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RESPIRATION AND ENERGY-DEPENDENT MOVEMENTS OF CHLORIDE AT PLASMALEMMA AND TONOPLAST OF CARROT ROOT CELLS

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SUMMARY

1. The effects of oligomycin, *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) and anaerobiosis on Cl^- fluxes and on respiration in carrot root cells have been investigated, in order to elucidate how energy is supplied to the energy-utilising active Cl^- fluxes. The inhibitors were used in the absence of organic solvents.

2. The active influx of Cl^- at the plasmalemma is unaffected by concentrations of CCCP and oligomycin which affect respiration and other Cl^- fluxes in the cell, but is reduced under N_2. This suggests that there is a close link between the plasmalemma Cl^- influx and respiratory redox reactions. The stoichiometric relationship between respiration rate and the rate of salt accumulation in previous studies is re-examined.

3. The active influx of Cl^- at the tonoplast is reduced under N_2 and in the presence of CCCP, probably *via* their effects on respiration. Oligomycin also reduces this Cl^- flux, but does not appear to do this *via* an effect on respiration. These effects suggest that energy may be supplied to this pump from respiratory high energy intermediate without the mediation of ATP.

4. Oligomycin does not inhibit respiration in water or in salt, whereas CCCP stimulates respiration in both. The implications of this discrepancy are briefly discussed.

INTRODUCTION

The previous paper[1] justified the use of a simple compartmental analysis of tracer fluxes in obtaining estimates of the two one-way fluxes at the plasmalemma and tonoplast and the cytoplasmic and vacuolar contents of carrot root cells. In conjunction with measurements of electrical potential differences, results so obtained indicated that Cl^- was probably actively transported inwards at both plasmalemma and tonoplast. The object of the work reported in this paper is to determine how these Cl^- pumps are related to energy supply processes.

There is an extensive literature on the effects of various inhibitors on ion movements in plant tissues[2], but so far little systematic distinction of different transport processes and their inhibitor sensitivities (giant algal coenocytes excepted[3]). Since

Abbreviation: CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone.

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there are at least two distinguishable transport processes in plant cells, one can only begin to make unambiguous interpretations of inhibitor effects on ion movements if these transport processes are taken into account.

This paper reports the effects of anaerobiosis, a respiratory uncoupler (*m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) and oligomycin on individual Cl^- fluxes and on Cl^- distribution in carrot root cells. The effects of these treatments on isolated plant and animal mitochondria (including carrot root mitochondria) are well characterised[4–6]. Oligomycin inhibits the formation of ATP but does not directly affect high energy intermediate formation or electron flow. CCCP discharges the high energy intermediate and consequently also stops ATP formation, but does not stop electron flow. In N_2 electron flow to O_2 will not occur, and there will be no coupled ATP or high energy intermediate formation. The effects of these treatments on energy-utilising processes give some indication as to whether energy is supplied to them *via* ATP, or whether there is some more direct coupling to high energy intermediate formation or to respiratory redox reactions.

ATKINSON *et al.*[7] have shown that the effects of these treatments on O_2 uptake and on total ATP level in carrot root tissue are similar to their effects on isolated carrot root mitochondria[6]. As a working hypothesis it will be assumed that O_2 taken up by carrot tissue is used in respiration in the mitochondria.

The results show that Cl^- movements across the plasmalemma and the tonoplast in carrot root cells are linked in different ways to respiratory energy supply.

METHODS

1-mm thick discs of carrot (*Daucus carota*, L.) root tissue were cut and washed in several changes of distilled water for 4 days to bring them to a state in which they will accumulate salts[2], and then put in a standard solution and allowed to accumulate salt for 2 to 3 days. The two one-way fluxes between the external solution and the cytoplasm (M_{oc} and M_{co}) and between the cytoplasm and the vacuole (M_{cv} and M_{vc}), and the cytoplasmic and vacuolar contents (Q_c and Q_v), were estimated, as described in the previous paper[1], in the presence and absence of inhibitors.

Transient changes in net tracer influx or tracer efflux were also examined. Net tracer influx was measured by counting samples of tissue at intervals, and tracer efflux was measured by counting the activity washing into successive aliquots of inactive solution, as described in the previous paper[1]. When these tracer fluxes were steady (*i.e.*, when the cytoplasmic specific activity was steady during loading or washing out) the inhibitor was added, and the tracer fluxes followed until a new steady state was reached. It is assumed that when these two overall fluxes are steady, the fluxes at the plasmalemma and the tonoplast are steady and the cytoplasmic content is constant, as is required for the application of the compartmental analysis. Also, by working at low or high external concentrations, when either the plasmalemma or the tonoplast flux limits the overall flux across the cytoplasm[1], some conclusions can be drawn about the relative speeds of effects of inhibitors on individual one-way fluxes at the plasmalemma and at the tonoplast.

The standard solution contained 5 mM Cl^-, 1.25 mM Ca^{2+}, and 1.25 mM Na^+ and K^+. The pH was adjusted to 6.0 in the CCCP solutions (pK_a of CCCP = 5.95 (ref. 8)) and in their controls by adding NaOH or HCl. Otherwise the pH was about 6, but

was not accurately adjusted, since it has little influence on ion movement in other plant tissues over the range 5.5–7.5 (ref. 9).

Anaerobiosis was maintained by bubbling O_2-free N_2 through the solutions. Anaerobic solutions were transferred through a syringe which was flushed with O_2-free solution before each transfer. CCCP (Calbiochem) and oligomycin (80% oligomycin B, Sigma Chemical Co.) solutions were prepared without ethanol, since a preliminary experiment showed that the smallest amount of ethanol with which loading solutions with inhibitor could be prepared without preliminary treatment (1 drop of ethanol in 30 ml of solution, about 8 mM ethanol) had considerable effects on K^+ fluxes. The required amount of the ethanolic solution of the inhibitor was added to the vessel and the ethanol evaporated off in a stream of air. The solution was added, the bottom of the vessel covered in small glass beads, and the vessel shaken. Solutions with inhibitor could in this way be made up in a few minutes, but were generally used after having been made up for an hour or more. There was no significant increase in the inhibitor activity of such a solution after being made up for 2 h.

Respiration measurements were made in Warburg flasks, at the same temperature and in the same solutions as for flux measurements. The effects of the various treatments on the Cl^- fluxes have been taken to be *via* their effects on respiration if the time course of the effects on tissue respiration and on fluxes are the same. This is a necessary, though not sufficient, condition for a close linkage of the two processes.

The electrical potential difference between the vacuole and the external solution was measured using glass microelectrodes filled with 3 M KCl and having a resistance of 5–10 MΩ, as described in the previous paper[1].

RESULTS

The uncoupler CCCP

4 μM CCCP stimulated the O_2 uptake in salt by about 25%, the new steady respiration rate being reached in less than 20 min, in agreement with previous workers[10].

A new steady state with regard to Cl^- movements is reached after about 2 h in CCCP (Fig. 1). In this state first order cytoplasmic and vacuolar components of the efflux of Cl^- appear as usual, and the compartmental analysis can therefore be applied. The estimated values of the plasmalemma and tonoplast fluxes and of the cytoplasmic and vacuolar contents in the presence and absence of 4 μM CCCP are shown in Fig. 2. 4 μM was chosen as the lowest concentration having nearly maximal effect on net tracer influx, and also as being the concentration used by ATKINSON *et al.*[7].

The most important observation is that M_{oc}, which on thermodynamic grounds is probably an active movement[1], is not inhibited by CCCP. However, M_{cv}, M_{vc} and Q_c are smaller and M_{co} is larger in the presence of CCCP. To distinguish the primary effect(s) of CCCP, these results can be compared with the transient changes in net tracer influx and tracer efflux (Fig. 1) and respiration.

If the primary effect of CCCP were a stimulation of M_{co} (leading to reduced Q_c and M_{cv}) one would expect that the tracer Cl^- efflux would rise initially to a peak after CCCP was added, and would subsequently fall to a new steady level higher than that before CCCP was added (*cf.* the effect of external Cl^- on loss of tracer Cl^-, ref. 1). In fact there is no such peak, although tracer efflux appears to begin rising almost immediately after CCCP has been added. Also, the rise in M_{co} (tracer efflux in water is

very nearly equal to M_{co}[1]), as shown in Fig. 1, is not synchronous with the fall in net tracer influx; and further, the ratio M_{cv}/Q_c falls in the presence of CCCP, which suggests that the fall in Q_c is not the cause of the fall in M_{cv} (although the fall is only just significant ($P<0.05$)).

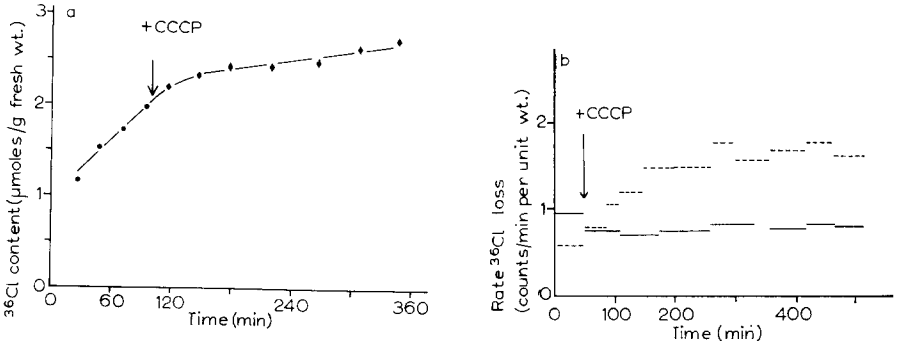

Fig. 1. a. Effect of CCCP on net influx of $^{36}Cl^-$. The sample of tissue was removed from the loading solution at intervals and the activity in it counted under an end-window counter. 4 μM CCCP was added as indicated. Vertical bars show 95% limits of error due to counting uncertainties. Controls (not shown) remained approximately linear over the whole period. The experiment was performed once over a range of concentrations of inhibitor, and repeated 3 times at 4 μM CCCP. The inhibitions had the same time course. pH 6.0. 28°. b. The effect of CCCP on the efflux of $^{36}Cl^-$. Tissue was loaded with $^{36}Cl^-$ and washed into water until the cytoplasmic component of the efflux had fallen to zero. 4 μM CCCP was added (– – – –) at arrow. CCCP also stimulated $^{36}Cl^-$ efflux in salt solution, with approximately the same time course. The experiment was repeated 3 or 4 times in salt and in water. The stimulations had the same time course. pH 6.0. 22°.

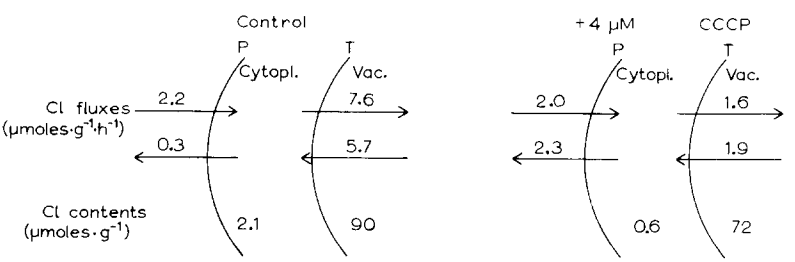

Fig. 2. Estimated values of plasmalemma (P) and tonoplast (T) Cl^- fluxes and cytoplasmic and vacuolar Cl^- contents in the presence and absence of CCCP. The tissue was pretreated in 4 μM CCCP to bring it to a new steady state, loaded with $^{36}Cl^-$ for about 1 h and washed out in unlabelled solution in the presence of CCCP, and the fluxes and contents estimated from the wash-out data[1]. Controls were run in parallel without CCCP. Values shown are means of two or three samples. Differences between controls and treated tissue discussed in the text are significant at the 1% level. Other fluxes not significantly different. pH 6.0. 22°.

It is more likely that CCCP directly inhibits M_{cv}, which is also probably an active flux[1]. If this were the case one would expect that after CCCP had taken effect there would be a transient continued influx of tracer Cl^- as the cytoplasmic content continued to rise (*cf.* the observation of ROBERTSON, WILKINS AND WEEKS[10], discussed by MACROBBIE[11]). An increase in the cytoplasmic content would lead to an increase in the plasmalemma efflux which would account for the fall in net tracer influx. The transient changes observed in tracer efflux and in net tracer influx (Fig. 1), and in respiration, are consistent with this picture.

One still has to account for the fall in Q_c and M_{vc}, and for the continued rise in tracer efflux after net tracer influx has apparently reached a new steady level. One must conclude that CCCP affects other fluxes as well as M_{vc}, and that these are more apparent in tracer efflux than in net tracer influx. It is difficult to account for the fall in Q_c unless CCCP directly stimulates M_{co} as well as inhibiting M_{cv}. (Such an effect on M_{co} must be relatively slow since no peak in the efflux is observed.) However, no effect of CCCP on the electrical potential difference between the vacuole and the external solution has been detected over a range of external concentrations and pH's with several salt solutions, and it therefore seems unlikely that CCCP alters the passive permeability of the plasmalemma to Cl^-, although a small change in Cl^- permeability alone might not change the carrot cell potential difference significantly[34].

The conclusions that can be reached are that CCCP does not inhibit the active flux M_{oc}, but probably inhibits the active flux M_{cv} at the same rate as respiration is affected. CCCP also affects other, putatively passive, fluxes, probably more slowly; but the absence of any detectable electrical response to CCCP suggests that these fluxes may not be simple diffusional movements.

Oligomycin

12 μM oligomycin does not affect the O_2 uptake of carrot tissue in salt or in water over a period of 8 h, in agreement with, and in extension of, previous obser-

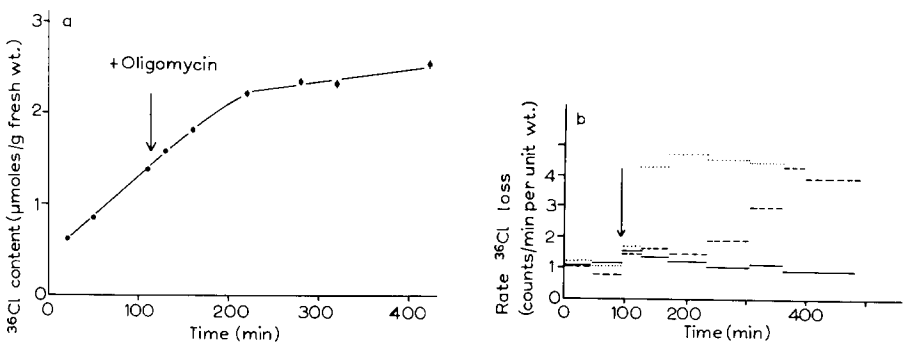

Fig. 3. a. Effect of oligomycin on net influx of $^{36}Cl^-$. Details as in Fig. 1a, but with 50 μM oligomycin added at arrow. pH 6. 22°. b. Effect of oligomycin and anaerobiosis on efflux of $^{36}Cl^-$. Details as in Fig. 1b, but 12 μM oligomycin added (– – – –) or transferred to anaerobic conditions (.....) at arrow. Both treatments also stimulated $^{36}Cl^-$ efflux in salt solution, with approximately the same time course. pH 6. 22°.

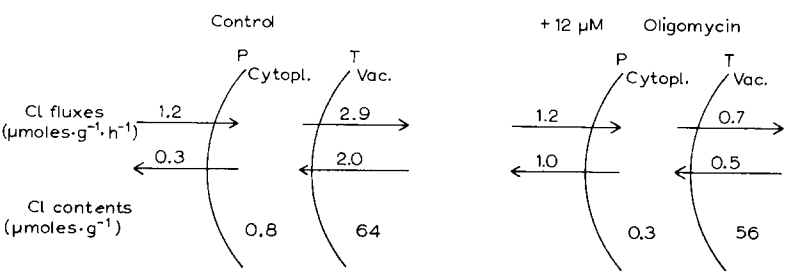

Fig. 4. Estimated values of plasmalemma (P) and tonoplast (T) Cl^- fluxes and of cytoplasmic and vacuolar Cl^- contents in the presence and absence of oligomycin. All details as in Fig. 2, but with 12 μM oligomycin instead of CCCP.

vations on other plant tissues[7,12,13]. However, ATKINSON *et al.*[7] showed that total ATP in washed carrot tissue fell to a new level within 30 min after the addition of 12 μM oligomycin.

A new steady state with regard to Cl^- movements is reached after about 4 h in oligomycin (Fig. 3), and first order components of the Cl^- efflux are observed in the new steady state. The estimated values of the plasmalemma and tonoplast fluxes and of the cytoplasmic and vacuolar contents in the presence and absence of 12 μM oligomycin are shown in Fig. 4. 12 μM oligomycin was chosen as having a near maximum effect on net tracer influx, and as being the concentration used by ATKINSON *et al.*[7].

The final effects of oligomycin are qualitatively the same as those of CCCP. M_{oc} is unaffected, M_{cv} and M_{vc} and Q_c are reduced, and M_{co} is increased. Following the same lines of argument as for the CCCP results, it is most likely that oligomycin inhibits net tracer influx by inhibiting M_{cv}, and one must also suppose that oligomycin affects some of the other Cl^- movements.

However, oligomycin differs from CCCP in the rate at which it affects M_{cv}. The results of ATKINSON *et al.*[7] show that both oligomycin and CCCP complete their effects on respiration, and in particular on total ATP level, within 30 min. The results in the previous section suggest that CCCP inhibits M_{cv} about as fast as it affects respiration, and show that when M_{cv} is inhibited as fast as this the net tracer influx falls to a new steady rate within about 40 min. In contrast to this, oligomycin does not reduce net tracer influx to a new steady rate until about 2 h after it has been added, although it acts on respiration about as fast as does CCCP. Therefore oligomycin does not inhibit M_{cv} *via* its direct effect on respiration.

Nitrogen

On transfer to N_2 all aerobic respiratory pathways must almost immediately cease to function, but glycolysis continues[14]. Carrot tissue maintains its internal electrolytes for up to 2 weeks under anaerobiosis[15]. Again, first order cytoplasmic and vacuolar components of the efflux of Cl^- are observed at the new steady state in N_2. The estimated values of the plasmalemma and tonoplast fluxes and of cytoplasmic and vacuolar contents in air and N_2 are shown in Fig. 5.

Under N_2 M_{cv}, M_{vc} and Q_c are reduced and M_{co} is increased, as in CCCP. (This strengthens the argument that the effects of CCCP are consequent upon its effect on respiration.) But in addition anaerobiosis leads to a reduction in M_{oc}.

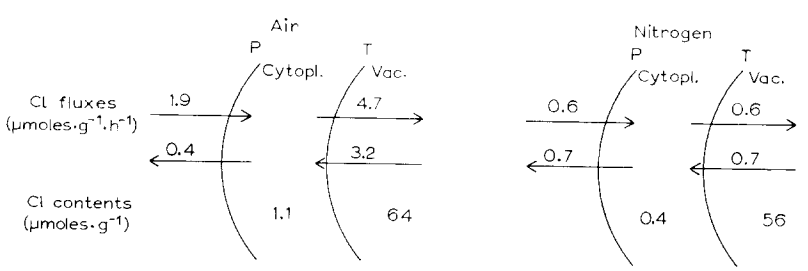

Fig. 5. Estimated values of plasmalemma (P) and tonoplast (T) Cl^- fluxes and of cytoplasmic and vacuolar Cl^- contents in air and under N_2. All details as in Fig. 2, but treated tissue was under anaerobic conditions instead of with CCCP.

It is simplest to suppose that the effect on M_{oc} is primary, *via* an effect on respiration. ATKINSON *et al.*[7] observed an immediate inhibition of net salt accumulation on transfer to N_2, which is consistent with this, but the experiment needs to be confirmed with tracer Cl^-.

The ratio M_{cv}/Q_c in N_2 is significantly ($P<0.01$) less than the ratio in air, which indicates that there is also a primary inhibition of M_{cv} in N_2. The relatively slow rise in tracer efflux (Fig. 3b) after transfer to N_2 suggests that some of the other Cl^- movements are also directly affected in N_2, as in CCCP.

After transferring to anaerobiosis, in the standard solution or in water, the potential difference between the vacuole and the external solution remains constant for at least 29 min. In some experiments a slow fall of potential difference was observed (about 0.5 mV per min), which was reversed on transfer back to O_2. This suggests that there are no electrogenic pumps in carrot cells, or that the potential difference so generated is almost completely short-circuited by passive ion movements; and that passive permeabilities to the potential determining ions are not altered under N_2.

DISCUSSION

The influx of Cl^- across the plasmalemma in normal, washed, carrot cells is up a free energy gradient[1], and therefore must be linked to some energy supply. In this paper it has been shown that the Cl^- tracer influx across the plasmalemma is not inhibited by oligomycin or CCCP at concentrations which affect respiration and other Cl^- movements in the cell, but is inhibited under N_2. It seems therefore that the Cl^- influx pump at the plasmalemma is more closely linked to the redox reactions of the respiratory chain than is ATP or respiratory high energy intermediate formation. In giant algal coenocytes, similarly, Cl^- influx across the plasmalemma appears to be related to photosynthetic electron flow rather than to photophosphorylation[11,16]. These observations raise problems of structural relationships between the mitochondrion or chloroplast and the plasmalemma.

If the influx of one Cl^- across the plasmalemma in carrot cells is linked to the movement of 1 equivalent along the whole of the respiratory redox chain[17,18], then the ratio (anions pumped/linked O_2 uptake) cannot be greater than 4. One can make some estimate of the ratio (plasmalemma Cl^- influx/salt stimulated O_2 uptake) from the data of ROBERTSON AND WILKINS[19] and the results presented in the previous paper[1]. The estimated active component of the Cl^- influx at the plasmalemma[1] and the salt stimulated respiration[19] both increase with external Cl^- concentration up to 10 mM. Under the conditions of the experiments of ROBERTSON AND WILKINS (low vacuolar content) the plasmalemma influx is much less than the tonoplast influx, and the plasmalemma efflux is low[2,20]. Therefore the net influx and the plasmalemma influx are approximately equal. Hence the maximum ratio (active plasmalemma Cl^- influx/salt stimulated O_2 uptake) would appear to be approximately equal to ROBERTSON AND WILKINS' ratio at an external concentration of 10 mM, *i.e.*, about 3. This is consistent with a close linkage of this Cl^- pump to respiratory redox reactions, and is very similar to values found for the ratio of H^+ secretion to linked O_2 uptake in the gastric mucosa[21–23].

The influx of Cl^- across the tonoplast in normal, washed, carrot cells is probably also up a free energy gradient[1], and must therefore also be linked to an energy supply.

In this paper it has been concluded that the influx of Cl^- at the tonoplast is reduced by CCCP and anaerobiosis *via* their effects on respiration, and that oligomycin probably does not inhibit this flux *via* an effect on respiration. CCCP and oligomycin, at the concentrations used in this work, reduce the total cell ATP to approximately the same level[7]. L-Ethionine also reduces the total cell ATP in carrot without reducing salt accumulation concomitantly[14]. The effect of CCCP in inhibiting the tonoplast influx is therefore not *via* reduction of total cell ATP. This seems to imply that energy from respiration is supplied to the tonoplast pump *via* respiratory high energy intermediate, without the mediation of ATP. This conclusion has also been reached, on similar grounds, for several cellular and mitochondrial energy-utilising processes in animal systems (*e.g.*, refs. 5, 25, 26). There is no evidence as yet to indicate what the intermediate stages might be.

It is difficult to account for the slow inhibition of the tonoplast influx by oligomycin. A direct inhibition of the tonoplast flux by oligomycin (*cf.* ref. 27) seems more likely than an explanation in terms of pools of ATP (*cf.* further discussion below), although it is difficult to see why oligomycin should not act at the tonoplast as fast as it acts at the mitochondrion.

Although the Cl^- influx across the plasmalemma in carrot cells has all the characteristics to be expected of a close link to respiratory redox reactions, when overall salt accumulation in the vacuole is measured this will not be apparent. It follows that attempts to reconcile the accumulation/salt stimulated O_2 uptake stoichiometry with uncoupler sensitivity in the same step[18] may be unnecessary; and further, that the question of whether "salt uptake" by higher plant tissues is linked to ATP-high energy intermediate or to redox reactions may be inappropriate. So long as the overall influx, or net influx, to the vacuole is measured, the answer would appear to be — to both.

Other authors have produced evidence that the influx of Cl^- at the plasmalemma and the tonoplast of higher plant cells are both energy dependent but are linked in different ways to energy supply processes[28,29]. These two methods are not quantitative, but they do give weight to the idea that in both photosynthetic and non-photosynthetic higher plant cells the energy supply to the tonoplast and the plasmalemma transport processes may be different (*cf.* DISCUSSION in ref. 11).

In Nitella, on the other hand, the tonoplast influx appears to be linked to the plasmalemma influx under all the conditions so far examined[3]. No such linkage has appeared in this work on carrot.

The effects of these three treatments on the other fluxes and on the cytoplasmic content cannot be simply interpreted, except insofar as they suggest that the outward fluxes may not be simply passive diffusional movements. Since there appears to be some effect on the outward fluxes, one cannot feel confident in taking the inhibited portions of M_{oc} and M_{cv} as minimal estimates of the pump component of these fluxes, although this interpretation would be the simplest.

The absence of an effect of oligomycin on O_2 uptake by plant tissues is unexpected. In plant and animal mitochondria in State 4 (refs. 4–6, 30), and in animal tissues (*e.g.* refs. 26 and 31), oligomycin inhibits O_2 uptake at least to some extent, whereas oligomycin has not been found to affect O_2 uptake in plant tissues[7,12,13]. CCCP causes a fall in ATP and an increase in O_2 uptake in carrot tissue, which cannot be due to an increased rate of glycolysis following a lowered ATP/ADP ratio (*cf.* ref. 32), since

oligomycin causes a similar fall in ATP[7] without stimulating O_2 uptake. O_2 uptake by carrot root mitochondria *in vivo* therefore appears to be at least in part limited by the rate of high energy intermediate utilisation. As oligomycin does not inhibit O_2 uptake, it would appear that ATP production is not a rate limiting factor in the utilisation of high energy intermediate in carrot. This leads to the conclusion either that ATP is not the main energy transducer from the mitochondrion *in vivo*, or that the action of oligomycin *in vivo* and/or the nature of respiration in carrot tissue are not the same as in extracted mitochondria.

(If the interpretation of these results in terms of the activities of extracted mitochondria is rejected, the simplest alternative would be to begin with the hypothesis that both active Cl^- fluxes in carrot cells are driven by ATP. The salient features of such a scheme are that it would involve at least two pools of ATP in the cytoplasm, both dependent on aerobic metabolism; that the two active Cl^- fluxes would depend on a single small pool of ATP; and that the differential effect of CCCP would be the result of different relationships of the two fluxes to the ATP level in this pool. This scheme would lead one to expect that the two active Cl^- fluxes might compete for the energy source under some circumstances (*cf.* the fall in tonoplast influx with rise in plasmalemma influx as the external Cl^- concentration is raised, ref. 1, Fig. 6). The stoichiometry between the plasmalemma influx and salt stimulated respiration would then have to be interpreted not as a tightly linked energy sink–energy source relationship, but possibly as some sort of control mechanism. Such a scheme necessitates more postulates than does the more inductive scheme in the body of the text, and will therefore be rejected for the present in favour of the latter[33].)

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