Comparison of Rates of Proton Ejection and Oxygen Consumption within 300 msec after Oxygenation of Beef Heart Mitochondria†

John T. Penniston‡

ABSTRACT: Suspensions of beef heart mitochondria, in the presence of appropriate inducing reagents, showed a rapid burst of respiration and of proton ejection. A continuous flow apparatus with a time resolution of 10 msec was utilized to measure the rates of proton ejection and oxygen consumption on a single batch of mitochondria under constant conditions. Fluorescein mercuric acetate and gramicidin were used as inducing agents, and H^+: O ratios were determined during the first 300 msec after oxygenation, and also over the first several seconds after oxygenation. The longer time experiments showed that the rapid phases of oxygen consumption and proton ejection ran concurrently. When oxygen consumption slowed, proton ejection slowed even more drastically, so that the H^+: O ratio fell after the first second. H^+: O ratios measured during the first 300 msec were higher than those measured at longer times.

The theoretical importance of the ratio of proton ejection to oxygen consumption in mitochondria has been developed extensively (Mitchell and Moyle, 1965, 1967a) and their point of view has been critically discussed (Slater, 1967; Racker, 1970). Mitchell emphasized heavily the stoichiometry of protons ejected to oxygen consumed as an experimental basis for the chemiosmotic theory. While other studies have also emphasized the stoichiometry of either proton ejection (Hinkle and Horstman, 1971) or ion accumulation (Harris *et al.*, 1967a), all of these studies have been made with rather poor time resolution. These measurements have been made by means of electrodes or chemical assays, and the time resolution has ranged from a few seconds to a minute. The processes involved are in fact quite rapid, and the parameters which control the rate of process are continually changing. For example, in experiments of the type performed by Mitchell (Mitchell and Moyle, 1967a), the pH change occurs so rapidly that only the rate of response of the pH electrode is observed, necessitating extrapolations back to zero time. Previous papers have discussed the kinetics of the proton ejection by mitochondria (Penniston *et al.*, 1971) and the kinetics of oxygen consumption by mitochondria (Penniston, 1972), both of these being measured immediately after oxygenation of the mitochondrial suspension. It was clear from these papers that conventional methods could not measure the rates of proton ejection and oxygen consumption. This article presents the results of measurements of both oxygen consumption and proton ejection *vs.* time. In every case both types of measurement were made on a single preparation of beef heart mitochondria within 5 hr of preparation, allowing the direct comparison of the rate of oxygen consumption with the rate of proton ejection.

Experimental Procedures

Preparation of mitochondria and measurements utilizing the continuous flow apparatus were performed as previously described (Penniston *et al.*, 1971; Penniston, 1972). The following modifications were made in order to accurately measure the pH changes and changes in oxygen tension which occurred.

An accurate primary standard was used for calibration of the oxygen electrode; an analyzed mixture of oxygen and nitrogen of the appropriate composition (Matheson Corporation) was equilibrated with water at a constant pressure, by means of a Matheson Model 71S low pressure regulator. The regulator valves were kept open, so that as water was removed from the calibration bottle, gas at a constant composition flowed into the bottle and the partial pressure of oxygen above the water was kept constant. Thus water with a known dissolved oxygen content was available, and the oxygen electrode was calibrated against this primary standard every few minutes throughout each experiment.

Similarly it was necessary to calibrate the buffer power of the suspension. This was done by making the aerobic medium 100 μM in HCl and adding to the mitochondrial suspension 1 μg of antimycin/mg of mitochondrial protein. The continuous flow experiment was then performed both with the medium made 100 μM in HCl and with medium to which no HCl had been added. The difference in pH between these two experiments was taken as the pH difference caused by making the mitochondrial suspension (after mixing) 50 μM in HCl. This titration in the continuous flow apparatus was performed using the shortest tubes, which gave measurements at a time of about 25–40 msec, using a moderate flow rate. Thus the titration utilized was a short time titration, which presumably does not include the titration of the inside of the mitochondria (Mitchell and Moyle, 1967b). This is the appropriate parameter to measure for this type of experiment, since the acidification of the outside space by mitochondria upon energization was being measured, therefore making it undesirable to include the titration of the inside of the mitochondria in the calibration measurements. This titration experiment was carried out at the end of each pH run reported, in order that an accurate calibration of the pH changes would be available for

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‡ Established Investigator, American Heart Association. Request reprints from the Chapel Hill address.

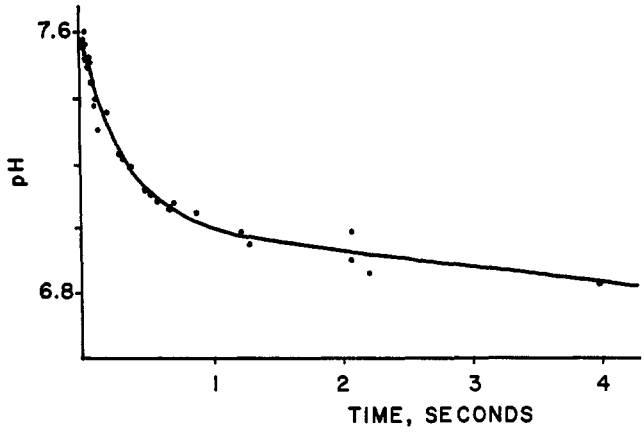

FIGURE 1: pH *vs.* time after oxygenation; mitochondria treated with 30 μM FMA. Temperature was 24.5; oxidizable substrates were 1.5 mM potassium pyruvate and 1.5 mM potassium malate. The solid lines in Figures 1–4 represent the best fit of the equation (given under Experimental Procedures) to the data. *A* was −0.562 pH unit, *B* was 0.00298 $msec^{-1}$, and *C* was −0.0000465 pH unit/msec.

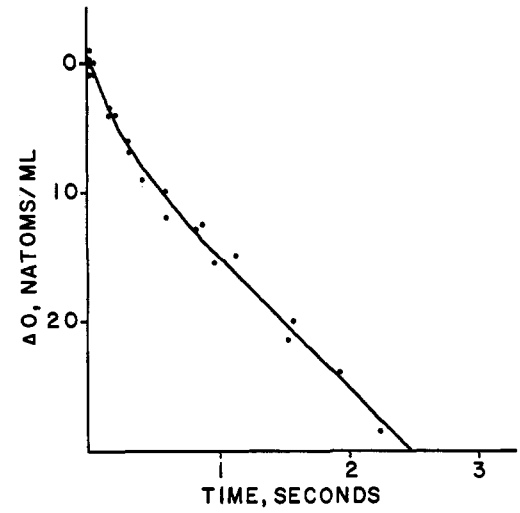

FIGURE 2: Oxygen tension *vs.* time after oxygenation; mitochondria treated with FMA. Same batch of mitochondria and same conditions as Figure 1. Initial oxygen concentration was 50 natoms/ml. For these data, *A* was 5.78 natoms/ml, *B* was 0.00374 $msec^{-1}$, and *C* was 0.0100 natom ml^{-1} $msec^{-1}$.

each experiment. The buffer power of the medium increased sharply below pH 7, reducing the accuracy of proton ejection measurements made at lower pH. This was due to the buffering action of the carboxylic acid groups of the oxidizable substrates present. In order to obtain greater accuracy most of the measurements were made at a pH above 7.

All experiments had mitochondria present at a concentration of 3 mg of mitochondrial protein/ml, and the solutions were 0.15 M in sucrose, 0.05 M in KCl, and about 50 μM in oxygen.

The experimental points presented in Figures 1–4 were fitted to an equation of the form

$$Y = Y_0 + A(1 - e^{-Bt}) + Ct$$

The fitting was performed by means of a computer, which performed successive approximations by varying the constants *A*, *B*, and *C*. The best fit was chosen by the criterion of least squares. The lines generated are shown in the figures.

In order to calculate the H^+:O ratios, the experimental points were fitted to a straight line by the least-squares method and the standard deviation of the slope of the line was determined. The ratio of the slope of the proton ejection plot to that of the oxygen consumption plot gave the H^+:O ratio, and the standard deviation of this ratio was calculated from the standard deviations of the slopes by means of the general equation for the propagation of errors.

Results

Figures 1 and 2 show the oxygen consumption and proton ejection by a suspension of mitochondria to which FMA[1] had been added. In this experiment the substrates utilized were pyruvate and malate, and no inhibitors of respiration were added. It is clear from these two figures that both respiration and proton ejection occurred at a very rapid rate during the first half-second and both processes then slowed down concurrently.

In the quantitative fitting of a line to the experimental points, the time constant of the transient phase is isolated in the constant *B*. The value of *B* obtained from the oxygen consumption data can be compared directly with the value of *B* obtained from pH *vs.* time data, since the units of scale are expressed in the constants *A* and *C*. The values of *B* for proton ejection and oxygen consumption were 0.0030 and 0.0038, respectively, for the experiment performed in the presence of FMA. This would suggest that the rapid phase of the proton ejection was slightly slower than that for oxygen consumption. The two values of *B* were rather close to one another, and the value of *B* for the oxygen consumption data was probably artificially increased by the nature of the curve fitting process. This occurred because of the relatively rapid constant rate of respiration which followed the initial burst in Figure 2. The tail of the exponential function disappeared more quickly into this rapid rate of respiration, so that a "shorter lived" exponential with a higher value of *B* produced the best fit in this case. In any case, it was clear that the rapid phases of the two processes ran concurrently.

Similar results, observed when proton ejection by mitochondria was induced by addition of gramicidin to the medium, are shown in Figures 3 and 4. In this case, too, the biphasic nature of both curves was observed and the points at which respiration and proton ejection slowed down coincided quite well. The values of *B* were 0.0017 and 0.0015 for proton ejection and oxygen consumption, respectively. These two values were identical within the limits of experimental error.

Figures 1 and 2 and also Figures 3 and 4 represent pairs of experiments in which each pair was done on a single batch of mitochondria, and thus it is valid to calculate H^+:O ratios from these data. This was done both for the short time points and for the longer time points and results are shown in Table I. It was quite clear that the H^+:O ratio was high during the first few hundred milliseconds after aeration, and that it subsequently declined. For simplicity in calculation, straight lines were fitted to the data in order to obtain H^+:O ratios. Over the limited time ranges utilized this was not significantly less accurate than the use of exponential functions.

It was recognized that it would be desirable to obtain results similar to those shown in Figures 1–4, utilizing valinomycin as

[1] Unusual abbreviations: FMA, fluorescein mercuric acetate; H^+:O ratio, molar ratio of proton ejection to oxygen consumption.

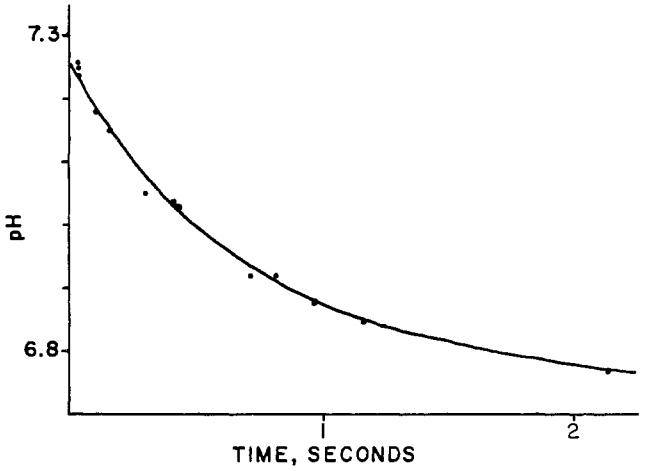

FIGURE 3: pH *vs.* time after oxygenation; mitochondria treated with gramicidin. After mixing, the suspension was 1 ng/ml in gramicidin; oxidizable substrates were pyruvate and malate at the same concentrations shown for Figure 1. Temperature was 24.5°; oxidizable substrates were pyruvate and malate. For these data, A was −0.436, B was 0.00166, and C was −0.0000284. The units of A, B, and C were the same as for Figure 1.

the inducing reagent. However, the pattern of acidification with valinomycin varied with time for an hour after the valinomycin was added, making it impossible to carry out continuous flow experiments utilizing valinomycin.

In order to evaluate more carefully the validity of the measurements of the buffer power, measurements were made both at short times, as was routinely done in the H^+: O measurements, and at longer times as well. When the amount of HCl added was only 50 μM, as was true for the routine calibration, any changes in the pH, between 25 msec and several seconds after mixing, were so small as not to be accurately measurable. In an effort to measure more accurately the pH changes which ensued after the rapid phase of titration of the mitochondria, the amount of acid utilized for the titration was increased. Table II shows measurements of the buffer power at both short (less than 50 msec) and long (about 6 sec) times after the titration. Even though a threefold greater amount of HCl was used, the pH changes after the titration were still quite small. Even after a sudden pH drop of about 0.5 pH unit, the subsequent increase in pH is almost negligibly small.

In order to obtain an accurate measure of the maximum possible extent and of the kinetics of the small pH increase which followed the buffer titration, both the protein concentration and the HCl concentration were further increased, and the mitochondria were treated with both an ionophore and an uncoupler. This combination of reagents should maximize the permeability of the mitochondrial membrane to protons (Mitchell and Moyle, 1967b). Thus changes which may occur in the apparent buffer power of the medium during the first few seconds after mixing should be exaggerated, if these changes are due to titration of the interior of the mitochon-

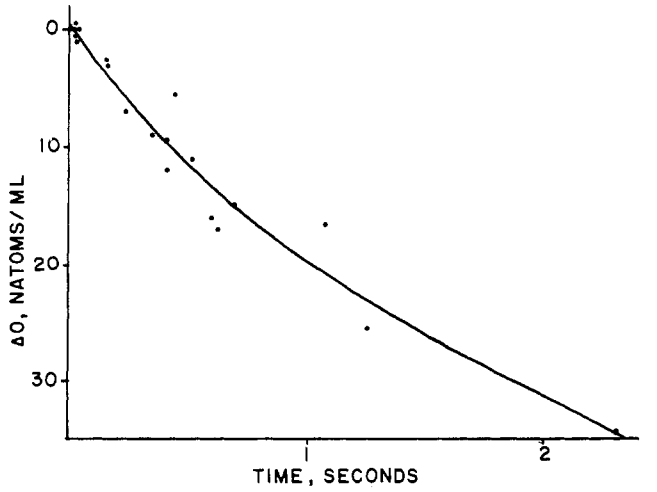

FIGURE 4: Oxygen tension *vs.* time after oxygenation; mitochondria treated with gramicidin. Same batch of mitochondria and same conditions as Figure 3. Initial oxygen concentration was 41 natoms/ml. A was 14.5, B was 0.00150, and C was 0.0091. The units of A, B, and C were the same as for Figure 2.

TABLE I: Comparison of H^+:O Ratios for Short and Long Times after Oxygenation of Mitochondria.[a]

Times Analyzed (msec)	Reagent Added	Rates (natoms, ml^{-1} sec^{-1}) H^+ Ejection	O Consumption	H^+:O	S[b]
<450	FMA	145	23	6.2	±0.6
>450	FMA	28	10.5	2.7	±0.3
<700	Gramicidin	166	25	6.7	±0.6
>650	Gramicidin	55	12.4	4.4	±1.1

[a] These data are from the same experiments shown in Figures 1–4. Since NADH-linked substrates were employed, the theoretical H^+: O ratio was 6.0. [b] S is the standard deviation of the H^+:O ratio, calculated from the standard deviations of the slopes of the H^+ ejection and O consumption curves.

TABLE II: Measurements of Buffer Power.[a]

Additive	Time after Mixing (msec)	pH	Buffer Power ($\Delta\mu$M H^+/ΔpH)
None	26	6.81	288
	47	6.80	283
	6410	6.81	288
FMA	26	6.92	300
	5930	6.945	316

[a] Conditions were as already described, except that antimycin was added to the mitochondrial suspension; the concentration of antimycin after mixing was 2.4 μg/ml; the HCl was added to the medium, and its final concentration after mixing was 150 μM. When mitochondria treated with antimycin were mixed with medium, the pH showed no trend with time over a period of 4 sec after mixing, and the initial pH of the mixture was 7.33. The corresponding pH for mitochondria treated with antimycin and FMA was 7.42. The buffer powers calculated for this experiment are higher than those given for a comparable initial pH in Figure 6, because the larger HCl concentration caused the titration range to extend to a more acid pH.

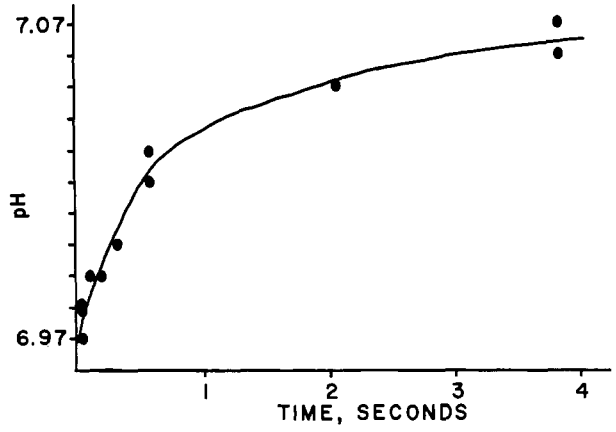

FIGURE 5: Rebound of pH after titration under extreme conditions. The concentrations of acid and of mitochondria were increased fourfold, and both ionophore and uncoupler were present to maximize membrane permeability to protons. The suspension medium was the same as in the other experiments, but the concentrations of other reagents were as follows: mitochondria, 12 mg of protein/ml; antimycin, 10 μg/ml; potassium succinate, 5 mM; HCl, 200 μM; gramicidin, 15 ng/ml; pentachlorophenol, 30 μM.

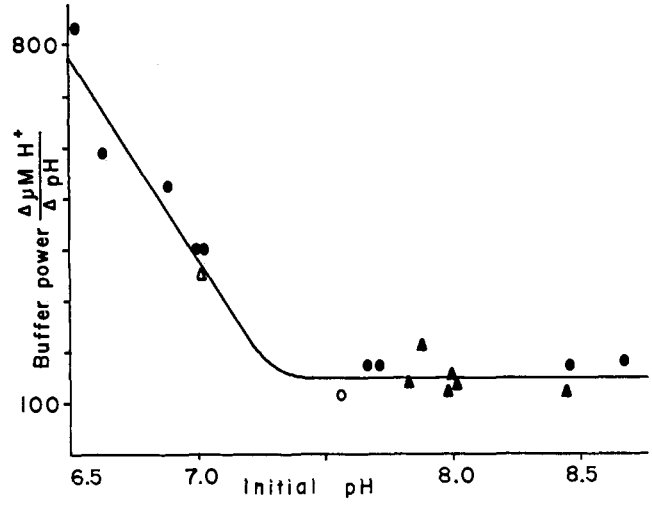

FIGURE 6: Dependence of buffer power on pH. Buffer power was determined as described in the text. The initial pH was the pH obtained upon mixing antimycin-inhibited mitochondria with medium containing no HCl. Circles represent values measured in the presence of FMA, triangles represent values measured in the presence of gramicidin. Open symbols have the same meaning as closed symbols, except that pyruvate and malate were the substrates instead of succinate.

drion. Figure 5 shows the time course of the pH rebound observed under these conditions. In another experiment, utilizing exactly the same conditions as for the experiment shown in Figure 5, the buffer power of the suspension was determined, and values of 277 and 268 $\Delta\mu$M H^+/ΔpH were obtained at a time of 23 msec after mixing; this buffer power rose to 294 at 228 msec and to 299 at 660 msec. This 10% increase in the buffer power at longer times certainly sets the maximum variation which could occur under rather extreme conditions. The data shown in Table II and Figure 5, when taken together with the observation of a negligible change of pH with time when the titration was done under the conditions used for the H^+:O determinations, show the validity of the short time titrations utilized for determination of the buffer power.

Figure 6 shows the variation of the short time buffer power with the initial pH, for the experiments reported here and others of the same type. Clearly the buffer power of the system does not vary with pH if the pH is above 7.5. Values of pH above 7.5 were utilized for the short time points taken for all of the experiments, except the experiment shown in Figure 3, for which the initial pH was 7.25 and the pH after 650 msec, 6.92. For this experiment, and for the longer time points shown in Figures 1 and 3, the buffer power was determined by use of Figure 6. It was assumed that the buffer power increased linearly as pH decreased, using the slope shown in the figure. Since the buffer power is a derivative ($\Delta\mu$M H^+/ΔpH), the number of nanomoles of protons/milliliter for each point was determined by integration along the line. The resulting values were fitted to a straight line for determination of the proton ejection rates and H^+:O ratios reported in Table I.

With a view toward measuring the higher H^+:O ratios of which the system apparently was capable during the first few hundred milliseconds, results were obtained in subsequent experiments utilizing only points in the first 300 msec. Figure 7 represents the data from one such experiment, in which oxygen consumption and proton ejection were plotted on the same graph. Several sets of experiments such as the one shown in Figure 7 were carried out. In each case, proton ejection and oxygen consumption measurements were made on a single batch of mitochondria, within a short period after the mitochondria were prepared. Straight lines were fitted to the points by the linear least-squares method, and the data shown in Table III were thus obtained. The H^+:O ratio predicted by the chemiosmotic theory was 4.0 with succinate as substrate and 6.0 with NADH-linked substrates, and it is clear that the

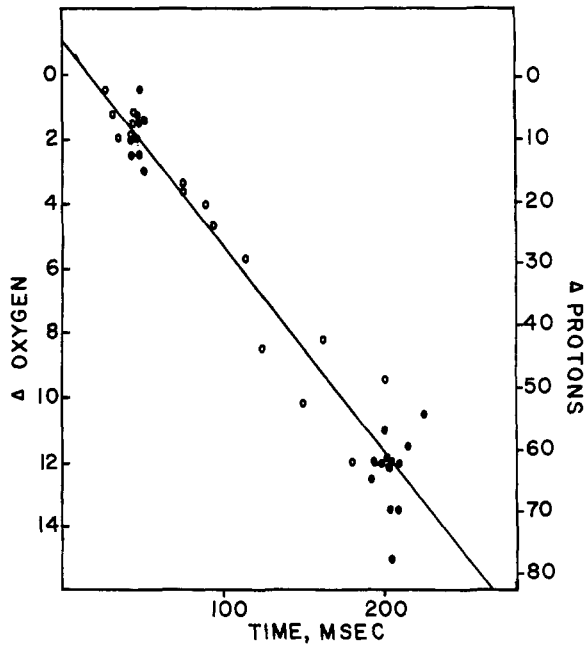

FIGURE 7: Proton ejection and oxygen consumption *vs.* time after oxygenation; mitochondria treated with FMA. Temperature was 24.5°, oxidizable substrate was succinate (5 mM) and rotenone (10 μM) was present. Initial pH was 8.68 and oxygen concentration was 42 natoms/ml. Each set of data was separately fitted with a least-squares straight line, and the figure axes were then adjusted so that the two lines coincided. For proton ejection, the slope was 336 ± 27 natoms ml^{-1} sec^{-1} (standard deviation of slope given). For oxygen consumption, it was 64.2 ± 2.6 natoms ml^{-1} sec^{-1}. The open circles indicate measurements of pH; the closed circles, of oxygen concentration. Both axes are calibrated in natoms/ml.

TABLE III: H^+: O Ratios for Times of Less than 300 msec after Oxygenation.[a]

Reagent Added	Initial pH	Temp (°C)	Rates (natoms, ml^{-1}, sec^{-1}) H^+ Ejection	O Consumption	H^+:O	S
FMA	7.8	24	291	33	8.9	±2.4
FMA	7.6	24.5	161	31	5.2	±0.9
FMA	8.4	24.5	422	70	6.0	±0.5
FMA	8.6	24.5	336	64	5.2	±0.5
Gramicidin	7.9	27	142	20	7.2	±0.6
Gramicidin	7.9	26	173	40	4.4	±0.4
Gramicidin	8.6	22.5	165	34	4.9	±0.8
Gramicidin	7.7	23	128	34	3.7	±0.5
Gramicidin	7.9	23.5	93	37	2.5	±0.3

[a] The number of points taken to obtain each value of the rate of proton ejection or oxygen consumption varied from 11 to 37. Succinate (5 mM) was the oxidizable substrate, and rotenone (10 μM) was present. The H^+: O ratio predicted by the chemiosmotic theory was 4.0.

H^+: O values obtained show a preponderance of values higher than those predicted, particularly the results obtained in the presence of FMA.

The experiments performed in the presence of FMA all gave values of H^+: O higher than predicted by the chemiosmotic theory, and three of the five values obtained (including those reported for short times in both Tables I and II) were more than two standard deviations above the theoretical value, indicating a greater than 97% probability that the true value was greater than the theoretical value for each of these three cases.

Four of the six experiments performed in the presence of gramicidin gave high values of H^+: O, and one of these was more than two standard deviations above the theoretical value.

It was also clear that H^+: O ratios varied from one preparation of mitochondria to another, and that this variation was far outside the limits of accuracy of the measurements. The time range and time resolution available were quite adequate for accurate calculations of the ratio of the rate of proton ejection to the rate of oxygen consumption, and it is thus necessary to explain how such high H^+: O ratios came about.

Discussion

The results presented here show the accurately determined rates of proton ejection and of oxygen consumption by mitochondria during the first 300 msec after oxygenation. The results may most adequately be discussed in terms of a generalized proton pumping model (Massari and Azzone, 1970; Montal *et al.*, 1970a). In such a model, the observed stoichiometry between proton ejection and oxygen consumption is explained in terms of a "proton pump" which moves protons from the inside of the mitochondrion to the outside in a definite stoichiometric ratio. This proton pump could be the electron transfer chain, as proposed by Mitchell, or it could be a pump of some other nature.

In measuring the stoichiometry of such a pump, it is important to measure the *rate* of proton ejection and of oxygen consumption. Measurement of the total amounts of these parameters raises other questions, because the rates of both are rapidly changing, and so also is the ratio H^+: O, as is evident from Table I. Thus the data reported here represent a substantial advance in technique over the techniques previously used to measure H^+: O ratios.

The experiments on antimycin-inhibited mitochondria make it clear that small changes in the buffer power do occur during the first few seconds after an acid titration of antimycin-inhibited mitochondria. However, these changes are of almost negligible magnitude. Under conditions considerably more extreme than those used for the buffer calibrations, changes occurred which would, at most, lead to a 10% increase in the calculated H^+: O ratios. Thus, it can be concluded that the simple short time buffer power measurements are a valid measure of the actual buffer power for all the points measured. The problem of buffer power does not arise in the oxygen measurements; the number of oxygen binding sites in the mitochondrion is very small. This was evident from the experimental observation that the oxygen tension of antimycin-treated mitochondria mixed with medium was routinely half that of the medium alone.

Both of the reagents utilized encourage the movement of cations across the membrane. The action of gramicidin on mitochondria has been thoroughly investigated. At the level utilized in this experiment it acts as an ionophore, complexing univalent cations such as Na^+ or K^+, and allowing them to move across the membrane in exchange for H^+ moving in the opposite direction. It also has a weak tendency to catalyze movement of H^+ across the membrane (Montal *et al.*, 1970b). At considerably higher levels than utilized in this study, gramicidin uncoupled oxidative phosphorylation even in the absence of Na^+ or K^+, but at a level comparable to that used here, this effect is very small (Harris *et al.*, 1967b).

The effect of FMA on mitochondria is less well understood. However, the action of other mercurials on mitochondria has been studied more extensively (Brierley *et al.*, 1968). It was concluded from these studies that low levels of mercurial block uptake; higher levels stimulate potassium acetate uptake, and yet higher levels allow permeability to potassium chloride and release of endogenous potassium ions (Scott *et al.*, 1970). The level of FMA utilized in these experiments was high enough to inhibit phosphate transport (Lee *et al.*, 1969). It also stimulates potassium acetate uptake, and probably allows some permeability to potassium chloride as well.[2] However, mitochondria treated with this level of FMA show a normal amount of matrix material and a normal ultrastructural appearance when suspended in a sucrose medium similar to that employed in the experiment described here (Lee *et al.*, 1969).

It seems clear that the primary effect of both of these reagents is to increase the permeability of the membrane so that positively charged ions may move inward, or negatively charged ions outward, in compensation for the movement of protons outward. Of all the secondary effects which gramicidin and FMA have on the mitochondrial membrane, the one most relevant to these experiments is the increase of permeability to protons. Gramicidin increases this permeability slightly, and FMA may also do so. However, since a net acidification of the exterior is observed in direct response to respiration, any increased permeability of the membrane to protons would tend to lower the observed H^+: O ratio, due to leakage of protons back through the membrane. Thus, the ob-

[2] Personal communication from Dr. James H. Southard.

served high H^+:O ratios occur despite the presence of reagents which have a slight tendency to conduct protons back through the membrane. Increase in proton permeability is not responsible for the proton ejection, since reagents which do not affect proton permeability, such as valinomycin, still induce proton ejection by mitochondria.

These new data show clearly that the initial rapid ejection of protons coincides with the transient burst of respiration, and that the H^+:O ratios observed are higher during this transient period than they are subsequently.

It is possible to explain the H^+:O ratios observed in two alternative ways: either the high H^+:O ratios observed during the first few hundred milliseconds represent the true stoichiometry of the proton pump, or they are caused by extra protons generated during the transient burst of respiration. In the first case, the lower H^+:O ratios observed at longer times, and sometimes even at the shorter times, could be attributed to return of some protons through the membrane by weak acids or other proton carriers. In the second case, the extra protons would be generated only during the transient phase, and the stoichiometry of the proton pump could be lower than the ratios observed during the initial burst. It is not possible to make a definitive choice between these alternatives, but it can be suggested that the latter explanation is the more probable one.

The most important fact supporting this conclusion is the coincidence of the high H^+:O ratios with the transient burst of respiration. This burst of respiration represents the oxidation of respiratory chain components, mainly coenzyme Q and nonheme iron, from a fully reduced initial state to a partially reduced steady state (Penniston, 1972). It would not be at all surprising if this net change in the oxidation state of the components of the chain caused a release of extra protons, over and above the cyclical pumping out of protons which occurs in the steady state. Such an effect would be similar to the Bohr effect in hemoglobin, except that the oxidation state rather than the liganding state would be the controlling factor. Once the steady state had been reached, the true stoichiometry of the pump could be observed, but the existence of species which carry protons back through the membrane might lower the H^+:O ratio below the theoretical value once the steady state has been achieved.

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