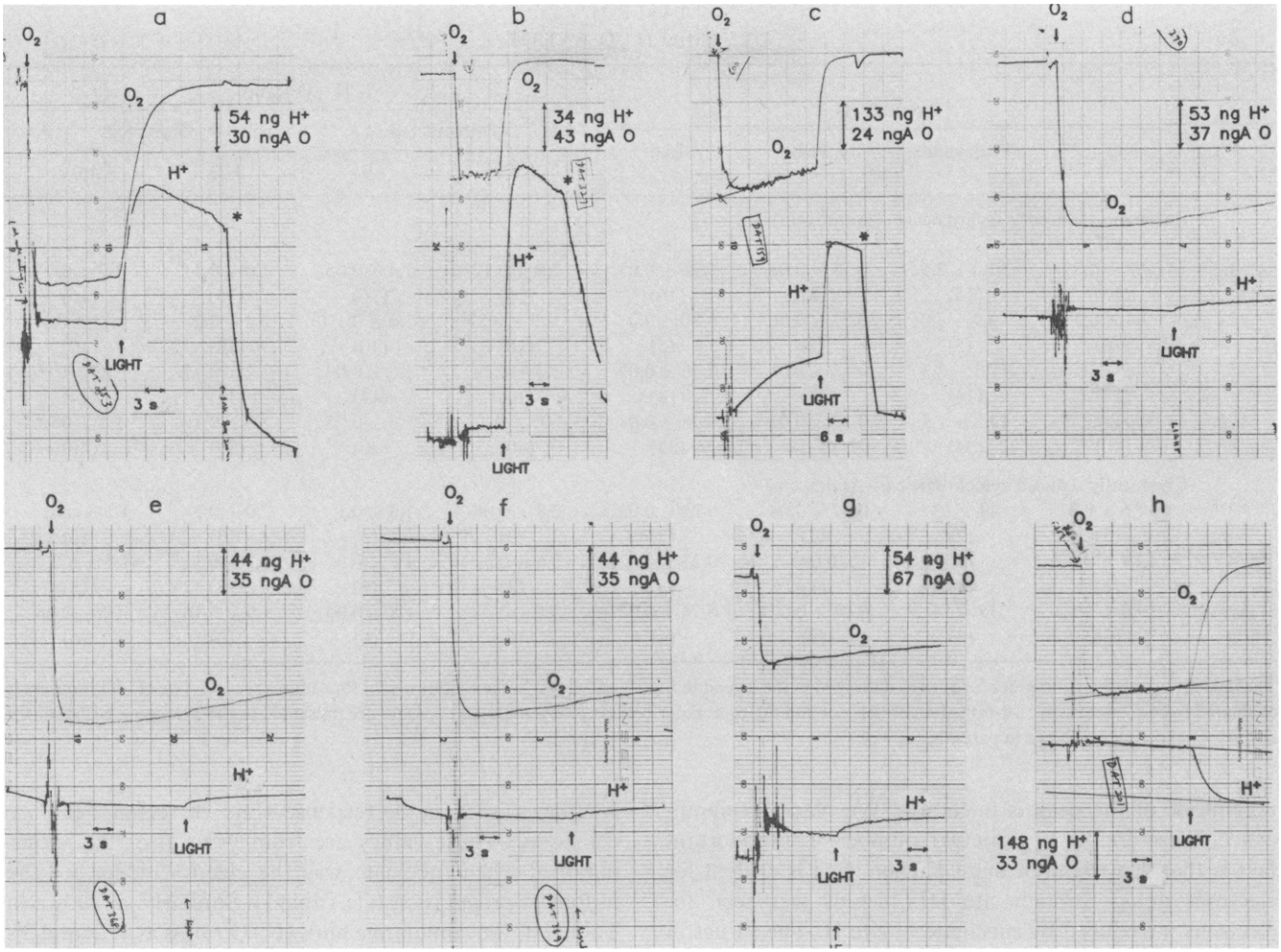

FIGURE 7 Control experiments. All panels show the raw $[O_2]$ and $[H^+]$ data collected from the electrodes. The respiratory pulse was initiated by opening a photographic shutter to illuminate the suspension and photolyze the CO complex at the time indicated by the arrow. The medium and conditions of incubation were as described in the Methods section and in the legends to Figs. 1 and 2 except for specific changes as noted here. (a) Incubation at 10°C with in situ reduced cytochrome *c* as electron donor. (*b*) Incubation at 10°C with chemically reduced cytochrome *c* as electron donor. (*c*) Incubation at 25°C with succinate as electron donor in the absence of antimycin. (*d*) Incubation at 10°C with succinate as electron donor in the presence of 0.25 μM antimycin. (*e*) Incubation at 10°C in the absence of added electron donor. (*f*) Incubation at 10°C in the absence of added electron donor in the presence of 15 μM FCCP. (*g*) Incubation at 10°C after a prior pulse had exhausted the in situ reduced cytochrome *c*. This confirms the finding shown in *d*, that antimycin prevents electrons from succinate passing through sites II and III during the course of the respiratory pulse. (*h*) Incubation at 10°C with cytochrome *c* reduced in situ as electron donor in the presence of 15 μM FCCP. The asterisks in *a*, *b*, and *c* denote where the chart speed was decreased to 1 cm per 3 min, 36 s, and 6 min, respectively.

there was negligible light-stimulated respiration H^+ translocation (*e*). The same indication of a lack of endogenous electron donors and cytochrome *c* is seen in *f* where in the presence of FCCP, there is no light-stimulated O_2 uptake or alkalinization. *g* is similar to *d* in showing that the large excess of succinate, which remains after the reduction of cytochrome *c* in situ, does not serve as a reducing pool for cytochrome *c* when antimycin is present. In this case, the cytochrome *c* reduced in situ was oxidized by a prior respiratory pulse. Subsequent illumination did not lead to further O_2 uptake and caused only a minor amount of medium acidification. Finally, it is shown in *h* that a respiratory pulse, with cytochrome *c* reduced in situ in the presence of FCCP, leads to prompt net alkalinization of the medium with a stoichiometry close to -2.0, which represents the stoichiometry of the reduction of O_2 to 2 H_2O.

Summary of Results

Four preparations of mitoplasts were examined for rates and stoichiometries of proton fluxes in relation to O_2 uptake. Table II shows a summary of data obtained from measurements of O_2 and pH at 10-ms intervals. The results are quite similar with either cytochrome *c* in situ reduced or cytochrome *c* reduced exogenously as the electron source. The only indicated difference is that the peak with exogenously reduced cytochrome *c* may be more narrow (i.e., ½ peak at ~0.08 s compared with ~0.17 s). The actual

TABLE II
DYNAMIC H^+/O RATIOS

Prep. No.	Burst H^+/O				H^+/O Ratio			
					Uncorrected		Corrected	
	O_2	Peak value	½ Peak	End	0.5 s	1.0 s	0.5 s	1.0 s
	Succinate-reduced cytochrome *c* (+ Antimycin)							
	μM		*s*	*s*				
1	9.8 ± 0.5	14.0 ± 2.5	0.17 ± 0.05	0.26 ± 0.03	2.9 ± 0.17	1.4 ± 0.05	1.6 ± 0.11	1.2 ± 0.07
	(16)	(15)	(15)	(16)	(16)	(16)	(15)	(16)
2	7.4 ± 0.5	9.9 ± 3.0	0.23 ± 0.06	0.30 ± 0.0	2.9 ± 0.23	1.6 ± 0.1	2.2 ± 0.2	1.3 ± 0.1
	(10)	(5)	(5)	(5)	(10)	(10)	(10)	(10)
3	9.2 ± 0.3	17.3 ± 2.3	0.18 ± 0.01	0.30 ± 0.02	3.3 ± 0.28	1.6 ± 0.09	2.2 ± 0.17	1.23 ± 0.07
	(14)	(14)	(14)	(14)	(14)	(14)	(14)	(14)
Avg.	9.0 ± 0.25	14.8 ± 1.5	0.17 ± 0.02	0.28 ± 0.01	3.05 ± 0.13	1.5 ± 0.05	2.0 ± 0.1	1.2 ± 0.04
	(40)	(34)	(33)	(33)	(40)	(40)	(39)	(40)
	Chemically reduced cytochrome *c* (−Antimycin)							
2	7.6 ± 0.5	47 ± 13	0.07 ± 0.04	0.28 ± 0.02	2.8 ± 0.64	1.8 ± 0.1	2.0 ± 0.3	1.5 ± 0.01
	(3)	(2)	(2)	(3)	(3)	(3)	(3)	(3)
4	12.7 ± 0.6	11	0.09	0.23 ± 0.02	3.6 ± 0.5	1.7 ± 0.0	1.7 ± 0.1	1.5 ± 0.0
	(3)	(1)	(1)	(3)	(3)	(3)	(3)	(3)
Avg.	9.5 ± 0.8	35 ± 14	0.08 ± 0.02	0.26 ± 0.02	3.2 ± 0.41	1.8 ± 0.06	1.9 ± 0.16	1.5 ± 0.06
	(6)	(3)	(3)	(6)	(6)	(6)	(6)	(6)

The incubation medium and procedure are as described in the legend to Figs. 1 and 2 and in the Experimental Procedures section. The H^+/O ratios were computed from the successive 10-ms values of dH^+/dt and $d0/dt$ as described in the legend to Fig. 3. Data are presented as the average ± SEM for the number of experiments shown in parentheses.

magnitude of the peak is uncertain. We place no significance, therefore, on a particular number. The important fact is that some kind of burst phenomenon is evident for electrons going through site III, just as was seen for electrons introduced by succinate at site II. The values of H^+/O ratios seen at 0.5 and 1.0 s from cytochrome *c* reduced in situ or exogenously are essentially the same whether raw data or response time corrected data are compared. These values are from ½ to ⅓ of the values obtained when succinate was the electron donor for the mitoplast preparation (cf. Table I). Similarly, as shown in Table III, the cumulative bulk H^+/O ratios at 1, 2, and 3 s are the same for the series with in situ reduced cytochrome *c* as for those using chemically reduced cytochrome *c*. The

TABLE III
BULK H^+/O RATIOS

Prep. No.	Peak H^+/O		H^+/O Ratio			Total changes during burst	
	Value	s	1 s	2 s	3 s	H^+	0
	Succinate-reduced cytochrome *c* (+Antimycin)					*ngA/mg protein*	
1	8.7 ± 1.1	0.11 ± 0.01	2.0 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.1 ± 0.14	0.24 ± 0.04
	(16)	(16)	(16)	(16)	(16)	(15)	(15)
2	9.1 ± 2.4	0.16 ± 0.05	2.7 ± 0.2	1.9 ± 0.1	1.7 ± 0.1	2.3 ± 0.2	0.44 ± 0.09
	(8)	(8)	(11)	(11)	(11)	(8)	(8)
3	10.9 ± 1.1	0.13 ± 0.02	2.9 ± 0.21	2.0 ± 0.1	1.6 ± 0.1	1.7 ± 0.2	0.23 ± 0.03
	(14)	(14)	(14)	(14)	(14)	(14)	(14)
Avg.	9.6 ± 0.8	0.13 ± 0.01	2.5 ± 0.1	1.8 ± 0.05	1.5 ± 0.04	1.6 ± 0.12	0.28 ± 0.03
	(38)	(38)	(41)	(41)	(41)	(37)	(37)
	Chemically reduced cytochrome *c* (−Antimycin)						
2	20.0 ± 0.8	0.09 ± 0.02	3.1 ± 0.5	2.1 ± 0.1	1.9 ± 0.0	2.5 ± 0.4	0.26 ± 0.13
	(2)	(2)	(3)	(3)	(3)	(3)	(3)
4	24 ± 18	0.11 ± 0.02	2.4 ± 0.2	1.9 ± 0.1	1.7 ± 0.0	1.2 ± 0.1	0.19 ± 0.15
	(2)	(2)	(3)	(3)	(3)	(3)	(2)
Avg.	21.9 ± 7.4	0.10 ± 0.01	2.7 ± 0.3	2.0 ± 0.06	1.8 ± 0.04	1.8 ± 0.32	0.23 ± 0.09
	(4)	(4)	(6)	(6)	(6)	(6)	(5)

The data obtained in the experiments summarized in Table II were recomputed as described in the legend to Fig. 4 to determine the cumulative bulk $H^+/0$ ratios shown here. Data are presented as the average ± SEM for the number of experiments in parentheses.

H^+/O ratios are also from ½ to ⅓ of those seen when succinate was the electron donor (cf. Table I).

Zero Time H^+/O Ratios Obtained by Back Extrapolation of Single Exponential Plots

There has been considerable controversy as to whether the zero time (i.e., "level flow," "mechanistic") H^+/O ratio is 4.0 or 2.0 for electrons going through site III to O_2. The numbers obtained from different laboratories have involved extrapolations of data obtained either during the acidification phase or during the subsequent alkalinization phase which accompanies a respiratory pulse, on the assumption that the kinetics of H^+ fluxes and O_2 uptake adhere to single exponential laws. When we fit the O_2 and pH data from 0.8 to 3.0 s assuming single exponential behavior, we obtained a ratio of 2.1 ± 0.21 for nine experiments using cytochrome *c* reduced in situ and 2.2 ± 0.15 for five experiments using chemically reduced cytochrome *c*. In our earlier papers, we discussed the weaknesses of such extrapolation methods (1, 2). There are theoretical reasons why neither the O_2 consumption behavior nor the pH changes would be expected to obey single exponential kinetics. There are also experimental observations that show that the data are not properly expressed by single exponentials (2). This is very easily demonstrated in the case of the pH data. For illustration, an experiment is taken in which the H^+/O ratio extrapolated to zero time was 2.7 when data in the range of 0.8–3 s were used. As shown in Fig. 8 *a*, the value depends on the range of data that is fit to the one exponential expression. Either by extending the later time point (*solid line*) or the earlier time point (*dashed line*), a variety of zero time extrapolation values can be obtained. In this approach, uncorrected electrode data are used. Therefore, the curves shown in Fig. 8 *a* will be shifted and distorted when different O_2 and pH electrodes (with different time constants) are used. A single laboratory repeating the experiment using the same electrodes will then be expected to confirm its own values anywhere in the spectrum of possible values indicated in the figure. The reason for this behavior is that the experimental curve for pH vs. time contains more information than can be accounted for by a single exponential. Forcing a single exponential fit to different portions of the curve will emphasize local kinetic information in the selected part of the curve, which will dominate and distort the value of the single exponential constant. If a single exponential O_2 consumption process is responsible for a concomitant proton translocation process, the single exponential constants for the two processes should be the same (i.e., $k_{H+}/k_O = 1$). We find, however, that the ratio of the two constants varies with the portion of the pH curve used for fitting. There is a smooth relationship between the value of the k_{H+}/k_O ratio and the value of the zero time extrapolation for the H^+/O ratio (Fig. 8 *b*). The solid and dashed portions of the curve shown in Fig. 8 *b* were taken from the

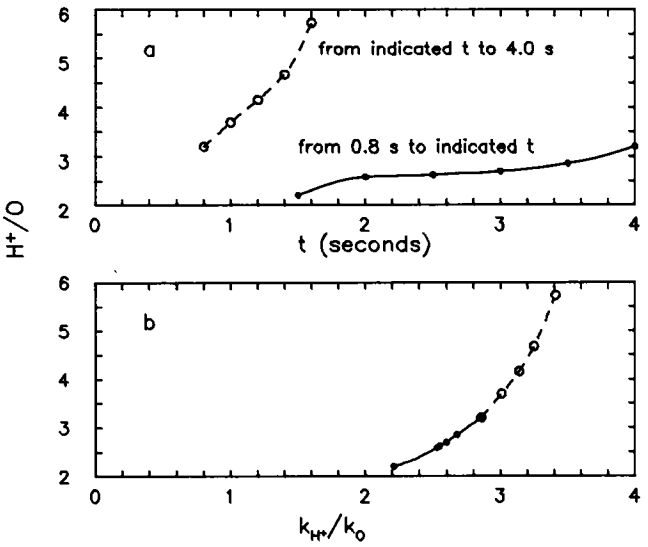

FIGURE 8 The pH data in an experiment with cytochrome *c* reduced in situ as substrate were fit to the one exponential expression $[H^+]_t = [H^+]_0 e^{-k'H'}$ using data collected over different time periods and then the theoretical (fitted) curve was extrapolated back to zero time to obtain the initial dH^+/dt. The ratio of this value to the extrapolated zero time dO/dt obtained from a one exponential fitting of the O_2 data from 0.8 to 5 s was taken as the zero time H^+/O ratio. In *a*, the solid line shows the H^+/O ratios obtained when the pH data used for fitting were from 0.8 s to the indicated time. The dashed line shows the H^+/O ratios when the pH data used for fitting were from the indicated time to 4.0 s. The variations in H^+/O ratio result from the fact that the $[H^+]$ vs. time curve is not a true single exponential function and this is revealed by the fact that the value of k_H obtained in the process varies with the different time frames used for fitting. In *b* the "zero time" H^+/O ratio is shown as a function of k_H/k_O obtained using the k_H values in the one exponential fittings of the H^+ data shown in *a* with the K_o obtained in the one exponential fitting of the O data.

values of the constants from the fittings for the solid and dashed line curves in Fig. 8 *a*. A corresponding case can be made for the values of the zero time extrapolation values for H^+/O ratio obtained with succinate as electron donor to sites II and III in the rate liver mitochondria system (2).

Experiments in the Presence of FCCP

When 15 μM FCCP was present, the bulk H^+/O ratio at 5 s was −1.94 ± 0.17 (seven experiments), which is what would be expected from the stoichiometry of the net reaction at site III. However, at earlier times such as at 2 and 1 s, the ratio was more negative. The greater deficit in H^+ disappearance at the earliest times is also seen in the dynamic H^+/O ratios which peaked at a ratio of −13.1 ± 2.9 at <50 ms and in the bulk changes in H^+ and O during the time course of the burst peak phenomenon, which was 3.3 ngA H^+ disappearing for 0.6 ngA oxygen consumed per milligram of mitochondrial protein. 2.1 ngA more of H^+ disappeared then would have been expected from the amount of oxygen consumed. This is ~10 ngA H^+ per nmol of 200,000-mol-wt enzyme (i.e., 0.2 nmol enzyme/mg

mitochondrial protein). If protons are translocated by site III, it is generally accepted that ionizable groups of the enzyme may be involved in taking up protons on one side of the membrane and discharging them on the other. If the process of dissociating the protons requires the formation of a $\Delta\tilde{\mu}_{H+}$, then the presence of the FCCP may allow the binding of protons to the enzyme when oxygen is bound but prevent the subsequent release of bound protons which normally would accompany the intramolecular reduction of oxygen. It is possible, therefore, that the techniques used in this study are able to record the initial loading of the enzyme with protons.

DISCUSSION

In the current investigation, we have applied recently described techniques that enable the instantaneous start of a respiratory pulse in a completely mixed suspension of electron donor, mitoplasts, and O_2, and the continuous monitoring of $[O_2]$ and pH by rapidly responding electrodes. Electrode responses have been corrected for initial delays using experimentally determined relaxation constants. In our previous paper, we described a phenomenon in which there was an initial burst in medium acidification which peaked within 50 ms of the start of respiration with succinate as substrate in a rat liver mitochondrial system (1). The initial burst in acidification was complete by 300 ms, after which the time course of acidification followed an apparent first order course until close to the "turn around," where alkalinization was caused by the net return of extruded protons to the mitochondria. Similar observations are reported here for electrons passing through site III to O_2, although the magnitude of the burst is ~⅓ to ½ of that seen for electrons passing through sites II and III. This same phenomenon was discovered by Penniston et al. in 1971 (3) and confirmed by Papa and co-workers (4, 5). Both of these groups used rapid mixing and continuous flow techniques capable of sampling pH at a series of fixed times after mixing anaerobic mitochondria or submitochondrial particles with substrate and oxygenated buffer. Using a different approach, we have obtained a continuous record of pH readings from zero time onwards. Our results are entirely consistent with the earlier findings in that we see an initial burst in medium acidification appears to peak within 50 ms and then to decay to a slower rate of acidification within 300 ms.

It is important to discuss the possible influence electrode relaxation phenomena might have on the data obtained in this and our previous studies. In this connection, certain experimental controls described in our earlier paper (1) will be briefly reviewed. The electrode behavior displayed by both electrodes during their first second of response was well represented by a first order equation, and there was no indication of an initial lag in the response of the O_2 electrode (see also reference 7). When both electrodes were placed in a flow cell and subjected to a simultaneous step change in H^+ and O_2 concentrations, there was no artifactual initial burst in the H^+/O ratio of the corrected responses from the two electrodes. The pH data obtained from the respiratory pulse experiments showed an initial rapid phase of acidification during the first 0.3 s followed by a slower rate of acidification. It was shown that the first order equation used to correct the pH data could not introduce such biphasic behavior to an electrode response which was truly monophasic. It is recognized that the corrected values become more uncertain as the correction is applied to data obtained in times that are small compared with the relaxation time of the electrode (~0.2 s for each electrode). For this reason, we do not place any emphasis on particular numerical values during the first two-tenths of a second. However, that the H^+/O ratios continued to decline from values well above 6 or 8 when succinate was the electron donor (1) and above 2 or 4 when cytochrome *c* was the electron donor (this paper) out to three-tenths of a second, seems to be well established and significant.

Although our results on the initial rates of acidification agree with those of Penniston and of Papa et al., our results on initial rates of O_2 consumption are markedly different from those two groups and from those of Reynafarje (8, 9) and of West et al. (10), all of whom found an initial burst in $-dO/dt$. The most obvious possible explanation is that the O_2 electrodes we used show an initial delay in response that is not properly accounted for by the correction procedures. However, we can discount this on two grounds. First of all, the O_2 electrodes that we used were the same as used by Reynafarje and were prepared in his laboratory. Second, as described in detail in our previous paper (1) and briefly above, a variety of controls were performed to show that neither the pH nor O_2 electrodes displayed any peculiar start-up phenomenon that could account for our observations. We can also discount the possibility that either the mitochondria or mitoplasts that were used were in some way different, because the same results were obtained with mitochondria prepared in Dr. Reynafarje's laboratory as in our own and with mitoplasts prepared in Dr. Reynafarje's laboratory. Therefore, another explanation is required, and we propose the following. It is appreciated that cytochrome oxidase is an exceedingly complex enzyme that is not thoroughly understood. The enzyme can exist in different forms, one of which is enzymatically more active and known as the "pulsed" form (11). The resting enzyme is converted to the pulsed form by cycling through a state of oxidation and reduction. In the studies where there was an initial rapid phase of O_2 consumption, the enzyme went from air-oxidized to substrate-reduced before initiation of the respiratory pulse. In our procedure, the reduced enzyme was liganded to CO in the presence of O_2 and substrate before the initiation of respiration by photolysis of the CO complex. The absence of an initial burst in O_2 consumption may be attributed to a different form of cytochrome oxidase at the start. In any

case, under the conditions we have used, the burst in acidification has been separated from the burst in O_2 consumption.

In addition to the initial burst in acidification accompanying a respiratory pulse with reduced cytochrome *c* as electron donor in the absence of uncoupler, a negative burst (i.e., alkalinization) of the same magnitude was seen in the presence of FCCP. This kind of behavior might be expected if cytochrome *c* oxidase functions as a proton pump. The binding of O_2 might enable the enzyme to bind translocatable protons from the matrix side and move them to the cytoplasmic side. The local $\Delta\tilde{\mu}_{H+}$ developed could be an important factor in the release of the translocated protons on the cytoplasmic side, if for example the pKs of the binding groups were influenced by $\Delta\psi$ (see reference 12). During the first turnover in the absence of uncoupler, the full load of bound H^+ could be released. The rapid build-up of $\Delta\tilde{\mu}_{H+}$ attending further turnover would hasten the back-leak processes, and eventually, upon exhaustion of the O_2 pulse, the familiar alkalinization phase would follow. In the presence of FCCP, the translocatable protons would be bound but not released. The first turnover, therefore, would produce a deficit in the number of protons equal to the number bound by the enzyme plus the four scaler protons used in the reduction of O_2 to H_2O. During subsequent turnovers, as long as O_2 is present, four protons would be removed for each O_2 consumed. After exhaustion of the O_2, the bound protons might be slowly released, leading to a H^+/O ratio that is less negative than -2.0. We have frequently seen the H^+/O ratio in the presence of FCCP change from more negative than -2.0 in the early phases to less negative than -2.0 in later phases. A similar shortfall in the negative H^+/O stoichiometry has been discussed recently by West et al. (10).

As a result of the studies reported in this and our two previous papers, we suggest that mechanistic H^+/O ratios determined by extrapolation procedures based on assumptions of single exponential behaviors are of questionable validity. The initial proton burst phenomenon, seen in this work with cytochrome *c* as electron donor and previously with succinate as electron donor, as well as similar phenomena described previously (3–5), indicates a pre-steady state phenomenon of potential significance in the mechanism of proton pumping and/or scaler proton release.

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