Cation Accumulation during the Anaerobic–Aerobic Transition in Brain Mitochondria*

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ABSTRACT: The proton jump accompanying the anaerobic–aerobic transition of mitochondria as demonstrated by Mitchell (Mitchell, P. (1962), *Symp. Biochem. Soc. 22*, 142) is shown to be related to the reaccumulation of mono- and divalent cations. In our experiments, the anaerobic–aerobic transition is achieved by addition of either H_2O_2 or molecular oxygen to the anaerobic system. K^+ ions are shown to be lost from the mitochondria during the approach to, and more extensively during the period of anaerobiosis, and are reaccumulated upon oxygenation. Chelators of divalent cations (EGTA and EDTA) are used to demonstrate the role of divalent cations in the observed proton release from the mitochondria, their addition resulting in a substantial decrease in the proton release. Gramicidin or valinomycin potentiates the reaccumulation of K^+ ions and the extrusion of H^+ ions during the aerobic transition. The reversal of these processes at the onset on anaerobiosis is also potentiated.

The initial phase of the proton release, *i.e.*, the rapid (or jump) phase, is inhibited by the addition of CN^- or antimycin A. The pH change (alkalinization) of the mitochondrial membrane(s) during the transition to aerobiosis as monitored spectrophotometrically reaches its maximum in approximately 5 sec, whereas the oxidation changes in the components of the electron-transport chain (cytochromes *a* and *b* and flavoproteins) occur within the first 180 msec. This and other chronological discrepancies as well as the significance of the findings are discussed.

There are two theories of energy conservation during electron transport in mitochondria. In the one instance, there is the possibility of chemical intermediates between the electron-transport components, be they hydride ions, free radicals, or some other high-energy compounds, and the ultimate resultant formation of ATP[1] (Griffiths and Chapman, 1962a,b; Cooper and Lehninger, 1956a,b; Chance and Williams, 1956a). In the other instance, it has been proposed (Lundegårdh, 1945; Davies and Ogston, 1950; Mitchell, 1961, 1962) that there is a proton gradient mechanism whereby energy is conserved. In this proposal, there is no need for chemical intermediates between the electron-transport components and ATP synthesis. Instead, the ion-impermeable mitochondrial membrane is visualized as creating a barrier for a chemical gradient developed during electron transport with a separation of electrons and hydrogen ions, with a resultant energy buildup sufficent to drive ATP synthesis.

In spite of the work of Racker and coworkers (Pullman and Racker, 1956; Racker, 1962, 1963; Conover *et al.*, 1963) and of other investigators (Sanadi, 1963; Green *et al.*, 1963; Ernster and Lee, 1964) in isolating coupling factors which participate in the phosphorylation reaction, no high-energy intermediate of oxidative phosphorylation has been characterized or identified. Brodie and coworkers (Brodie *et al.*, 1959, 1960, 1961) and Grabe (1957) demonstrated the incorporation of ^{32}P into ATP from phosphorylated derivatives. From his work with napthohydroquinone monophosphate, Wieland and Patterman (1958) hypothesized that a similar compound might be functioning in phosphate-transfer reactions in mitochondria. On the other hand, the recent papers of Mitchell and coworkers (Mitchell, 1961, 1962; Mitchell and Moyle, 1966) would presume that there is no need for any chemical intermediate. There is proposed instead a hydration–dehydration process with a splitting of HOH into OH^- and H^+. The extramitochondrial space thereby becomes acidic and the intramitochondrial space or mitochondrial membrane basic. This process driven by oxidation–reduction mechanisms within the mitochondria, would result in the formation of ATP. Remembering that this is a mixed anhydride between ADP and inorganic phosphate, it is necessary to predict that for such a process to occur at any significant rate in a biological system, an enzymatic process must be postulated. This would require either the binding of one or both of the reactants, or that there is a sufficient supply of energy available to drive the synthesis of ATP in such a fantastic chemical milieu.

One of the salient features of a discussion by Cockrell *et al.* (1966) was the improbability of the creation of the high pH gradient necessary to drive the ATP synthesis, in the manner postulated by the Mitchell hypothesis (Mitchell, 1961, 1962; Mitchell and Moyle, 1966). Mitchell and coworkers have shown that during the transition of mitochondrial preparations from a state of

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[1] Abbreviation used: ADP and ATP, adenosine di- and triphosphates; BTB, bromothymol blue.

anaerobiosis to aerobiosis, there is an acid shift which reaches a rapid equilibrium and derives its driving force from oxidation–reduction processes. If the basis of such a shift were due strictly to oxidation–reduction processes it could lend some support to their hypothesis, not withstanding the valid criticism of Cockrell *et al.* (1966). There was, however, no consideration of an alternative basis for such an acid shift.

Since it is known that cation shifts are accompanied by compensatory hydrogen ion movements, such a possibility could eliminate the need for a new hypothesis, and would point to an electrogenic balance occurring with an aerobic–anaerobic cycling of cations and hydrogen ions. If such cyclic shifts do occur, it would be important to determine if they are separable from one another, and if they are related at all to the transport of electrons or the energy derived therefrom. In order to clarify these points, we studied the anaerobic–aerobic transition as it pertains to proton and cation shifts, and the dynamic redox states of the components of the cytochrome system.

Materials and Methods

The pH measurements were made using the Beckman micro blood pH electrode; K^+ was monitored with the Beckman cation electrode, and modified Corning 12 pH meters were used. Changes in oxygen concentration were followed polarographically with a modified Clarke oxygen electrode (Chappell, 1961). Cytochromes *a* and *b* were followed according to the methods of Chance and coworkers (Chance, 1957; Chance and Williams, 1956b) using the Phoenix precision dual-wavelength-scanning spectophotometer. The pH of the mitochondrial membrane was followed by the BTB method as described by Mela (1966) and Chance (1957). Rat brain mitochondria were prepared as described elsewhere (C. L. Moore, submitted for publication), and kept at 0° until used soon after preparation. Unless otherwise stated, the basic assay medium was made up of 220 mM sucrose–10 mM choline chloride as described by Chappel and Crofts (1965), and was buffered with 2.0 mM of either Tris-HCl, Tris-acetate, or Tris-phosphate (pH 7.4). The substrate, a mixture of Tris-succinate, Tris-malate, and Tris-glutamate (pH 7.4), was present in all cases at a final concentration of 2.0 mM. Changes in H^+ concentration were estimated by titration with standard acid and base.

The anaerobic–aerobic transition experiments were carried out in a closed vessel fitted with H^+, K^+, and O_2 electrodes and a small magnetic stirring bar. The total volume of the system was 3 ml, and the temperature was maintained at 20°. The anaerobic–aerobic transition was accomplished either by introducing a bubble of pure oxygen at one end of the chamber and allowing it to escape at the other, or by the introduction of 0.5–1.0-μl quantities of appropriate solutions of H_2O_2 which in the presence of catalase releases oxygen into the system.

For the BTB mitochondrial membrane pH indicator system, mitochondria were incubated with BTB at a concentration of 0.35 mμmole/mg of mitochondrial protein at 0° for 3 min, and after centrifugation, the mitochondrial pellet was resuspended to its original volume in sucrose–choline chloride solution at 0°, and used in the experiments as required and described in the text.

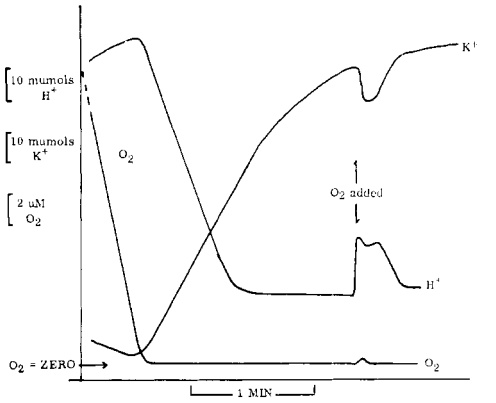

FIGURE 1: The release of K^+ ions from and the uptake of H^+ ions by brain mitochondria after the onset of anaerobiosis, and the reversal of this change by introduction of oxygen to the system. O_2 was measured polarographically, K^+ with Beckman glass electrode no. 39047, and H^+ with Beckman glass electrode no. 39045. The reaction mixture (3 ml) consisted of 220 mM sucrose, 10 mM choline chloride, and 2.0 mM Tris-HCl (pH 7.4), and 30 mg of brain mitochondria at 20°. An upward deflection in K^+ or H^+ is indicative of a release of that ion from the mitochondria.

Results

When 30 mg of brain mitochondria was incubated at 20° in the basic medium buffered with 2.0 mM Tris-HCl (pH 7.4) and containing 180–240 mμmoles of O_2, there was a rapid transition to anaerobiosis at a rate of 36–42 μmoles of O_2/min. This was accompanied by a loss of K^+ and H^+ ions from the mitochondria, possibly attributable to temperature equilibration. Upon depletion of the oxygen, there was an uptake of 120–200 mμmoles of H^+ and an extrusion of 100–150 mμmoles of K^+. The range in the values of H^+ and K^+ movements could be predicted by the amount of these ions displaced before the start of the experiment. These changes are presented in Figure 1, and are meant to show that there is a movement of ions dependent upon the transfer of electrons or the energy derived therefrom. In Figure 2, it is seen with the peroxide technique, where it is possible to control more reproducibly the amount of oxygen introduced into the system, the addition of 6 or 150 mμmoles of H_2O_2 resulted in a release of 2.1–2.5 and 54–60 mμmoles of O_2, respectively. The resultant proton jump at these two concentrations of peroxide, was 47–55 mμmoles of H^+ which is approximately 1.5 mμmoles of H^+/mg of mitochondrial protein. The characteristics of the phases of the proton changes were as seen in Figure 2. There was an uptake of 20–33 mμmoles of K^+ in the presence of 6 mμmoles of H_2O_2, and 45–52 mμmoles in the presence of 150 mμmoles of H_2O_2. It was also found that raising the

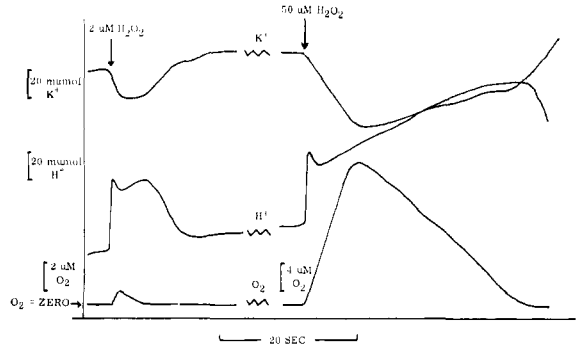

FIGURE 2: The use of H_2O_2 instead of molecular oxygen to cause the anaerobic–aerobic transition by the release of O_2 (by catalase) into the system. The two concentrations of H_2O_2 were added to the same system as indicated in the figure. An upward deflection of the H^+ or K^+ tracing indicates a release of that ion from the mitochondria. The reaction mixture was as described for Figure 1.

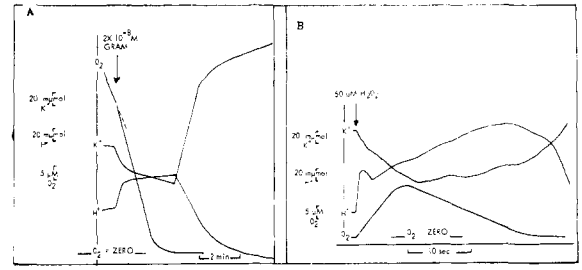

FIGURE 3: The potentiation of the proton jump by gramicidin. Gramicidin (2×10^{-8} M) is added during the transition to anaerobiosis (A) as described in the text. In B, H_2O_2 (150 mμmoles) in a 3-ml reaction mixture in the presence of catalase is used to introduce O_2 into the system. An upward deflection of the K^+ or H^+ tracing indicates a release of that ion into the medium. An upward deflection in the O_2 electrode tracing indicates a release of O_2 into the medium. The system was as described in Figure 1.

O_2 concentration to approximately 0.5 μmole resulted in a rapid ejection of 55–62 mμmoles of H^+ within the first 0.6–0.7 sec. This was followed by a slight cycling of the pH with the eventual incomplete reversal of the ejection. The initial pH change was observed before the change in the oxygen tracing was evident and was followed by an uptake of 20–25 mμmoles of K^+ which chronologically paralleled more closely the change in the oxygen electrode tracing. As the system became anaerobic, the K^+ ions were released and H^+ ions reaccumulated. A second pulse of oxygen to a concentration between 22 and 35 μM, resulted in a rapid expulsion of 45–57 mμmoles of H^+ within 0.8 sec. There was a slight reversal of the pH followed by a slower H^+ ejection which reversed upon depletion of the oxygen of the system. Here, as with the first pulse, the changes in the hydrogen ion tracing were evident before either the oxygen or potassium ion tracing had changed. There was an uptake of 35–45 mμmoles of K^+, reversing itself as the oxygen returned to zero. While the uptake of potassium increased as the amount of oxygen added to the system was increased, the initial hydrogen ion change did not. On the other hand, the secondary or slower release of hydrogen ions was a function of the oxygen pulse, and most likely depended upon the movement of cations (for the other experiments, only data obtained with H_2O_2 are presented).

The potentiation of the initial proton jump in the presence of gramicidin is shown in Figure 3. The addition of 2×10^{-8} M gramicidin during the transition to anaerobiosis (Figure 3) resulted in a 20% increase in the rate of respiration accompanied by an uptake of 32–36 mμmoles of K^+ and a release of 25–30 mμmoles of H^+. When the system became anaerobic, there was an uptake of 65–82 mμmoles of H^+ as compared to 47–55 mμmoles in the absence of gramicidin (Figure 2), and, as stated above, an efflux of 150–70 mμmoles of K^+ within the first 75 sec, after which the K^+ extrusion continued at a reduced rate. Upon addition of 150 mμmoles of H_2O_2 to this anaerobic system (Figure 3b), there was a rapid release of 65–68 mμmoles of H^+ within the first 0.8 sec followed by an uptake of 6–8 mμmoles and a slower release which reversed itself as the oxygen was depleted. There was also an uptake of 85–92 mμmoles of K^+, which reversed itself as the oxygen was depleted. When gramicidin was added to a system which had just become anaerobic, there was an extrusion of 110–120 mμmoles of K^+ and an uptake of 85–89 mμmoles of H^+; the addition of 150 mμmoles of H_2O_2 resulted in changes comparable to those described for Figure 3. The possibility that part of the rapid proton ejection at the onset of the anaerobic–aerobic transition could be related to an uptake of calcium or other divalent ions which are also prone to leak out of the mitochondria during anaerobiosis was checked by repeating the above experiments in the presence of EGTA or EDTA. While the procedure for preparing the mitochondria does include homogenization in sucrose solution containing 0.2 mM EDTA followed by two washings in the absence of EDTA, nevertheless, the possibility had to be considered that anaerobiosis could result in release of divalent cations from the mitochondria, and that during the anaerobic–aerobic transition these could be reaccumulated, resulting in the release of H^+ ions. The data of Vasington and Murphy (1962, 1963), Rossi and coworkers (Rossi and Lehninger, 1963a,b; Lehninger *et al.*, 1963), and of others (Brierley *et al.*, 1963a,b, 1962) are indicative of such an exchange under aerobic conditions.

As seen in Figure 4a, in the presence of 2.5 μM EDTA (or EGTA), there is upon addition of 150 mμmoles of H_2O_2 to an anaerobic system, a rapid release of only 10–15 mμmoles of H^+ in the initial phase, and a slow secondary phase which is also much decreased accompanying the pH change is an uptake of approximately 40 mμmoles of K^+ ions. In Figure 4b, the same experiment is carried out in the presence of 2×10^{-8} M gramicidin; there was

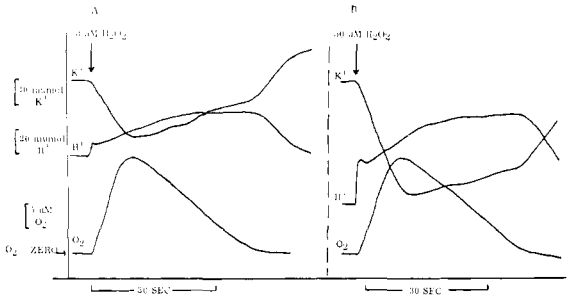

FIGURE 4: The effect of EGTA or EDTA on the proton jump. EDTA (or EGTA) (2.5 μM) was included in the respiration solution, and the anaerobic–aerobic transition was brought about by adding (A) 150 mμmoles of H_2O_2 to a 3-ml reaction mixture in the absence of gramicidin and (B) in the presence of 2×10^{-8} M gramicidin.

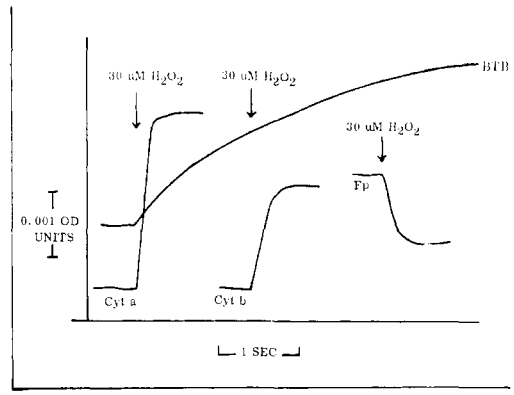

FIGURE 5: Changes in redox states of cytochromes *a* and *b*γ, and in flavoproteins, measured spectrophotometrically. Cytochrome *a* was followed at 450 mμ with 460 mμ as the reference wavelength. Cytochrome *b*γ was followed at 560 mμ with 575 mμ as the reference wavelength. Changes in the oxidation state of the flavoproteins were followed at 476 mμ with 500 mμ as the reference wavelength. An upward deflection indicates an increase in optical density. The pH of the mitochondrial membrane was monitored using the indicator BTB, and was followed at 618 mμ with 700 mμ as the reference wavelength. An upward deflection is indicative of alkalinization of the membrane.

a release of 35–38 mμmoles of H^+ upon addition of 150 mμmoles of H_2O_2, followed by the slight reversal and slower release phases of the pH tracing. There was also an uptake of 90–95 mμmoles of K^+ which was released as the system became anaerobic. Thus it would appear valid to propose that the transport of both monovalent and divalent cations is involved in the proton jump observed during the transition from anaerobiosis to aerobiosis. The addition of CN^- or antimycin A to the anaerobic system inhibited the initial pH jump and reduced the K^+ uptake by over 80%, the remaining activity being possibly due to the effect of the introduction of the H_2O_2 on the cation electrode. This would indicate that the developed pH gradient and ion translocations which accompany it are dependent upon oxidation–reduction reactions for their support and that the pH gradient *per se* is dependent upon ion translocation. However, whether the rate of oxidation of the cytochrome system is commensurate with the rate of development of the pH gradient must be established. The rate at which the changes in pH, K^+, and O_2 are recorded is a function of the electronic circuitry and the time constants of the electrodes. The rates of change observed spectrophotometrically are expected to be faster than the electrode changes. It should be expected therefore that if the oxidation reduction processes are occurring at an appropriate rate, the resultant changes should be observed sooner than with the electrodes.

Changes in the redox state of the cytochromes were therefore followed to correlate with the H^+ and cation shifts during the anaerobic–aerobic transition. The change in optical density of cytochrome a_3 was followed with 560 mμ as the measuring wavelength and 575 mμ as the reference. Change in the redox state of the flavoprotein was followed at 476 mμ with 500 mμ as the reference wavelength. When the pH of the mitochondrial membrane was determined, BTB-treated mitochondria were used, and the change in optical density of BTB was followed using 618 mμ as the measuring wavelength and 700 mμ as the reference. The data thus obtained are recorded in Figure 5. In Figure 5, it is seen that in the absence of gramicidin after a steady-state level at anaerobiosis is established, the addition of 130 mμmoles of H_2O_2 resulted in rapid oxidation of cytochrome *a* (0.0025 ODU), cytochrome *b*γ (0.0014 ODU), and flavoprotein (0.0011 ODU) within the first 180 msec and a slower increase of 0.0024 ODU in the BTB system indicating an alkaline change in the mitochondrial membrane reaching its maximum change in 5 sec, which is much slower than the rate of oxidation of the cytochromes. In the presence of gramicidin, the oxidation of the cytochromes and flavoprotein is no more extensive than in its absence, but the rate of oxidation is faster.

Discussion

The experiments reported here were carried out with the express purpose of evaluating the rapid proton ejection as indicative of or as being a correlary of the chemiosmotic theory of oxidative phosphorylation. We have shown that while there is an acid expulsion concomitant with the onset of the transition from anaerobiosis to aerobiosis, this pH gradient can be correlated with an uptake of divalent and monovalent cations extruded from the mitochondria during the transition to and during the brief period of anaerobiosis. During the preparation of this manuscript, Chance and Mela (1966) presented data showing that in liver mitochondria the reaccumulation of divalent cations can partly account for the pH jump reported by Mitchell (1961). Our data substantiate this finding, although more definitive experi-

ments are required to determine which divalent cations are involved. The inhibition of the H^+ jump by chelating agents and the potentiation by gramicidin (which induces rapid monovalent cation uptake by mitochondria) are added evidence that the acid extrusion which accompanied the anaerobic–aerobic transition is compensatory to mono- or divalent cation translocation, a phenomenon yet to be adequately explained. That the oxygen concentration does not have to exceed unit micromolar quantities in order to see the hydrogen ion shift is one aspect of the data difficult to explain, and could be related to the K_m value of O_2 for cytochrome a_3. However, one could presume that the amount of electron transporting energy necessary for ion translocation is extremely small, and that a small but rapid change in cation concentration in an environment close to the mitochondrial membrane could take place, with the resultant pH shift. A secondary change in cation uptake could account for the sustained pH change during the ensuing controlled respiration. It should also be pointed out, that the respiratory rate after the anaerobic–aerobic transition is slightly faster than the state 4 rate in the absence of gramicidin. The rapid changes in oxidation state of the components of the electron transport chain are indicative of a rapid release of the electron dam created during the period of anaerobiosis. In the presence of EDTA, and in the absence of gramicidin, under which conditions the pH gradient is inhibited by over 80%, there is no decrease in the electron flux. This would make it possible to separate the H^+ ejection from the electron flow. Whether there is a brief period of phosphorylative activity and an induced pH gradient sufficient to energize such a synthesis during the onset of aerobiosis, must await the results of investigations now being undertaken.

In conjunction with this last thought it is of interest that Reid *et al.* (1966) have reported that in an acid bath experiment with a gradient of 4.6 pH units, mitochondria were capable of synthesizing ATP. The amount of ATP was however very small, and its significance doubtful. It is hoped that future investigation would assist in clarifying this point.

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