This article looks at two *Biochemical Journal* papers that are frequently cited as the definitive statement of the quantitative support for Mitchell’s chemiosmotic hypothesis in relation to mitochondrial respiration. Both are rich in methodological detail; both contain elaborations of chemiosmotic and biophysical theory. With these two papers, chemiosmotic theory entered the mainstream of bioenergetic thought.

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 described in exhaustive detail how the buffering power of the inner and outer aqueous phases of anaerobic mitochondrial suspensions was 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 described with equal thoroughness the measurement of H+/O quotients (stochiometries) 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. With these two papers, chemiosmotic theory entered the mainstream of bioenergetic thought, and a number of new words entered the language.

Mitchell’s childhood, schooling and early scientific career have been well described in the biography by Preble and Weber. 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’. Preble and Weber 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 as both a 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 where she stayed until she retired in 1983.

Mitchell’s science in the 1950s could be described as a concept in search of an application. The germinal concept was that a chemical reaction, catalysed by an enzyme embedded 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. It was not a novel concept (see Kluyver and Robertson and Wilkins, and reviewed by Mitchell), but Mitchell developed it beyond existing thinking. The eventually fruitful application of this chemiosmotic concept was to the respiratory chain and reversible ATPase systems of mitochondria, chloroplasts and bacteria.

Mitchell’s ‘chemiosmotic type of mechanism’ for coupling phosphorylation to electron and hydrogen transfer was first clearly presented in a *Nature* paper in 1961, but made little impact at the time. Over the next 5 years, following wide reading, valuable conversations, and detailed theoretical work, the hypothesis was greatly supported, improved and republished in a *Biological Reviews* article and the famous, privately printed, ‘First Grey Book’. 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. Mitchell recruited two local technicians and a secretary, installed a butcher’s cold-room, and purchased a minimal amount of core equipment such as centrifuges, glass electrodes and electrometers. He fabricated the thermostatically controlled reaction vessels himself with glass-blowing skills acquired as an undergraduate, and much patient
grinding with carborundum powder. Although the laboratory was in one of the remotest corners of England, it was not out of touch; chemicals ordered before 5 p.m. one day arrived by 9:30 a.m. the next. It was, however, spared essentially all of the distractions of university life. No-one came except by invitation. Guests could be collected from the railway station at the foot of the mile-long drive, brought up to the Institute's guest room, and fed in the Mitchell's magnificently spacious and elegant living-room-cum-kitchen, where Helen Mitchell, bare-footed and long-skirted, worked her culinary magic. A Russian visitor to Glynn House reported waking in the middle of the night with an uneasy feeling which he eventually ascribed to the utter silence, and which he dispelled by placing his watch by his pillow.

Although the team gradually expanded with the introduction of a postdoctoral scientist, a second secretary, a third technician, etc., in the early days of these two classic papers, it numbered Mitchell, Moyle, a secretary and the two technicians. One of the technicians, Roy Mitchell (a Cornishman, and not a relation), progressed to conducting his own research and eventually gained a PhD from London University as an external student; more of him below. With Jennifer Moyle conducting essentially all of the experiments, they triumphantly confirmed three of the four main predictions of the hypothesis: (i) that bursts of respiration send pulses of acid (outwards) across the inner mitochondrial membrane, (ii) 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 (iii) 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 amount of a 'classical uncoupling agent' (e.g. dinitrophenol) is added to the suspension. This barrier is identified with the inner mitochondrial membrane. They concluded further that the hydrolysis of each ATP translocated two proton equivalents, whereas respiration from succinate and the NADH-linked β-hydroxybutyrate dehydrogenase translocated four and six protons respectively per oxygen atom reduced. This fitted nicely with the conventional understanding of the time that there could be a maximum of three ATP molecules synthesized per oxygen atom reduced, if the electrons passed the supposed three 'coupling sites', but only two ATP if they came from succinate.

This work was published by Mitchell and Moyle in *Nature* in October 1965. However, it was greeted as laughable nonsense by most of 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.

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 earlier 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, i.e. 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 become intelligible, and its complexities predictable from the simplest of postulates. 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. It is ironic then that the 'Ninth Sir Hans Krebs Memorial Lecture' was given by Mitchell in Dresden in 1978, the year he was awarded the Nobel Prize for Chemistry.

These two classic papers, 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. From then on, only idiots could ignore the fact
that mitochondria have an inside and an outside. There are
painstaking definitions of the buffering power 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,
$[H^+]f_{tit}$, the symbol $f_{tit}$ representing the abstract $H^+$
ion activity coefficient, which is approximately equal to
the mean ionic activity coefficient.….The value of $f_{tit}$ 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 pH corresponding approximately to:

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

The scientific claims of the two papers seem irresistible
even without understanding the definition of buffering
power, or the distinction being made between MacInnes's
and any other definition of pH, and even if you cannot
“readily show” that the buffering power is approximately
given by that formula, for 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 $\Delta p_H$.

The first paper 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. The slowness of that proton leak
is crucially important; the chemiosmotic hypothesis
postulated that there is a membrane (identified as the
inner mitochondrial membrane) separating an inner
aqueous ‘phase’ from the external medium that is rather
resistant to the ‘effective’ passage of protons. (‘Effective’
because protons moving one way cannot be distinguished
from hydroxyl ions moving the other way.) On adding
acid, the measured external pH fell (becoming acid), but
steadied after about 50 seconds and then, for the next 4
minutes, showed a slow, exponential, rise. Plotting the
logarithm of the external pH against time showed that
between 50 and 250 seconds, the leak followed a single
exponential, with a single rate constant, suggesting that
the same mechanism of effective proton movement
operated for the entire period. The paper also showed
that the ‘effective’ proton leak can be greatly sped up by
tiny concentrations of agents such as carbonyl cyanide
$p$-trifluoromethoxyphenylhydrazone (CFCCP) that
uncouple oxidation from phosphorylation.

The second paper establishes that small pulses
of oxygen (added as air-saturated medium, freed of
$CO_2$ held at 25°C at defined barometric pressure
and 50% relative humidity) cause acidification of the
suspension medium. Furthermore, and this is where our
attention turns to the very considerable experimental
skills of Jennifer Moyle who carried out all of the
experiments reported here, the paper shows with great
reproducibility that backward extrapolation of the
linearized (semi-logarithmic) plots of the collapsing
transmembrane $\Delta p_H$, can allow a correction for missing
protons leaking back into the matrix $\text{by that mechanism}$. Were a different, faster, mechanism of pulse-collapse to
operate during the dead-time of the equipment, it would
be impossible to account for the missing protons, but
such a hypothetical faster process would have to have
completely ended before the start of the linear portion of
the semi-logarithmic plot, i.e. by 7 seconds. 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. At the time it
seemed reasonable to regard the small scatter of results
as representing random errors symmetrically placed
about a ‘true’ value. In retrospect, it might seem that
there are more ways in which the results could be on the
low side, than on the high side. When $\beta$-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 too much oxygen. Again
there were some results higher than 6 and others lower.
It was concluded that the respiratory ‘coupling sites 1,
2 and 3’ (‘loops 1, 2 and 3’ in Mitchell’s Hypothesis$^{1,11}$)
contribute six protons per oxygen and thus three ATP
molecules per oxygen (a result expected from previous
work of others, and already shown in 1965$^{15}$).

The second paper 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 transmembrane
potential difference of 180–210 mV (inside negative),
it would be impossible to import anions such as ADP$^-$,
P$^-$, or succinate$^{2-}$, and equally impossible to exclude
ions such as K$^+$, Na$^+$ and, more particularly, divalent
Ca\(^{2+}\). 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. The idea that solute translocation across a membrane could be tightly coupled to similar translocation in the opposite direction (countertransport or antiport) was familiar from the late 1940s. The idea that sugar uptake could be linked to (and driven by) cation uptake in the same direction (co-transport or symport) was introduced in 1962 by the work of Robert Crane and his colleagues on Na\(^{+}\)–glucose co-transport (or symport) in guinea pig intestine; but the concept was instantly extended by Mitchell to the co-transport (or symport) of galactoside with protons into *Escherichia coli*. In the context of the mitochondrion, however, these ideas were novel.

Both cation/proton antiport and anion–proton symport were conclusively shown by Jennifer Moyle’s careful experiments described in this paper. 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 25°C) that some protons were irretrievably lost before the electrode could detect them. Only by cooling to 5°C could the semi-logarithmic plots be extrapolated back to suggest the H\(^+\)/O quotients found previously. 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\(^{15}\) and dogged Mitchell for the next 10 years until 1986\(^{16,17}\). It was undeniable that N-ethylmaleimide (NEM), and other agents known to inhibit phosphate influx, raised the apparent H\(^+\)/O quotient\(^{15}\). The result was repeated by Roy Mitchell at Glynn House, but Mitchell believed he had ruled out the phosphate porter and concluded that the effect of NEM on stoichiometry must be sought elsewhere. The very reproducibility of Jennifer Moyle’s experiments was misleading. To obtain the same result of 5.88±0.12 every time in a dozen experiments was very persuasive. Jennifer Moyle was very methodical, and always took a lunch break between 1 and 2 p.m., deliberately allowing the concentrated and quasi-anaerobic mitochondrial suspension to ‘stabilize’. In some imaginative experiments in the Glynn Laboratory, Roy Mitchell found that fresh mitochondria gave pulses bigger than 6 H\(^+\)/O, but that pulse size declined during the afternoon. It is now accepted that, in the classic papers of Mitchell and Moyle\(^{1,2}\), phosphate concentrations in these suspensions of mitochondria were greater than supposed by Mitchell and Moyle\(^{16,17}\), and rose progressively during the afternoon; protons were probably 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%\(^{15}\).

Nevertheless, there are few, if any, who would deny the enormous importance of Mitchell’s contribution to modern biology, or deny that these two papers remain classics, and fascinating (if somewhat baffling) reading. In the decade 1967–1977, the experts in the field came to accept the correctness of the fundamental chemiosmotic concept, and to appreciate the pervasive reach of chemiosmotic proton circuits in biology\(^{18}\). ■

References