Biochemical Journal Classic Paper: Mitchell & Moyle (1967a,b) Biochem. J. 104, 588-600, and Biochem. J. 105, p.1147-1162

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In 1967 the Biochemical Journal published two papers originating from what was essentially a private address — Glynn House, Bodmin, Cornwall — the home and private laboratory of Peter Denis Mitchell. The first paper [1] described in exhaustive detail how the buffering power of the inner and outer aqueous phases of anaerobic mitochondrial suspensions were measured, how the permeability of the cristae membrane to the translocation of protons was measured, and how that permeability was increased by dinitrophenol. The second paper [2] described with equal thoroughness the measurement of H^+/O quotients (stoichiometies) obtained by pulsing anaerobic suspensions of mitochondria with small amounts of oxygen, how respiratory pulses depended on substrate, size of pulse, and how the relaxation of the pH change in the suspension back to its starting value was accelerated by uncouplers, anionic substrates (e.g. succinate) and cations (e.g. by replacing K^+ with Na^+ or $Choline^+$). These papers became frequently cited over the years, probably as the definitive statement of the experimental support for Mitchell's chemiosmotic hypothesis in relation to mitochondria respiration. Both are rich in methodological detail, leaving nothing "to be explained elsewhere"; and both contain startling elaborations of chemiosmotic and biophysical theory. Let me elaborate.

Mitchell's childhood, schooling and early scientific career have been well described in the biography by Prebble and Weber [3]. His unusually original and self-confident mind, with an engineering and mathematical tendency and family background, was drawn to biology; but was constantly trying to understand it *'a priori'* as a mathematician, or mechanistically as an engineer; he was searching always for what he called 'general principles'. Prebble and Weber [3] also give an excellent account of how Mitchell resigned his Readership at Edinburgh University, and bought the ruined Glynn House in Cornwall, which he restored to serve both as family home and a research facility for his tiny group of colleagues and assistants. His main colleague and assistant was the meticulous and gifted experimentalist Jennifer Moyle who had been his 'pair of hands' since completing her doctoral work with Malcolm Dixon in Cambridge, moving with Mitchell, first to Edinburgh and then to Cornwall. (More of this below.)

(Figure about here of Mitchell aged circa 50, and (if possible) Jennifer Moyle.)

Mitchell's science in the nineteen fifties could be described as a concept in search of an application [4]. The germinal concept was that a chemical reaction, catalysed by an enzyme imbedded in a topologically closed membrane, can deposit its products to a different side of the membrane from its substrates, thus catalysing an osmotically significant transfer of solutes across the membrane synchronously with the chemical reaction. The osmotic process is not ***linked*** to the chemistry of metabolism, it is essentially the same process; thus a ***chemi-osmotic*** process [5]. It was not a novel concept (See Kluyver [6], Robertson [7], etc. [8]), but Mitchell developed it beyond existing thinking. The eventually fruitful application of this chemi-osmotic concept was to the respiratory chain and reversible ATPase systems of mitochondria, chloroplasts and bacteria.

Mitchell's 'chemiosmotic type of mechanism' for coupling phosphoryltion to electron and hydrogen transfer was first clearly presented in a Nature paper in 1961 [9], but was greatly supported (and improved) by detailed theoretical work over the next 5 years, published in a Biological Reviews article [10] and the famous, privately printed, 'First Grey Book' [11]. As soon as the restoration of Glynn house had progressed sufficiently to allow experimental work, Moyle and Mitchell set to work to test this exciting but outrageously original hypothesis. They triumphantly confirmed three of the four main predictions of the hypothesis: (1) that bursts of respiration send pulses of acid (outwards) across the mitochondrial membrane, (2) that the hydrolysis of small amounts of ATP (added to resting mitochondrial suspensions) also causes acidification of the suspending medium

(partly because of the net synthesis of acid in the form of phosphoric acid, and partly by 'translocation' of protons from the matrix of the mitochondria), and (3) that there is a barrier separating the inside (matrix) phase from the outside (suspension) phase that is rather impermeable to protons (and OH^- ions) except when ruptured or when a small amounts of a 'classical uncoupling agent' (e.g. dinitrophenol) is added to the suspension. They further concluded that the hydrolysis of each ATP translocated 2 proton equivalents, while respiration from succinate and the NADH-linked β-hydroxybutyrate dehydrogenase translocated 4 and 6 protons respectively per oxygen atom reduced. This work was published in Nature [12] in October 1965.

Mitchell was invited to Oxford the following year to give a research seminar in the Biochemistry department, where Sir Hans Krebs was still Head of Department. To me as a young scientist, baffled by the complexity of mitochondrial respiration, but already familiar with the concepts of transmembrane transport, osmotic pressure, and Mitchell's work on coupled exchanges of phosphate in bacteria, this was most exciting stuff; not least by its mode of presentation, for Mitchell proposed to test, and attempt to falsify, one by one the theoretical planks of his 'chemiosmotic hypothesis'. But not one plank could he falsify. Suddenly, in the space of an hour, oxidative phosphorylation had became intelligible. Mitchell and Moyle's Nature paper [12], however, was greeted as laughable nonsense by the dozen or so international experts who had dedicated their working lives to mitochondrial respiration and who knew so much of the arcane complexities of the system: the fragility, the microscopic anatomy, the presumed and inferred intermediates (squiggles, phosphorylated and unphosphorylated), the actions of "uncoupling agents" and a generous handful of exotic poisons extracted from actinobacteria, thistles, derris root and the poisons cabinet. There is a story, which I have heard from two sources, that during Mitchell's Oxford seminar, Sir Hans Krebs turned to the departmental colleague sitting next to him and remarked that it was a scandal that he should have to sit and listen to such rubbish. But sit he did; as he may also have sat through the 9th Sir Hans Krebs Memorial lecture given by Mitchell in Dresden in 1978 [13], the year Mitchell was awarded the Nobel prize for Chemistry.

These two classic papers [1,2], published in the Biochemical Journal in 1967, seem to be Mitchell's response to this contemptuous dismissal. They seem designed to show that these results cannot be ignored, that the thinking behind the experiments is extremely thorough, that anyone who tries but fails to confirm this work is probably overlooking some subtlety carefully analysed and circumvented in the Glynn Laboratory and meticulously described in the protocols presented here. There are painstaking definitions of the buffering power of the of the matrix (proteins and solutes), of the supporting medium and the protonatable groups in rapid diffusional equilibrium with the medium, and of the entire suspension treated as a single, topologically continuous, phase. Our authors state: "We have adopted the operational definition of pH given by MacInnes (1939) which, in the present studies, is practically equivalent to that based on H^+ ion activity, $[H^+] \times f_{H+}$, the symbol f_{H+} representing the abstract H^+ ion activity coefficient, which is approximately equal to the mean ionic activity coefficient….The value of f_{H+} has been taken as 0.75 in the 150 mM-KCl medium of the present studies…". They also suggest that "it can readily be shown that the concentration of a buffer group gives a buffering power at the pK corresponding approximately to:

$$\partial H^+/\partial pH = -2.3 \times [\text{buffering group}]/4$$ "

The scientific claims of the two papers seem irresistible even without understanding the definition of buffering power, or the distinction being made between MacInnes' and any other definition of pH, and even if you cannot "readily show" that the buffering power is approximately given by that formula. These rarefied points do not affect the conclusions; but they do show that Mitchell has considered them, and mastered them. On the other hand, it is undoubtedly important that anyone trying to reproduce these experiments should know the oxygen permeability of plastic, Teflon, and glass; and they should know when and why the electrical capacitance of a mitochondrion can be ignored, and how to combine, and correct for, the rate constants of the relaxation of glass-electrode, the electrometer, and the transmembrane ΔpH.

The first paper [1] establishes that the equipment and technique can detect small acidifications of the suspending medium, and can follow the partial collapse of that acidification as protons leak into the matrix to titrate groups there, that the 'effective' proton leak can be greatly speeded by tiny concentrations of agents like carbonyl cyanide p-trifluoronmethoxyphenylhydrazone (CFCCP) that uncouple oxidation from phosphorylation. ('Effective' because protons moving one way cannot be distinguished from hydroxyl ions moving the other way). The second paper [2] establishes that small pulses of oxygen (added as air-saturated medium, freed of CO_2, held at 25º at defined barometric pressure and 50% relative humidity) cause acidification of the suspension medium. Further, and this is where our attention turns to the very considerable experimental skills of Jennifer Moyle who did all the experiments reported here, the paper shows with great reproducibility that backward extrapolation of the linearized (semi-logarithmic) plots of the collapsing transmembrane ΔpH, can allow a correction for missing protons leaking back into the matrix ***by that mechanism.*** Mitchell and Moyle concluded that the H^+/O quotients in the presence of rotenone and succinate average 3.92 with some results a little above 4 and some a little below. When β-hydroxybutyrate was present and rotenone absent the pulses extrapolated back to an average of 5.88 H^+/O, provided the pulse contained neither too little, nor two much oxygen. (Again there were some results higher than 6 and others lower.) It was concluded that the respiratory 'sites 1, 2 & 3' ('loops 1, 2 & 3' in Mitchell's Hypothesis) contribute 6 protons per oxygen and thus 3 ATP/O (a result expected from previous work of others.)

The second paper [2] went on to explore a further very important aspect of the Chemiosmotic Hypothesis, one that became the fourth of the four fundamental postulates. If the cristae membrane is impermeable to protons and hydroxyl ions, and has a trans-membrane potential difference of 180 to 210 mV (inside negative) it would be impossible to import anions such as ADP^-, Pi^-, $succinate^{2-}$, etc. , or to exclude cations such as K^+, Na^+, and more particularly the divalent Ca^{2+}, etc. Mitchell postulated proton linked 'symport' as a means whereby the anions could be carried inwards, 'effectively' with protons, as an electrically neutral process, but thereby providing an extra means whereby the transmembrane ΔpH could collapse more rapidly than by uncatalysed leakage. Similarly, he postulated that cations might be carried out of the matrix by an electroneutral process 'effectively' in exchange for protons (antiport). The presence of an ion normally excluded from the matrix, such as Na^+, would in that case open an extra route by which the transmembrane ΔpH could collapse more rapidly. Both of these effects were conclusively shown by Jennifer Moyle's careful experiments. Collapse of the transmembrane ΔpH developed by a respiratory pulse was clearly faster when the suspending medium contained NaCl, but it was possible to believe that the swifter decay still extrapolated back to the same H^+/O quotients as reported for the standard 150 mM KCl medium. Phosphate, however, even at 0.1 mM, catalysed such a rapid collapse of the pulse (at 25º) that some protons were irretrievably lost before the electrode could detect them. Only by cooling to 5º could the semi-logarithmic plots be extrapolated back to suggest the previously found H^+/O quotients. This phosphate effect was so thoroughly anticipated, understood, tested, and allowed for, that Mitchell felt he could safely dismiss all further talk of phosphate, rapid pulse collapse and lost protons.

But in that it seems Mitchell was wrong, for this problem reared its head again 9 years later in work from Lehninger's group [14] and dogged Mitchell for the next 10 years till 1986 [15,16]. The very reproducibility of Jennifer Moyle's experiments was misleading. To get the same result of 5.88±0.12 every time in a dozen experiments was very persuasive. But Jennifer Moyle was very methodical, and always took a lunch break between 1 and 2 pm. In some imaginative experiments in the Glynn laboratory, Roy Mitchell (no relation) found that fresh mitochondria gave pulses bigger than 6 H^+/O, but that pulses declined during the afternoon. It is now accepted that, in the classic papers of Mitchell and Moyle [1,2], protons were lost within the resolution time of the equipment, or (indeed) never appeared outside the outer membrane, and that the H^+/O quotients, and H^+/ATP quotient, are higher than concluded there; perhaps by as much as 50% [14]. These two papers nevertheless remain classics, and remain fascinating (if somewhat baffling) reading.

REFERENCES

1 Mitchell, P. and Moyle, J. (1967) Acid-base titration across the membrane system of rat-liver mitochondria: catalysis by uncouplers. Biochem. J. 104, 588-600.

2 Mitchell, P. and Moyle, J. Respiration-driven Proton Translocation In Rat Liver Mitochondria. Biochem. J. 105, p.1147-1162.

3 Prebble, J. and Weber, B. (2003) Wanderings in the Garden of the Mind: Peter Mitchell and the Making of Glynn. Oxford University Press, Oxford.

4 West, I.C. (1992) In memoriam: Peter Mitchell, 1920–1992. Molecular Microbiology (1992) 6(23), 3623-3625.

5 Mitchell, P. and Moyle, J. (1958) Enzyme catalysis and group translocation. Proc. Royal Physical Soc. Edin. 27, 61-72

6 Kluyver, A. J. (1956) Microbial Metabolism: further evidence for life's unity. In The Microbe's Contribution to Biology(A.J. Kluyver and C.B. Van Niel), pp. 31-72, Harvard University Press, Cambridge, Mass.

7 Robertson, R.N. and Wilkins, M.J. (1948) Quantitative relation between salt accumulation and salt respiration in plant cells. Nature, Lond. 161, 101.

8 Mitchell, P. (1976) Vectorial chemistry and the molecular mechanics of chemiosmotic coupling: power transmission by proticity. Biochem. Soc. Trans. 4, 399-430.

9 Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature, Lond. 191, 144-148.

10 Mitchell, P. (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. 41, 445-502.

11 Mitchell, P. (1966) *Chemiosmotic coupling in oxidative and photosynthetic phosphorylation*. Glynn Research, Bodmin, Cornwall, UK.

12 Mitchell, P. and Moyle, J. (1965) Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. Mature, Lond. 208, 147-151

13 Mitchell P. (1979) The ninth Sir Hans Krebs lecture. Compartmentation and communication in living systems. Ligand conduction: a general catalytic principle in chemical, osmotic and chemiosmotic reaction systems. Eur J Biochem. 95, 1–20.

14 Brand, M.D., Reynafarje, B. and Lehninger, A.L. (1976) Re-evaluation of the H^+/site ratio of mitochondrial electron transport with the oxygen pulse technique. J. Biol. Chem., 251, 5670-5679.

15 Mitchell, R., West, I.C., Moody, A.J., Mitchell, P. (1986) Measurement of the proton-motive stoichiometry of the respiratory chain of rat liver mitochondria: the effect of N-ethylmaleimide. Biochim. Biophys. Acta, 849, 229-235.

16 West IC. (1986) A theoretical analysis of the effect of phosphate on apparent H^+/O stoichiometries in oxygen-pulse experiments with rat liver mitochondria. Biochim. Biophys. Acta, 849, 236-243.

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