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CHLORIDE TRANSPORT AND PHOTOSYNTHESIS IN CELLS OF GRIFFITHSIA

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SUMMARY

1. A convenient and sensitive method for making oxygen measurements on single giant algal cells is described.

2. Fluxes of Cl^- and rates of apparent photosynthetic oxygen evolution and respiratory oxygen consumption have been measured in cells of the marine algae *Griffithsia monile* and *Griffithsia flabelliformis*. The Cl^- influx was active and light-stimulated, but there was no compulsory linkage between photosynthetic electron flow and Cl^- influx. The energy required to support the light-dependent Cl^- influx in Griffithsia is estimated at approx. 0.3 % of that generated in photosynthesis.

3. The metabolic linkages of the light-stimulated and dark components of Cl^- influx have been investigated in *G. monile* by studying the effects of O_2 and N_2, and of the metabolic inhibitors 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and phlorizin on the Cl^- influx. The effects on carbon fixation, apparent rates of photosynthetic oxygen evolution, respiratory oxygen consumption, and membrane electrical properties were also determined.

4. The results suggest that the light-dependent component of Cl^- influx in *G. monile* is associated with the rate of non-cyclic photophosphorylation. The dark component of the Cl^- influx, which may be part of a Cl^- exchange-diffusion system and not active transport, may be associated with mitochondrial respiration.

INTRODUCTION

Many of the active transport systems in giant algal cells are partially dependent on light[1]. One approach to the study of the mechanism of active transport has been to identify the metabolic energy supply for these processes. An active ion transport system that is partially dependent on light may derive energy, not necessarily directly, from reductant generated by the photosystems, or from photophosphorylation.

The two principal active ion-transport systems found in giant algal cells[1] have been reported to have differing metabolic dependence. In both *Nitella translucens*[2,3] and *Hydrodictyon africanum*[4–8], the coupled Na^+–K^+ transport system is considered to

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

be linked to photophosphorylation and the Cl^- influx system to be linked to photosynthetic electron flow.

The main difficulty in interpreting the effects of metabolic inhibitors on active ion fluxes in giant algal cells has been due to uncertainties about the way in which some inhibitors act on these cells *in vivo*[10]. Most investigators have used carbon fixation to measure the effectiveness of metabolic inhibitors, although NISHIZAKI[11] has measured changes in photosynthetic oxygen evolution in *Chara braunii*. In no experiments so far have both electron transport and photophosphorylation been measured directly on giant algal cells. Changes in the rates of electron flow and of photophosphorylation may be estimated in intact cells by measuring the rates of both photosynthetic oxygen evolution and of carbon fixation.

The giant coenocytic cells of the red marine alga, Griffithsia, have a large active Cl^- influx[12,13]. It seemed that the measurement of Cl^- influx, photosynthetic oxygen evolution, and carbon fixation in Griffithsia, and the effects of metabolic inhibitors on these, should give useful information on the metabolic dependence of Cl^- influx in these cells.

MATERIAL AND METHODS

Material

Strands of cells of *G. monile* were collected from intertidal rock platforms at Robe, and at Cape du Couedic and Pennington Bay on Kangaroo Island, South Australia. *Griffithsia flabelliformis* was obtained from the sea-bed of the tidal inlet to American River, on the northern coast of Kangaroo Island. The material was stored in the laboratory for up to five weeks in aerated sea-water at 12° under low-intensity natural light.

Experimental media and inhibitors

The basic experimental medium was an artificial sea water, containing 10 mM KCl, 490 mM NaCl, 11.5 mM $CaCl_2$, 25 mM $MgCl_2$, 25 mM $MgSO_4$, 1 mM NaBr and 2.5 mM $NaHCO_3$ (final pH 7.6). In the medium from which $NaHCO_3$ was omitted, 1 mM sodium phosphate (pH 7.6) was also added.

Solutions of the metabolic inhibitors 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (E.I. DuPont de Nemours and Co.), phlorizin (Sigma), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Calbiochem) were prepared without added ethanol. Absorbance measurements showed that the inhibitors dissolved completely at the concentrations used.

$Na^{36}Cl$ and $NaH^{14}CO_3$ were supplied by the Radiochemical Centre, Amersham. The oxygen and nitrogen gases used contained less than 5 vol. per million CO_2, and the nitrogen contained less than 10 vol. per million O_2. All experiments were done at 22–23°.

Cell surface area and volume

The surface area and volume of cells of *G. monile* were determined by approximation to a prolate spheroid, as previously described[12]. Cells of *G. flabelliformis*, however, are waisted in shape, although radially symmmetrical about the major axis. The shape of these cells was treated as being made up of two end-hemispheres and two right

cone frustums (Fig. 1). The accuracy of estimates of surface area and volumes by this approximation was checked, for several cells, by comparison with values measured from an enlarged image of the cell circumference containing the major axis. The calculated and measured values, for both surface area and volume, agreed within 5 %.

Oxygen measurements on single Griffithsia cells

A Radiometer type E5046 oxygen electrode was connected to the circuit shown in Fig. 2. The electrode was normally positioned so that the oxygen-sensitive area of the electrode membrane was just touching the wall of a Griffithsia cell (Fig. 3). The electrode could be moved away from the cell wall into the artificial sea water for occasional standardising of the output and then returned to precisely the same position against the cell by means of a calibrated micromanipulator. The artificial sea water was gently stirred. The cell being measured was illuminated from underneath the perspex container by heat-filtered light provided by a bank of six 8-W, white fluorescent tubes. The light was of saturating intensity with respect to photosynthetic oxygen evolution.

The output of the oxygen electrode, when used in this manner, reached a steady level which appeared to be proportional to the rate of oxygen evolution or consump-

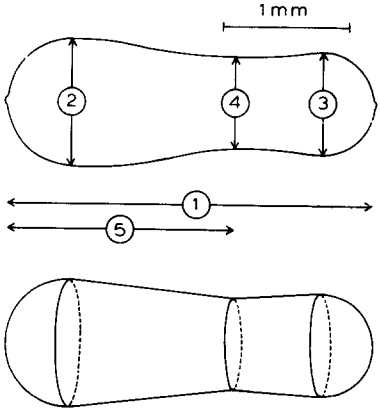

Fig. 1. Determination of surface area and volume of cells of *G. flabelliformis*. The five measurements were made on each cell (above) and the approximated shape (below) used for calculation.

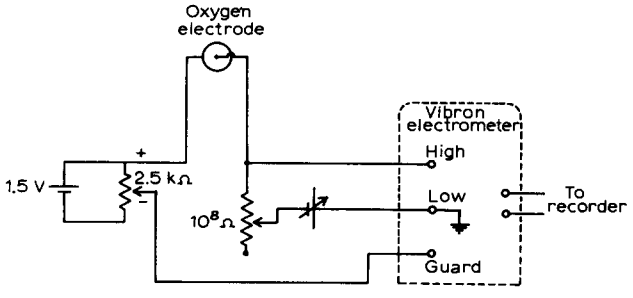

Fig. 2. Oxygen electrode measuring circuit. The potential of the guard terminal of the Vibron electrometer (Electronic Instruments Inc., Surrey, U.K.) follows that of the high resistance input lead, and prevents voltage drop across the oxygen electrode when current flows. The polarising voltage was adjusted to 630 mV.

tion, and was very sensitive to changes in this rate. This open system was calibrated by comparison with a closed system in which the increase in oxygen concentration of the artificial sea water due to evolution by a single Griffithsia cell was measured. This allowed calculation of the rate of oxygen evolution by the cell as pmoles of oxygen per sec per cm^2 of cell surface area.

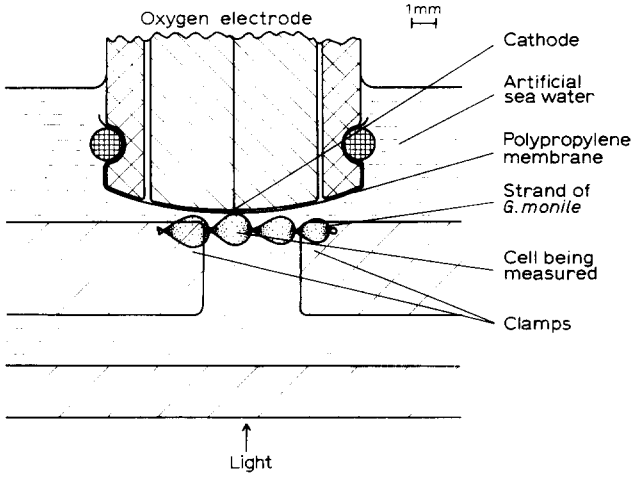

Fig. 3. The relationship between oxygen electrode and cells during measurements on *G. monile*. For explanation see text.

Alternate measurements were made on a single cell in both systems, under identical conditions of light and temperature, using the same oxygen electrode. It was found that in the open system, a change in output of the electrode of 1 mV corresponded to a change in rate of oxygen evolution by the cell of 1.2 pmoles$\cdot cm^{-2} \cdot sec^{-1}$.

Measurement of Cl^- fluxes

The rates of influx and efflux of Cl^- were estimated for single cells of Griffithsia by the ^{36}Cl tracer method described previously[13]. Routine influx measurements were made on batches of 7–10 cells using a 1-h pretreatment period in inactive solution followed by tracer for a further hour. The cells were then washed for 2 min with artificial sea water at 2°, and total cell radioactivity determined. The light source was a bank of four 40-W white fluorescent tubes.

Experiments, in which the effect of atmospheres of N_2 and O_2 on the Cl^- influx was determined, were done in tubes with loose-fitting plugs. The solutions (artificial sea water *minus* bicarbonate) were bubbled vigorously with air (control), oxygen, or nitrogen. Preliminary experiments showed that there was no significant difference in Cl^- influx between cells in unstirred artificial sea water, and cells in artificial sea water stirred and bubbled vigorously with air. All solutions were pre-saturated with the gas to be used.

Measurement of carbon fixation

The rates of carbon fixation by single cells of Griffithsia were determined with $NaH^{14}CO_3$, using the same treatment times and conditions as for Cl^- influx measure-

ments. At the end of the fixation period, cells were washed for 2 min in artificial sea water at 2° and then each cell was cut open and the contents dispersed in 0.5 ml of distilled water on a planchette. The heating during drying was sufficient to remove unfixed ^{14}C, as the count rate was not affected by acid treatment of the cell contents. Standard samples (1 μl) of the $NaH^{14}CO_3$-labelled artificial sea water used in each experiment were also dried on planchettes in 0.5 ml of distilled water with 10 μl of saturated $Ba(OH)_2$ solution which prevented any loss of $^{14}CO_2$.

The counting efficiency of ^{14}C was reduced by about 25 % by the contents of an average-size Griffithsia cell, compared with that of the standard samples. This was corrected by a factor determined from experiments in which standard amounts of ^{14}C radioactivity were added to the contents of Griffithsia cells on planchettes and counted in the normal manner.

Electrical measurements

The electrophysiological methods employed to measure the electrical potentials and resistances across the plasmalemma and tonoplast have been described previously[12].

RESULTS

Oxygen electrode measurements on Griffithsia

A typical recorder trace from the oxygen electrode with a Griffithsia cell is shown in Fig. 4. Typical rates for cells of *G. monile* and *G. flabelliformis* were in the range 45–80 pmoles $O_2 \cdot cm^{-2} \cdot sec^{-1}$ for apparent photosynthetic oxygen evolution and 5–20 pmoles $O_2 \cdot cm^{-2} \cdot sec^{-1}$ for respiratory consumption.

Cl^- fluxes

The influx of Cl^- to cells of *G. monile* in the light was in the range 20–70 pmoles $\cdot cm^{-2} \cdot sec^{-1}$, and in the dark, 5–15 pmoles $\cdot cm^{-2} \cdot sec^{-1}$, although values as high as 80 pmoles $\cdot cm^{-2} \cdot sec^{-1}$, and about equal to influx in the light, were occasionally observed

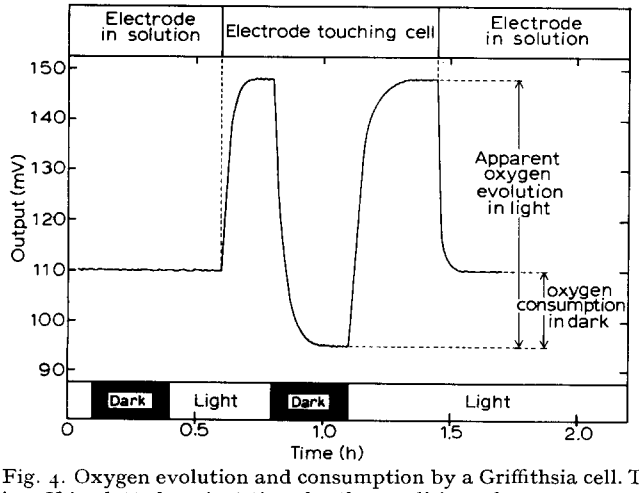

Fig. 4. Oxygen evolution and consumption by a Griffithsia cell. The output of the electrode circuit in mV is plotted against time for the conditions shown.

with fresh material during the first few days of storage. There was no significant difference in Cl^- influx with cells from diploid, haploid female, or haploid male plants, and all three types of cells were used interchangeably in subsequent experiments.

Table I illustrates the light-dependence of Cl^- influx usually observed with Griffithsia. A net influx of Cl^- took place in the light in both *G. monile* and *G. flabelliformis*. The efflux in both species was almost unaffected by light.

The results of an experiment with *G. flabelliformis*, in which the time-course of Cl^- influx was measured after a dark-to-light transition, are shown in Fig. 5. The light-dependent Cl^- influx took more than 15 min after turning on the light to reach the steady-state level. Similar results were obtained with *G. monile* for which the time taken to reach the steady-state value varied between 5 and 15 min.

Table II shows the effect of oxygen and nitrogen on Cl^- influx to cells of *G. monile* in light and dark. For these experiments, bicarbonate was omitted from the

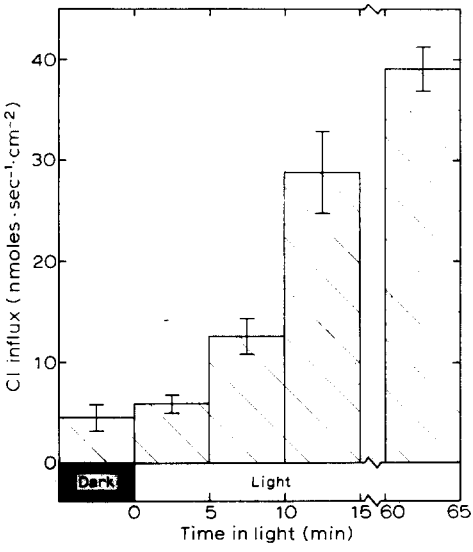

Fig. 5. Cl^- influx in *G. flabelliformis*, plotted against time, following dark-light transition. Cells were pretreated in the dark for 60 min, and the tracer uptake periods were 5 min. The length of the bars on each rectangle represents the standard error of the mean of 10 cells.

TABLE I

CHLORIDE FLUXES IN GRIFFITHSIA

The values for each species were determined on cells from the same plant and are means ± S.E., with the number of cells used in each batch given in parentheses.

		Rate (pmoles $Cl^- \cdot cm^{-2} \cdot sec^{-1}$)	
		Influx	*Efflux*
G. monile	Light	23.4 ± 5.9(9)	6.3 ± 1.1(3)
	Dark	10.4 ± 2.7(6)	9.6 ± 2.6(3)
G. flabelliformis	Light	39.1 ± 2.4(5)	15.1 ± 2.8(5)
	Dark	4.5 ± 1.3(10)	14.4 ± 2.8(5)

artificial sea water to help suppress photosynthetic oxygen evolution. The light-dependent component of the Cl^- influx to cells of *G. monile* was not significantly affected by oxygen or nitrogen. In the dark, however, a marked effect was observed, and the influx under oxygen was significantly higher than that under air, while the influx under nitrogen was lower.

Carbon fixation

The rates of carbon fixation by *G. monile* measured in the light were in the range of 20–50 pmoles·cm^{-2}·sec^{-1}. The rates of fixation in the dark were less than 3 % of those in the light.

Electrical measurements

Values for the electrical potentials and resistances across the plasmalemma and tonoplast membranes of *G. monile* in the light were found to be similar to those reported by FINDLAY *et al.*[12]. The transition from light to dark had no effect on the potential across the plasmalemma, which was in the range —80 to —85 mV. The potential across the tonoplast (+23 to +35 mV) usually depolarised by a few millivolts on darkening. The resistances of both membranes were increased reversibly by a factor of 2–3 in the dark.

The effect of $1 \cdot 10^{-6}$ M DCMU in the light was very similar to that of darkness. The effects of 5 μM CCCP were measured over periods of at least 2 h. This compound caused a small hyperpolarization of the tonoplast by up to 5 mV, and an increase in its resistance by a factor of up to 2.

TABLE II

EFFECT OF NITROGEN AND OXYGEN ON CHLORIDE INFLUX IN *G. monile*

	Rate (pmoles Cl^-·cm^{-2}·sec^{-1})		
	Light	*Dark*	*Light-dependent component*
Air	66.5 ± 6.3(30)	9.0 ± 2.6(28)	57.5 ± 6.8
Oxygen	71.9 ± 8.1(30)	21.2 ± 4.5(30)	50.7 ± 9.2
Nitrogen	56.9 ± 6.0(30)	3.6 ± 0.6(25)	53.3 ± 6.0

Inhibitor studies

DCMU. At a concentration of 3 μM DCMU, Cl^- influx was reduced to about that in the dark. By subtraction of the value for the dark from that for the light, a plot of the percentage inhibition by DCMU of the light-dependent component of the Cl^- influx was obtained (Fig. 6). The apparent rate of photosynthetic oxygen evolution by *G. monile* cells was also inhibited by DCMU (Fig. 6). The full inhibitory effect of a given concentration of DCMU was exerted within a few minutes suggesting that this inhibitor permeates the cell rapidly.

These results show that the inhibition of the light-stimulated component of the Cl^- influx follows very closely the inhibition curve for apparent photosynthetic oxygen evolution. 3 μM DCMU depressed the rate of carbon fixation to 5 % of the control value. DCMU had no significant effect on the rate of respiratory oxygen consumption,

and the rate of oxygen consumption was unaffected by light when $5 \cdot 10^{-5}$ M DCMU was present.

CCCP. In Fig. 7, the effects are compared of CCCP on the light-dependent component of Cl^- influx, on carbon fixation, and on the apparent rate of photosynthetic oxygen evolution. This compound also affected oxygen evolution by the cells almost immediately after addition to the medium.

The apparent rate of photosynthetic oxygen evolution was slightly stimulated by CCCP in the range 1–5 μM, but was inhibited by higher concentrations. The rate of carbon fixation was more sensitive, and at 5 μM CCCP was inhibited to 38 % of the

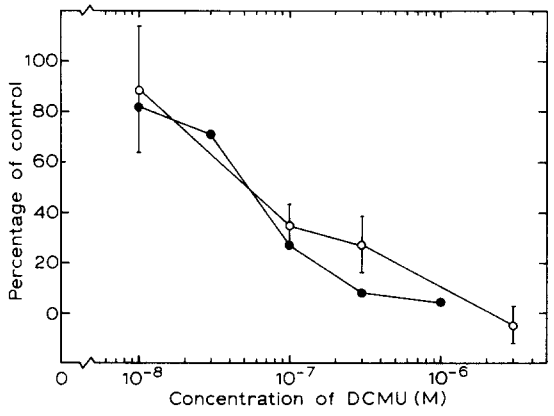

Fig. 6. Effects of DCMU on *G. monile* in light. ●——●, Apparent photosynthetic oxygen evolution, average for 2 cells; control rate was 61.7 pmoles $O_2 \cdot cm^{-2} \cdot sec^{-1}$. ○——○, light dependent component of Cl^- influx, average for 10 cells; control influx was 25.8 ± 2.7 pmoles $Cl^- \cdot cm^{-2} \cdot sec^{-1}$.

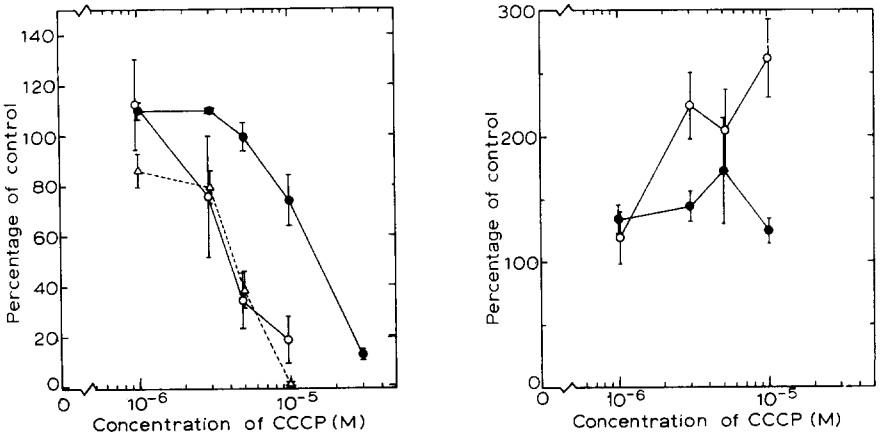

Fig. 7. Effects of CCCP on *G. monile* in light. ●——●, apparent photosynthetic oxygen evolution, average values for 4–6 cells; control rate was 63.1 ± 2.1 pmoles $O_2 \cdot cm^{-2} \cdot sec^{-1}$. ○——○, light-dependent component of Cl^- influx, average for 20 cells, control influx was 32.0 ± 3.9 pmoles $Cl^- \cdot cm^{-2} \cdot sec^{-1}$. △---△, carbon fixation, average for 20 cells; control rate was 48.8 ± 3.0 pmoles carbon $\cdot cm^{-2} \cdot sec^{-1}$.

Fig. 8. Effects of CCCP on *G. monile* in dark. ●——●, respiratory oxygen consumption, average values for 4–6 cells; control rate was 10.9 ± 1.4 pmoles $O_2 \cdot cm^{-2} \cdot sec^{-1}$. ○——○, Cl^- influx, average values for 20 cells; control influx was 4.5 ± 1.2 pmoles $Cl^- \cdot cm^{-2} \cdot sec^{-1}$.

control rate while apparent photosynthetic oxygen evolution was not significantly different from the control rate. The light-dependent component of the Cl^- influx was also more sensitive to CCCP and the inhibition curve appears to follow closely that for carbon fixation, with the possible exception of the flux at 1 μM CCCP.

The rate of respiratory oxygen consumption and Cl^- influx in the dark, however, were stimulated by CCCP in the range 1–10 μM (Fig. 8). The dark Cl^- influx appeared to be stimulated to a greater extent than respiratory oxygen consumption.

Phlorizin. In concentrations up to 1 mM, phlorizin had no significant effect on Cl^- influx into cells of *G. monile*. This compound, however, also had no effect on carbon fixation and on the rates of apparent photosynthetic oxygen evolution and respiratory oxygen consumption, showing that the cells may be impermeable to this glucoside. *Chara corallina* may also be impermeable to phlorizin[10].

DISCUSSION

Oxygen measurements

The rate of apparent photosynthetic oxygen evolution in Griffithsia has been determined from the difference between the rate of evolution in the light and the rate of respiratory consumption in the dark. Although light had no effect on the respiratory rate in the presence of $5 \cdot 10^{-5}$ M DCMU, it is possible that the rate of respiratory oxygen consumption is normally greater in the light[14,15] due to either the interaction of photosynthetic products with mitochondrial respiration, or to photorespiration. The values measured for apparent photosynthetic oxygen evolution may therefore be underestimates. The occurrence of pseudocyclic electron flow is a further potential source of underestimation.

Cl^- fluxes

The estimation of light-dependent Cl^- influx in Griffithsia also involves the necessary assumption that the dark component is unchanged in the light. However, the differing responses of the light-dependent and dark components of Cl^- influx to metabolic inhibitors and to anaerobic conditions appear to justify its separation into the two components.

An estimation of the fraction of the free energy of photosynthesis that is required to support the light-dependent component of Cl^- influx can be made from the relative rates of apparent photosynthetic oxygen evolution and Cl^- influx. Typical rates measured in comparable cells of *G. monile* were 70 pmoles $O_2 \cdot cm^{-2} \cdot sec^{-1}$ for apparent photosynthetic oxygen evolution and 20 pmoles $Cl^- \cdot cm^{-2} \cdot sec^{-1}$ for the light-dependent Cl^- influx. The approximate free energy of photosynthesis under normal conditions is 112 kcal per mole of oxygen evolved[16]. From this, the free energy of photosynthesis in *G. monile* can be calculated: $70 \cdot 10^{-12} \cdot 112 \cdot 4.19 \cdot 10^{10} = 328$ erg $\cdot cm^{-2} \cdot sec^{-1}$.

The energy required to transport one mole of chloride against an electrochemical gradient $\Delta\bar{\mu}_{Cl}$ is $F \cdot \Delta\bar{\mu}_{Cl}$ joules if $\Delta\bar{\mu}_{Cl}$ is in volts (F is the Faraday). The difference in electrochemical potential for Cl^- between the outside and the vacuole of *G. monile* is about 57 mV, and from this the energy required for an influx of 20 pmoles $Cl^- \cdot cm^{-2} \cdot sec^{-1}$ is: $20 \cdot 10^{-12} \cdot 0.057 \cdot 96500 \cdot 10^7 = 1.1$ erg $\cdot cm^{-2} \cdot sec^{-1}$. This shows that the energy required to maintain the light-dependent component of the Cl^- influx is a very small fraction (0.3 %) of the total free energy of photosynthesis.

The slow increase in the light-dependent Cl^- influx that is observed after a dark-light transition with both species of Griffithsia shows that this active Cl^- influx is not compulsorily linked to photosynthetic electron flow since the apparent photosynthetic oxygen evolution begins almost instantaneously. The energy for light-dependent Cl^- influx may therefore be derived from some high-energy compound generated in the chloroplasts by photosynthesis, which then takes a finite time to move from the chloroplasts to the site of the Cl^- active transport mechanism, or to build up an appreciable concentration there.

Effects of oxygen and nitrogen

The insensitivity of the light-dependent component of Cl^- influx to oxygen and nitrogen shows firstly that light-dependent Cl^- influx is not significantly affected by the presence or absence of CO_2, which was present in the controls in air but not in the oxygen or nitrogen. This again demonstrates that the rate of operation of the photosynthetic electron transport system, reduced in the absence of CO_2, is not directly linked to light-dependent Cl^- influx. Secondly, the indifference to the presence or absence of oxygen shows that this part of the influx is not dependent on any oxygen-consuming process such as light-stimulated respiration or pseudocylic photophosphorylation. In this respect, *G. monile* may differ from some of the fresh-water giant algal cells[3,5,17,18].

The dark component of the Cl^- influx in *G. monile*, however, appears to be affected by the concentration of oxygen present, suggesting that it may be influenced by mitochondrial respiration.

The effects of metabolic inhibitors

The effects of the substances DCMU and CCCP on the electrical properties of the plasmalemma and tonoplast of *G. monile* were not greater than the effects of darkness compared with light, so it is unlikely that the observed effects of these compounds were due to non-specific damage to the cell membranes. Both the light-dependent component of Cl^- influx and apparent photosynthetic oxygen evolution exhibited similar sensitivities to DCMU. This suggests that the Cl^- influx is not supported by any energy source related to cyclic electron flow or its associated photophosphorylation. Similar sensitivities of light-dependent Cl^- influx to DCMU have been demonstrated with fresh-water giant algal cells[2,5,9,17]. The Cl^- influx in the light into cells of *Chlorella pyrenoidosa*[19], however, is less sensitive to DCMU than is photosynthetic oxygen evolution.

The effects of 5 μM CCCP on carbon fixation and on apparent photosynthetic oxygen evolution confirm that this compound acted as an uncoupler of photosynthesis in cells of *G. monile in vivo*. The increase in respiratory oxygen consumption caused by CCCP suggest that oxidative phosphorylation was also uncoupled. Since the inhibition of light-dependent Cl^- influx by CCCP closely followed that of carbon fixation, the energy supply for this active Cl^- influx may be related to non-cyclic photophosphorylation rather than to photosynthetically generated reductant. Similar results have been obtained for *Chara corallina*[10], but in other characean cells light-dependent Cl^- influx is less sensitive to CCCP than is carbon fixation[8,17,20]. In the last three papers referred to, however, it was not directly demonstrated that uncoupling was actually taking place.

The marked stimulation of the dark component of Cl^- influx by CCCP is un-

likely to be due to an increased rate of energy supply to an active Cl^- influx mechanism. The Cl^- influx in the dark in Griffithsia may, however, be part of a passive exchange-diffusion system and coupled to an equal efflux[13]. In this case, the stimulation by CCCP may be the result of mitochondrial respiratory electron flow being associated in some unknown way with the Cl^- exchange-diffusion system, explaining why dark Cl^- influx is inhibited under nitrogen and stimulated under oxygen. The influx of Cl^- in the dark to cells of *C. corallina* may also be coupled to exchange-diffusion[18], and not be an active influx.

CONCLUSIONS

These results are consistent with the energy source for active Cl^- influx in cells of *G. monile* being derived from non-cyclic photophosphorylation rather than from photosynthetically generated reductant. This suggests that ATP or a high-energy compound derived from it moves from the chloroplasts to the site of the active Cl^- influx mechanism. The location of this site is not known for Griffithsia, but is likely to be in one or both of the cell membranes. Little is known about cytoplasmic levels of ATP in giant algal cells, but it has been shown that in leaf cells of higher plants, cytoplasm and chloroplasts share a common pool of ATP[21], while intracellular pyridine nucleotides exhibit compartmentation[22]. Similar information for giant algal cells would promote an understanding of the metabolic linkages to ion transport.

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