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ARTICLE

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Linking ion transport, electron flow and oxidative phosphorylation; one aspect of the research of Professor Sir Rutherford Ness Robertson, AC, KCMG, DSc, FAA, FRS (1913–2001)

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Abstract A summary is presented of the work on ion transport of Professor Sir Rutherford Ness Robertson.

Keywords Ion transport · Electron flow · Oxidative phosphorylation · Robertson RN

Introduction

Bob Robertson's interest in ion uptake started in 1936 when he went to the University of Cambridge on a "Scholarship of the Royal Commission of 1851". Professor G.E. Briggs had accepted him as a postgraduate student and suggested he investigate the movement of ions across a concentration gradient into plant tissue.

At that time it was being suggested that respiratory CO_2 would produce HCO_3^- (against which anions could enter) and H^+ for cation entry. Although the rates of ion accumulation and of CO_2 release were related (Robertson 1940), the uptake of ions was not due to exchange for HCO_3^- and H^+. He showed that the addition of methylene blue to plant tissue greatly increased the rate of CO_2 evolution but depressed the rate of salt accumulation (Robertson 1995). Nevertheless, Robertson and others had shown that the addition of salt could stimulate the rate of plant respiration. It was how the energy for salt accumulation, against a concentration gradient, was made available from respiration that interested and fascinated him (Robertson 1992). He was well acquainted with the research of H. Lundegårdh, a Swedish plant physiologist who was also investigating the link between salt accumulation and respiration. Lundegårdh realized that when a cytochrome was reduced by accepting an electron into its iron atom, a proton was simultaneously released into the medium. He suggested an electrochemical mechanism of ion uptake whereby the separation of charge might result in H^+ leaving the cell by one pathway and OH^- by another (Lundegårdh 1939); Robertson supported and defended (Robertson 1940) this idea.

In 1939, Bob Robertson returned to Australia to join the staff of the Botany Department, University of Sydney, where he continued his research.

Tissue experiments

His most often used experimental material was parenchyma tissue taken from the xylem of carrots. The material was cut into discs about 1 mm×8 mm, rinsed in several changes of distilled water and then aerated in distilled water for 4–5 days (this allowed the tissue to recover from "wound respiration", resulting in a steady rate of "ground respiration"). Addition of KCl to this tissue stimulated the rate of respiration and induced salt accumulation into the tissue (Robertson and Turner 1945). The salt-induced respiration, termed "anion respiration", was completely inhibited by KCN, as was the uptake of salt (Robertson and Turner 1945). Interestingly, the ground respiration was not inhibited by KCN whether salt was present or not, nor was the initial rapid absorption of salt which represented equilibration with the free space of the tissue. Similar inhibitory results were obtained with CO in the dark, establishing the involvement of cytochrome oxidase (Weeks and Robertson 1950).

He believed that if a biological system was understood, one should be able to make quantitative predictions about it. His scheme for this redox system of ion uptake (Robertson and Wilkins 1948) is shown in Fig. 1. Based on this and the equation for respiration using glucose as a substrate:

$$C_6H_{12}O_6 + 6O_2 + 6H_2O \rightarrow 6CO_2 + 12H_2O \quad (1)$$

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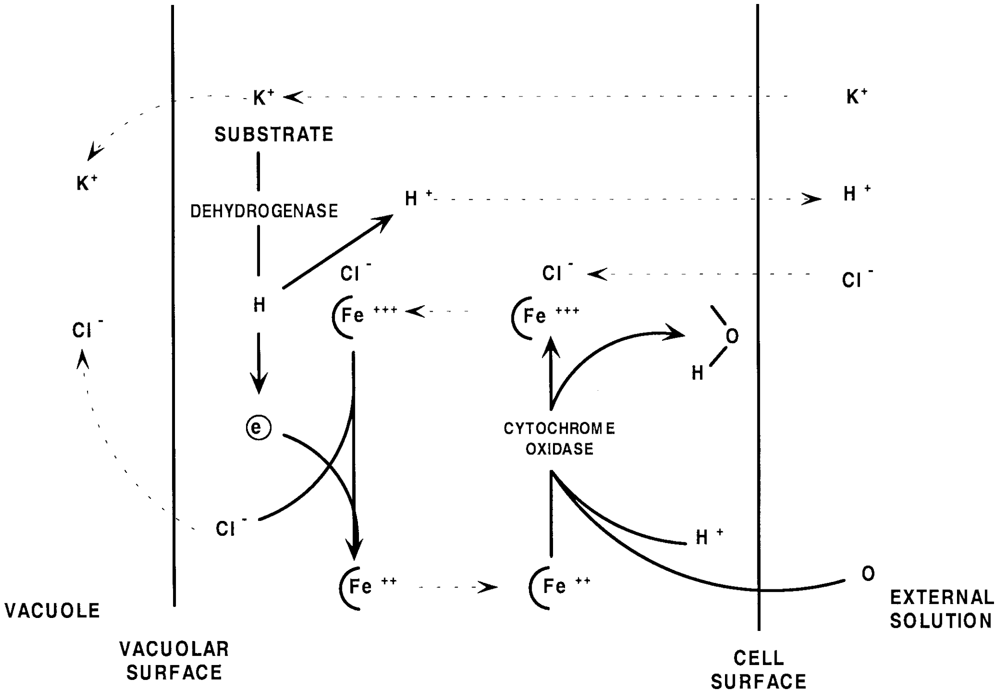

Fig. 1. Schematic representation of the electron and anion transport system. *Solid lines* represent chemical reactions; *broken lines* represent movements of substrances; (Fe^{+++} , oxidized cydochrome; (Fe^{++}, reduced cytochrome

it is apparent that each molecule of O_2 reduced to H_2O requires 4 electrons and 4 hydrogen atoms. Therefore, the maximum rate of salt accumulation should be 4 g mol monovalent salt accumulated per 1 g mol O_2 utilized, i.e. salt accumulation/salt respiration = 4. He showed this value to be correct (Robertson and Wilkins 1948), with the respiration becoming saturated at a much lower concentration of KCl (10–20 mM) than the accumulation ($>$60 mM) process. Lundegårdh had earlier arrived at the same ratio but failed to determine it experimentally, because most of Lundegårdh's experiments were performed with comparatively dilute salt solutions; hence the low ratios.

At about this time the concept of "uncouplers" was being established. These are compounds which separate the obligatory coupling system between ATP synthesis and electron flow in the mitochondrial electron transfer chain. We now know that the mitochondrial electron transfer chain develops a proton motive force by extruding H^+ across the inner membrane. It is this force which drives ATP synthesis and uncouplers are lipophilic weak acids which rapidly re-equilibrate this proton motive force, releasing the energy as heat (Mitchell and Moyle 1967).

Robertson et al. (1951) showed that: (1) 2,4-dinitrophenol (DNP – the "classic" uncoupler at that time) inhibited salt accumulation by carrot slices; (2) that it stimulated oxygen uptake of the tissue in both water and salt solution; and (3) that the stimulated respiration was proceeding via cytochrome oxidase because it was sensitive to KCN and to CO in the dark.

These results showing increased cytochrome oxidase turnover in the presence of KCl, without any salt accumulation, were clearly not consistent with the scheme shown in Fig. 1. Further, their data showed that the rate of outward leakage of salt could not explain the complete inhibition of salt uptake. A number of explanations of these results are offered in their paper (Robertson et al. 1951), including the possibility "that the cytochrome system plays no direct part in the accumulation mechanism"; if this were so, a new explanation of the quantitative relation between salt accumulation and salt respiration (Robertson and Wilkins 1948) would have to be sought.

Mitochondrial studies

By the 1950s it had been established that aerobic respiration involved mitochondria, which contained the cytochrome chain. Although it was known that cytochromes were also involved in photosynthesis in chloroplasts, the presence of cytochromes in other cellular membranes (plasma membrane and endoplasmic reticulum) had not been established. He had already implicated mitochondria as having a role in salt accumulation (Farrant et al. 1956), stating that "one effective way of putting ions through a lipid region would be to enclose them in a mobile particle predominantly lipid; mitochondria have the requisite mobility since they move with protoplasmic streaming". So it is not surprising that he began to investigate the properties of isolated plant mitochondria. The isolation technique was based on differential centrifuging in a sucrose medium, producing a washed 10,000×*g* pellet. He also began to use red beet tissue as well as carrot tissue because the former, being devoid of carotene-containing chromoplasts, produced "cleaner" preparations.

The information about plant mitochondria was very scant indeed and heavy reliance was placed on the knowledge obtained from studies with animal mitochondria. The beet preparations were examined with electron microscopy and although the cristae were somewhat swollen (which may have occurred during fixation) the mitochondria did appear to be intact (Farrant et al. 1956). A study of the respiratory activity and monovalent ion balance of these mitochondria revealed that both cations and anions were held in concentrations greater than in the supernatant. It was suggested that the cation concentration was due to a Donnan equilibrium (Robertson et al. 1955), based on immobile intra-mitochondrial anions. Since no simple Donnan equilibrium will account for the simultaneous concentration of both mobile cations and mobile anions, they suggested that the mobile anions might be accumulated by the cytochrome redox system. Robertson knew that plant tissues store the accumulated salts in their vacuoles but considered mitochondria as suitable vehicles for transferring ions across the cytoplasm (Robertson 1951). "The mitochondrion, actively oxidizing substrate in one part of the cell, may increase its concentration of mobile ions and then, in cytoplasmic streaming, be moved to another part of the cell, where if its oxidative capacity decreases, the ion concentration in the particle will also decrease" (Robertson 1951). Furthermore, they showed that salts would stimulate the rates of oxidation of NADH and of reduced cytochrome *c* of washed mitochondria in the presence of EDTA (Honda et al. 1957). These results were taken as an explanation of salt accumulation and of salt-stimulated respiration. We now know that NADH is oxidized via a dehydrogenase located on the outer surface of the inner membranes and that cations screen the fixed charges associated with the membrane. This decreases the repulsion of the negatively charged substrate ($NADH_2$) and increases the effective substrate concentration (Møller et al. 1981). There is also a specific role for Ca^{2+} in this externally facing NADH dehydrogenase and pretreatment of plant mitochondria with metal chelators decreases the rate of NADH oxidation.

It was at this stage that I joined his group as a postgraduate student on a project to understand the biochemistry of isolated plant mitochondria. During my tenure, Robertson went to U.C.L.A. as a visiting Professor and left me under the care of the late Professor R.K. Morton. We did describe the electron transport chain of isolated beet mitochondria and determined some of its general properties (Wiskich et al. 1960). Whilst at U.C.L.A., Robertson gave a lot of thought to the dilemma he had with the results obtained with uncouplers – which, as mentioned earlier, stimulated the rate of cytochrome path turnover but inhibited salt uptake, and suggested that ATP may be involved in the process. The process of oxidative phosphorylation was then expressed in algebraic terms of unknown intermediates (e.g. A$\sim$I, X$\sim$P) and based on the mechanism of substrate-level phosphorylation (Lehninger et al. 1958). Uncouplers were postulated to hydrolyse one of the high-energy intermediates. His review article (Robertson 1960) was published in 1960 and suggested the oxidation-reduction system (i.e. the electron transport chain) may result in a separation of negative and positive charge, which in turn could result in ATP production and/or ion uptake. The process of charge separation had inspired him during his student days in Cambridge, and had been influenced by Lundegårdh (1939). In fact, in those early days, Robertson felt that some of the criticism aimed at Lundegårdh's work and theory was unfairly based and wrote in support of Lundegårdh's ideas (Robertson 1940).

Robertson's review article was a timely publication because, shortly after it was published, Peter Mitchell's first paper on a chemi-osmosis type of mechanism of oxidative phosphorylation appeared in *Nature* (Mitchell 1961). The ideas were essentially the same, with a form of charge separation as the basis for energy conservation. However, the mitochondrial electron transport chain in Mitchell's scheme moved H^+ and not anions. Robertson has since written "in retrospect, I regret I did not have enough insight to make the ATP hydrolysis liberate H^+ on one side of the membrane and OH^- on the other, but I did not; Mitchell did and now, of course, the ATPase is a well-known transmembrane protein complex" (Robertson 1995). Nevertheless, Robertson's review (Robertson 1960) did contain the original suggestion that, after charge separation, ion uptake or phosphorylation of ADP are alternative consequences. It may seem surprising that Robertson, who had followed the suggestions of researchers studying HCl secretion across the gastric mucosa, did not consider H^+ translocation. Early hypotheses suggested that, in HCl secretion, H^+ ions (liberated from the respiratory process) were left on the outside of the membrane and that the Cl^- ions followed the potential established by these H^+. Robertson argued that in HCl secretion (as in salt accumulation in plants) it was the Cl^- ion that was actively transported (Robertson 1968) – hence his focus on the anion.

The University of Adelaide invited him to become Professor and Head of the Botany Department – a position he took up in 1962. The appointment gave him an opportunity to return to his research interests in salt uptake. He continued with his experiments on carrot slices and invited me to join him in some mitochondrial research. We showed that isolated beetroot mitochondria would accumulate magnesium and phosphate in high amounts (Millard et al. 1964) – the alkalinity inside the mitochondrial matrix led to a precipitation of magnesium phosphate. Nevertheless, the salt uptake was dependent on substrate oxidation, sensitive to electron transport chain inhibitors and to uncouplers, but insensitive to oligomycin. Further, it was shown that salt uptake competes unsuccessfully with ATP formation (i.e. no salt accumulated when ADP and inorganic phosphate were present). This result, together with the lack of inhibition of salt accumulation by oligomycin, confirmed the suggestion that ATP formation and salt uptake were alternative outcomes of a common process (Robertson 1960). As suggested from Robertson's earlier work (Robertson et al. 1955; Honda et al. 1957), the presence of monovalent cations in the medium inhibited the accumulation of magnesium phosphate (Millard et al. 1965). Although the accumulation of monovalent cations was not demonstrated, it was assumed these were competing with magnesium for uptake into the mitochondria.

Return to tissue experiments

While the research described above has shown that mitochondria can accumulate ions, it had not established that this was relevant to salt uptake in tissues. Before he left Adelaide, in 1969, Robertson did undertake the crucial experiment to determine the effect of oligomycin on salt uptake by tissues. The strategy was quite simple: add salt to aged carrot tissue with and without oligomycin present, and measure rates of salt uptake, oxygen consumption, and tissue levels of ATP. If salt uptake was dependent on ATP for energy, oligomycin should be inhibitory. The lack of any inhibition would suggest salt uptake was driven by the cytochrome redox flow. Unfortunately, the results were quite inconclusive (Atkinson et al. 1966). Oligomycin did lower the levels of ATP in the tissue (generally by about 25–50%), but it either had no effect or stimulated the rate of oxygen uptake. The latter was probably due to the ethanol in which the oligomycin was dissolved. However, the rate of salt accumulation was inhibited by oligomycin (by 40%). It makes one wonder whether oligomycin, being so water insoluble, was penetrating only the peripheral cells of the disks and not reaching the inner ones. This may explain the relatively minor effects of oligomycin on respiration and ATP levels, but not the effects on salt accumulation. It appears he may have missed some publications on this topic. It had been reported (Jacoby 1970) that Cl^- uptake by excised barley roots was significantly inhibited within 2 min of adding oligomycin, and that oligomycin inhibited plasmamembrane ATPases (Contessa and Bruni 1971).

Molecules, membranes, and imagination

In more recent times, Bob Robertson tried to tie up his original redox theory involving charge separation and ion transport. He reported on two electrogenic ion transport systems in carrot cells (Anderson et al. 1977), determined from cell membrane potentials as influenced by respiratory inhibitors and uncouplers. Again these were interpreted in terms of the mitochondrial cytochrome system. However, having become aware that a cytochrome system, and an H^+-translocating ATPase exist in the plasma membrane, he speculated that this may be where the ion-transporting redox system is located (Robertson 1991). He argued that in the mitochondrial cytochrome chain there are three H^+-translocating sites, whereas in the plasma membrane system there is probably only one. Is this why his original data of mol monovalent salt accumulation/mol oxygen consumed produced a value of 4? He wrote "the occurrence of the cytochrome system in the plasmalemma calls for a complete reinterpretation of our results, with the probability that the H^+ extrusion is from the plasmalemma redox system" (Robertson 1991). Although he accepted that H^+-translocating ATPases were involved in ion transport, he was reluctant to discard the idea of a redox-driven system. Of course, in the case of mitochondria, where ion uptake can be driven by either the ATPase or the electron transfer chain, he was right.

What kind of life

I have restricted this summary of Bob Robertson's research to his interest in ion accumulation by plant cells. Readers who wish to read about his own description of his science and life should consult his review articles (Robertson 1991, 1992, 1995). I conclude with an extract from his prefactory chapter in *Annual Reviews of Plant Physiology and Plant Molecular Biology* (1992), from which I have taken the title for this paragraph: "I have no doubt that the religious atmosphere of my home, my father's church, and of the two Protestant schools I attended set the ethical standards I still respect, though I no longer believe that the behaviour of men and women is directed by any supernatural power. I believe that the most satisfying philosophy of life encourages unselfishness, with concern for and tolerance of fellow humans. In this, science can play a vital role, helping us to leave the world a better place for our having lived. I cannot escape the simple, practical, and sobering fact of experience: it is difficult to live a happy contented life if surrounded by people who are discontented. Discontented neighbours may throw stones; discontented nations are liable to throw bombs. Scientific understanding of ourselves and of our environment should lead us to eventual peaceful interaction, irrespective of color, race, class or nationality. How would I like to be remembered? Perhaps, if it's not too much, as a generous man, who, despite human failings, talked sense and occasionally showed signs of wisdom."

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