A COMPARISON OF THE EFFECTS OF METABOLIC INHIBITORS ON CHLORIDE UPTAKE AND PHOTOSYNTHESIS IN *CHARA CORALLINA*

By F. A. SMITH* and K. R. WEST*

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Summary

A comparative study has been made of the effects of four metabolic inhibitors on chloride uptake and photosynthetic $^{14}CO_2$ fixation by cells of *C. corallina*, and on oxygen evolution by chloroplasts isolated from the cells. Low concentrations of phlorizin and Dio-9 inhibited chloride uptake, but this was not accompanied by an inhibition of photosynthesis *in vivo*, and could not be correlated with the measured inhibition of electron flow *in vitro*. Low concentrations of imidazole stimulated the chloride influx in light, but there was again no effect on photosynthetic $^{14}CO_2$ fixation, although imidazole did uncouple electron flow *in vitro*. The effect of imidazole was dependent on the pH of the external solution. Increasing concentrations of carbonyl cyanide *m*-chlorophenylhydrazone progressively reduced the chloride influx and $^{14}CO_2$ fixation, and uncoupled electron flow *in vitro*. The work provides no evidence to support the view that chloride uptake is directly linked to electron flow rather than phosphorylation.

The work is discussed with reference to previous studies of ion transport in giant algal cells, and the possible significance of direct effects of metabolic inhibitors on ion transport processes is stressed.

I. INTRODUCTION

MacRobbie (1965, 1966) showed that the active uptake of chloride and potassium ions by cells of *Nitella translucens* is greatly increased by light, under conditions where both photosynthetic light reactions (photosystems 1 and 2) are operative. When the short-wavelength system 2 is inhibited by using cut-off light filters or the chemical inhibitor *N'*-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea (DCMU), the light-promoted component of the chloride influx is also completely inhibited but the potassium influx is not. Raven (1967) obtained similar results with *Hydrodictyon africanum* and showed that the sodium extrusion pump in *H. africanum* is coupled to the inward potassium pump. Raven (1969) has also shown that the action spectrum for the chloride pump is similar to that of photosynthesis, whereas the action spectrum for the coupled potassium and sodium pumps does not show a pronounced "red drop" beyond 680 mμ wavelength. This work confirms that the potassium and sodium pumps can be supported by photosystem 1 alone. In several Characean species there is no detectable active potassium influx, but work with two of these species, *Chara corallina*† (see Hope, Simpson, and Walker 1966) and *Tolypella intricata* (see Smith 1968*a*), has indicated that the mechanism of the chloride pump is essentially similar to that in *N. translucens*.

* Department of Botany, University of Adelaide, S.A. 5001.

† The name *Chara corallina* is used in the present paper to replace *C. australis*, in accordance with the recently revised classification of the Characeae (Wood and Imahori 1965).

MacRobbie (1965, 1966) suggested that the chloride pump is linked to a non-cyclic electron transfer reaction, whilst the potassium pump is dependent on ATP, which may be supplied by cyclic photophosphorylation. This hypothesis is based on results obtained with chemical inhibitors known to be effective in several types of green plant material, and particular emphasis was placed on compounds known to act as uncouplers *in vitro*. However, the work provided no direct evidence as to the mode of operation of these compounds in the intact cells used in the studies of ion transport. Measurements of photosynthetic $^{14}CO_2$ fixation under the different conditions (see Raven 1967; Smith 1967*a*, 1968*a*) provide a useful check on the effectiveness of the different chemicals, but this technique can never conclusively demonstrate that a particular inhibitor has a specific effect on any part of the photosynthetic reaction sequence. Nevertheless, it has been found that the effects of some of the inhibitors *in vivo* seem incompatible with those to be expected *in vitro*. For example, an uncoupling of photophosphorylation *in vitro* has been obtained with ammonium salts (Krogmann, Jagendorf, and Avron 1959; Good 1960) and by imidazole (Hind and Whittingham 1963). These compounds inhibit potassium uptake and may stimulate chloride uptake in *N. translucens* (MacRobbie 1965, 1966), but do not inhibit photosynthesis (Smith 1967*a*). Furthermore, phlorizin has been shown to inhibit photophosphorylation and the accompanying electron transport *in vitro* (Izawa, Winget, and Good 1966), whereas it may stimulate $^{14}CO_2$ fixation by intact *H. africanum* cells by as much as 30% (Raven 1968). The same concentration (1 mM) inhibits the potassium and sodium pumps but has no effect on the chloride pump. By contrast, Coster and Hope (1968) reported that phlorizin at only 0·1 mM concentration greatly inhibited the chloride pump in *C. corallina*. (These workers did not investigate the effects of phlorizin on photosynthesis.)

In view of the importance of MacRobbie's hypothesis (1965, 1966) it is clearly necessary that direct information should be obtained about the mode of action of these supposedly specific inhibitors of photosynthesis, by carrying out parallel studies of ion transport and photosynthesis *in vivo* and of the biochemical effects *in vitro*. The present paper reports a series of such experiments with cells of *C. corallina*. Measurements of chloride uptake and photosynthetic $^{14}CO_2$ fixation were made using the now standard techniques. Chloroplasts were isolated and photosynthetic electron flow measured *in vitro*, using techniques developed in this laboratory by West and Wiskich (1968).* The inhibitors used in the experiments included Dio-9, which prevents photophosphorylation and the coupled electron flow *in vitro* (McCarty, Guillory, and Racker 1965), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which uncouples phosphorylation *in vitro* (Heytler 1963), as well as phlorizin and imidazole.

II. Methods

(*a*) *Growth of Cells*

Cultures of *C. corallina* were established in large-capacity (80 litres) plastic containers, with an artificial pond water composed of 1·0 mM NaCl, 0·1 mM KCl, and 0·1 mM $CaCl_2$ over a layer of garden soil 5 cm thick.

* For comparison, some results obtained with pea chloroplasts are also described.

(*b*) *Inhibitors*

Phlorizin was dissolved in ethanol to give a 250 mM stock solution. The required amounts from this solution were placed in small conical flasks from which most of the ethanol was evaporated before the experimental solutions were added. Dio-9 and imidazole were provided by Professor N. A. Walker, the Dio-9 being originally purchased from the Royal Netherlands Fermentation Industries Ltd., Delft. These compounds were dissolved in water to give stock solutions. The pH of experimental solutions containing imidazole was adjusted to the required value with dilute H_2SO_4. CCCP was a gift from Dr. P. G. Heytler (E. I. du Pont de Nemours & Co., Wilmington, Delaware) and was dissolved in ethanol to give a 0·3 mM stock solution. As with phlorizin, the required volumes of this solution were placed in small flasks and the alcohol was evaporated before the experimental solutions were added.

(*c*) *Measurements of Chloride Influx*

Experimental techniques were similar to those adopted for the previous studies of ion uptake into *N. translucens* (Smith 1967*b*) and *T. intricata* (Smith 1968*a*). Batches of 6–13 internodal cells of *C. corallina* were pretreated in solutions containing 1·0 mM NaCl, 0·1 mM KCl, and 0·5 mM $CaSO_4$, usually for 1 hr, before similar solutions labelled with ^{36}Cl were substituted for a further 1–2 hr. When used, inhibitors were present both during the pretreatment and the time over which the influx of ^{36}Cl was measured. In experiments with CCCP, the pretreatment period was only 30 min, and the influx was measured over the following hour.

Experiments were carried out at a controlled temperature (22°C), and illumination was provided by four 20-W fluorescent tubes about 40 cm from the cells. At the end of an experiment the cells were placed in non-radioactive solutions for 2 min to remove ^{36}Cl from the cell wall free space (Smith 1967*b*). Nodal cells were cut off and the internodes were dried down on planchets, the radioactivity being determined by end-window Geiger counting.

The mean chloride influx for each batch of cells was calculated, together with the standard error of the mean. The pH of experimental solutions was routinely checked.

(*d*) *Measurement of* $^{14}CO_2$ *Fixation*

Techniques used were those described previously (Smith 1967*a*). As described above, cells were pretreated in the basic solution (together with inhibitors, where appropriate). The radioactive experimental solution contained in addition 0·5 mM $NaH^{14}CO_3$ and 1 mM or 2 mM NaH_2PO_4–Na_2HPO_4 buffer at the required pH. The $NaH^{14}CO_3$ provided a supply of $^{14}CO_2$ sufficient to give high rates of photosynthesis. The aim in some experiments was to "saturate" the photosynthetic reactions so that the maximum effect of the inhibitors could be measured (Smith 1967*a*, 1968*b*).

(*e*) *Isolation of Chloroplasts*

Chloroplasts were isolated by a method similar to that described by West and Wiskich (1968). Briefly, a mass of *C. corallina* cells (internodal cells, nodes, and branch cells) was blended in a Waring blender at full speed for 5 sec in a medium which contained 0·4M sucrose, 1·0 mM EDTA, 1·0 mM $MgCl_2$, 0·03M TES* buffer at pH 7·2, and 1% bovine serum albumin. The homogenate was filtered through muslin and spun at 1000 *g* for 5 min in a Servall RC2 refrigerated centrifuge. The resulting pellet was resuspended in 1·0 ml of the isolating medium. The reaction cell used to measure chloroplast activity was constructed of Perspex and consisted of a small cell (2·5 ml volume) with an oxygen electrode and hole to admit reactants. The cell was enclosed by a water jacket which maintained the temperature at 25°C (Estabrook 1967).

Light from a 100-W tungsten source passed first through a 10-cm water-bath, then through a filter (filter I, as used by MacRobbie 1965), and was focussed on the reaction cell at an intensity of approximately 6×10^{-2} cal cm^{-2} min^{-1}. Chemicals were obtained from the Sigma Chemical Co., St. Louis, Mo. ADP was assayed enzymically (Wiskich, Young, and Biale 1964), and chlorophyll by the method of Arnon (1949). The oxygen concentration was lowered by bubbling nitrogen through the cell.

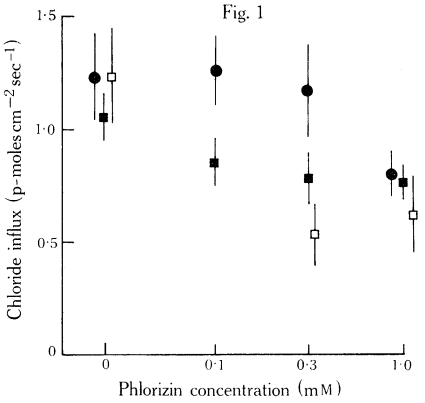
* Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

III. Results

(a) *Chloride Influx and Photosynthetic $^{14}CO_2$ Fixation*

The mean chloride influx in light from the basic solution varied in different experiments from 1·0 up to as much as 4·2 p-moles cm^{-2} sec^{-1}. The influx in the dark was less variable, the normal rate being about 0·5 p-moles cm^{-2} sec^{-1}.

Figure 1 shows the effects of phlorizin on the chloride influx in light. In the first of the two experiments shown, only the highest concentration of phlorizin (1 mM) produced a significant inhibition, the influx being reduced by about 35%. In the second experiment the chloride influx was more sensitive to phlorizin, and the influx from the basic solution was reduced by about 50% when 0·3 or 1·0 mM phlorizin was present. The addition to the solution of 1 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 6·7) considerably reduced the variability of the results, as is also shown in Figure 1.



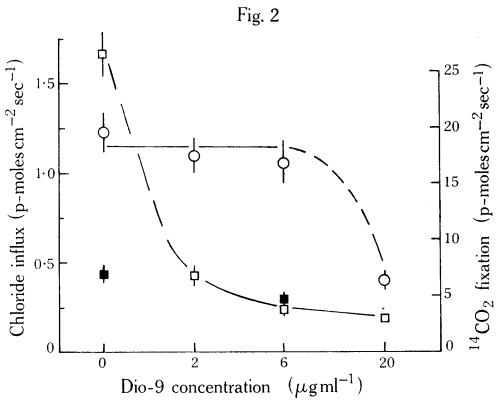


Fig. 1.—Effects of phlorizin on chloride influx in light. In experiment 1 (●) the experimental solution contained 1·0 mM $Na^{36}Cl$, 0·1 mM KCl, and 0·5 mM $CaSO_4$, together with phlorizin at the concentrations shown. In one set of treatments in experiment 2 the solution was similar (□), while in the other (■) 1 mM NaH_2PO_4–Na_2HPO_4 buffer (pH 6·7) was also present.

Fig. 2.—Effects of Dio-9 on chloride influx in light (□) and darkness (■), and on photosynthetic $^{14}CO_2$ fixation at pH 7·2 (○).

By contrast, phlorizin at concentrations up to 1 mM produced no effect on photosynthesis. For example, in one experiment the normal photosynthetic rate at pH 7·1 was 28·4±3·0 p-moles cm^{-2} sec^{-1}, whilst in the presence of 1 mM phlorizin the rate was 29·9±2·6 p-moles cm^{-2} sec^{-1}. In a second experiment at pH 6·7 the rates were 49·0±5·0 and 50·4±5·2 p-moles cm^{-2} sec^{-1} respectively.

Figure 2 shows the effects of Dio-9 on the chloride uptake and on $^{14}CO_2$ fixation. In the light, the chloride influx was greatly inhibited by Dio-9 at 2 μg ml^{-1}, and this inhibition was increased with higher concentrations. In the presence of 6 and 20 μg ml^{-1} Dio-9 the chloride influx in light was reduced to a value lower than the normal influx in the dark. It was shown that Dio-9 at 6 μg ml^{-1} also reduced the

chloride influx in the dark (Fig. 2). The two lowest concentrations of Dio-9 did not reduce photosynthetic $^{14}CO_2$ fixation, but a concentration of 20 μg ml^{-1} caused a marked inhibition.

Effects of imidazole on chloride uptake and photosynthesis are shown in Table 1. When 0·1 mM imidazole was added to the solution (at pH 7·3) the chloride influx in light was increased by about 30%, but there was no effect on the influx in darkness. With an imidazole concentration of 1·0 mM (at pH 7·2 or 6·7) the stimulation in light was even greater (about 125%). However, when the pH was increased to 7·8 the chloride influx in the presence of imidazole was slightly reduced. Photosynthetic $^{14}CO_2$ fixation was not reduced by 0·1 or 1·0 mM imidazole at pH 7·1 (Table 1).

TABLE 1

EFFECTS OF IMIDAZOLE ON CHLORIDE UPTAKE AND PHOTOSYNTHETIC $^{14}CO_2$ FIXATION

Conditions	Chloride Influx (p-moles cm^{-2} sec^{-1})	Percentage of Controls	Number of Cells
Light controls	1·66±0·15	100	7
+0·1 mM imidazole (pH 7·3)	2·15±0·24	130	8
+1·0 mM imidazole (pH 7·2)	3·76±0·40	226	8
+1·0 mM imidazole (pH 6·7)	3·73±0·37	224	7
+1·0 mM imidazole (pH 7·8)	1·36±0·20	82	8
Dark controls	0·44±0·04	100	7
+0·1 mM imidazole (pH 7·3)	0·45±0·05	102	8

Conditions	$^{14}CO_2$ Fixation (p-moles cm^{-2} sec^{-1})	Percentage of Controls	Number of Cells
Light controls (pH 7·1)	28·4±3·0	100	7
+0·1 mM imidazole (pH 7·1)	28·7±2·8	101	8
Light controls (pH 7·1)	25·0±3·8	100	6
+1·0 mM imidazole (pH 7·1)	25·3±2·6	101	6

Increasing concentrations of CCCP (from 1 to 5 μM) caused a progressively larger inhibition of the chloride influx in light (Fig. 3). In one of the experiments shown, the influx in the presence of 5 μM CCCP was 0·49±0·05 p-moles cm^{-2} sec^{-1}, which was not significantly different from the influx in the dark in the absence of CCCP (0·44±0·04 p-moles cm^{-2} sec^{-1}). In the second experiment, in which the control influx in light was higher, 5 μM CCCP produced an even greater inhibition;

the influx was $0 \cdot 09 \pm 0 \cdot 01$ p-moles $cm^{-2} sec^{-1}$, compared with a control influx in the dark of $0 \cdot 67 \pm 0 \cdot 16$ p-moles $cm^{-2} sec^{-1}$. (Effects of CCCP on the influx in the dark were not determined.) Increasing concentrations of CCCP also progressively reduced $^{14}CO_2$ fixation (Fig. 3). This experiment was carried out the day after the second experiment on chloride uptake, using cells from the same tank, and the degree of inhibition was clearly very similar.

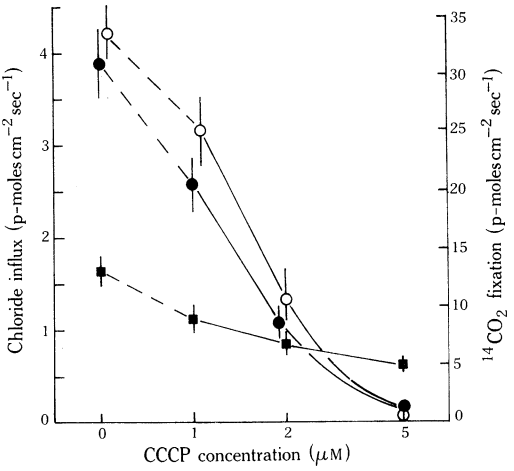


Fig. 3.—Effects of CCCP on chloride influx in light (■, experiment 1; ●, experiment 2), and on $^{14}CO_2$ fixation at pH 7·2 (○).

(*b*) *Isolated Chloroplasts*

The isolation technique described above yielded a preparation with a high proportion of intact chloroplasts as judged by phase contrast microscopy. The supernatant left after centrifugation at 1000 *g* was quite clear.

Light-induced oxygen evolution by isolated *C. corallina* chloroplasts, with ferricyanide as an electron acceptor, was coupled to phosphorylation, as was previously found with isolated pea chloroplasts (West and Wiskich 1968). Results from two preliminary experiments are shown in Figure 4. Oxygen evolution began at a slow rate when the chloroplasts were illuminated, but when ADP was added the rate increased sharply (Fig. 4, curve *A*). When the added ADP had been phosphorylated the rate of oxygen evolution decreased. A second addition of ADP again stimulated the rate.

The control of oxygen evolution during non-cyclic electron flow in isolated chloroplasts by the presence or absence of ADP has been termed photosynthetic control, and is equivalent to respiratory control in isolated mitochondria. The ratio of the rate of oxygen evolution in the presence of ADP (state III rate, Chance and Williams 1955) to the rate when ADP has been phosphorylated (state IV rate) is termed the "photosynthetic control ratio", and its value is a measure of how tightly photophosphorylation is coupled to electron flow. For the experiments shown in Figure 4 the values of this ratio were very similar—1·90 and 1·95. The values of the ADP : O ratio (i.e. moles of phosphate esterified to atoms of oxygen produced) are also given.

Figure 4, curve *A*, shows that the addition of 0·5 mM phlorizin decreased the stimulated rate of oxygen evolution, but this inhibition of non-cyclic electron flow caused by phlorizin could be relieved by the addition of the uncoupler ammonium chloride. The addition of ammonium chloride produced a stimulation of oxygen evolution which continued until the light was turned off.

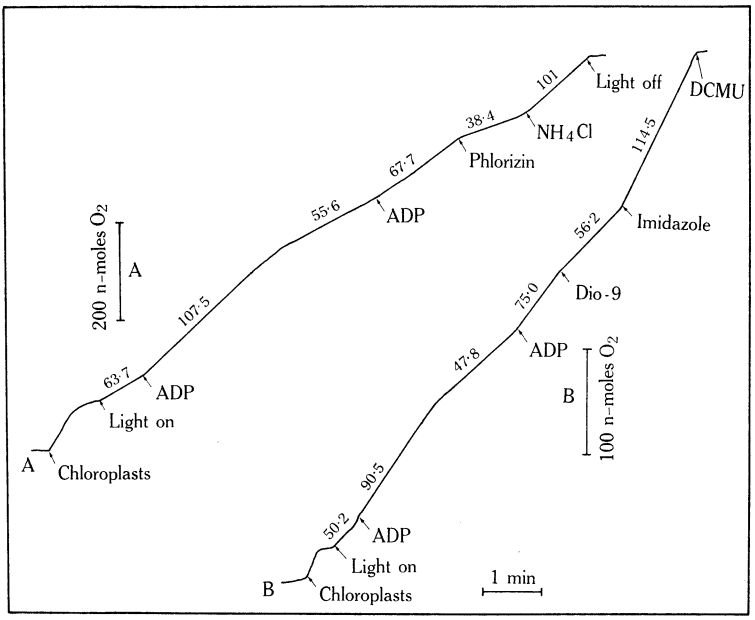


Fig. 4.—Polarographic tracing of oxygen evolution by isolated *C. corallina* chloroplasts with ferricyanide as electron acceptor. Curve *A*: the medium (4·8 ml) contained 225 mM sucrose, 8·9 mM K_2HPO_4–KH_2PO_4 buffer (pH 7·2), 0·89 mM EDTA, 4·5 mM $MgCl_2$, 2·1 mM potassium ferricyanide, and chloroplasts at a chlorophyll concentration of 0·07 mg ml^{-1}. Additions (indicated by arrows) were 0·1 mM ADP, 0·52 mM phlorizin, and 1 mM NH_4Cl. Curve *B*: the medium (2·35 ml) contained 234 mM sucrose, 9·3 mM Tris–phosphate buffer (pH 7·2), 0·93 mM EDTA, 4·68 mM $MgCl_2$, 2·0 mM ferricyanide, and chloroplasts at a chlorophyll concentration of 0·10 mg ml^{-1}. Additions were 0·1 mM ADP, 42·5 μg ml^{-1} Dio-9, 2 mM imidazole, and 42·5 μM DCMU. Numbers along the trace are n-moles O_2 min^{-1}. The photosynthetic control ratio was 1·95 and 1·90 for curves *A* and *B* respectively, and the ADP : O ratio was 1·00 and 1·30 respectively.

The antibiotic Dio-9 also acts as an inhibitor of non-cyclic electron flow, as is shown in Figure 4, curve *B*. In this experiment the inhibition of electron flow was relieved by imidazole, and oxygen evolution continued until DCMU, which blocks non-cyclic electron flow, was added.

Table 2 shows the effect of increasing concentrations of Dio-9 on the state III rate. The maximum inhibition was obtained with 42·4 μg ml^{-1}, while higher concentrations induced a progressive uncoupling. Exactly similar effects were obtained with tightly coupled pea chloroplasts.

The effectiveness of imidazole and CCCP as uncouplers of electron flow was tested by adding increasing amounts during ferricyanide-supported electron flow

TABLE 2

EFFECTS OF DIO-9 ON OXYGEN EVOLUTION BY ISOLATED CHLOROPLASTS OF *C. CORALLINA*

Concn. of Dio-9 (μg ml^{-1})	Rate of O_2 Evolution (n-moles min^{-1})	Percentage of State III Rate
Experiment 1		
0	63·7	100
4	63·7	100
Experiment 2		
0	45·0	100
21·2	33·4	74
42·4	28·8	62
63·6	42·4	94
108·8	50·8	113

without an addition of ADP (i.e. during the Hill reaction). Table 3 shows the increased rate of electron flow with increasing concentrations of imidazole.

TABLE 3

EFFECTS OF IMIDAZOLE ON OXYGEN EVOLUTION BY ISOLATED CHLOROPLASTS OF *C. CORALLINA*

Concn. of Imidazole (mM)	Rate of O_2 Evolution (n-moles min^{-1})	Percentage Stimulation of Hill Reaction or State IV Rate*
Experiment 1		
0	23·9	—
0·21	31·6	32
0·42	32·6	38
1·26	37·0	55
1·68	39·0	63
2·73	48·8	104
Experiment 2		
0	22·1	—
0·08	23·1	4
0·16	24·9	13
0·32	27·3	53·5
0·32, + 4 mM NH_4Cl	67·3	218

* Stimulation of the Hill reaction is observed in experiment 1, and of state IV in experiment 2.

Table 4 shows a similar experiment in which non-cyclic electron flow in *C. corallina* chloroplasts was progressively stimulated by increasing the concentration of CCCP. For comparison, a similar experiment with pea chloroplasts is also shown. These chloroplasts were more sensitive to CCCP.

An experiment designed to measure the effectiveness of CCCP as an uncoupler of photosynthesis *in vivo* is shown in Figure 5. Chloroplasts were isolated from cells which had been incubated in 2 μM CCCP for 40 min and the activity compared with that of chloroplasts isolated from control cells. Oxygen evolution by chloroplasts isolated from these CCCP-treated cells still responded to the addition of phosphate acceptor (ADP) and could be further stimulated by ammonium chloride. However, the photosynthetic control ratio and the ADP : O ratio were both lower than in the control cells, showing that 2 μM CCCP had partially uncoupled photophosphorylation.

TABLE 4

EFFECTS OF CCCP ON OXYGEN EVOLUTION BY *C. CORALLINA* AND PEA CHLOROPLASTS

Concn. of CCCP (μM)	Rate of O_2 Evolution (n-moles min^{-1})	Percentage Stimulation of Hill Reaction Rate
C. corallina chloroplasts		
0	20·1	—
0·6	20·1	0
1·2	20·5	2
1·8	21·8	8·5
3·0	24·2	15·5
4·2	26·5	27
4·2, + 4 mM NH_4Cl	63·0	214
Pea chloroplasts		
0	29·0	—
0·6	30·1	4
1·8	35·2	21·5
3·0	41·7	43·5
4·2	50·3	74
4·2, + 4 mM NH_4Cl	105·0	262

IV. DISCUSSION

(*a*) *General*

The measured values of the chloride influx under standard conditions (i.e. in light or darkness) were very similar to those reported by Coster and Hope (1968), although a different culture of *C. corallina* was used. It might be noted that the influx into *C. corallina* in darkness is considerably greater than in *N. translucens* or *T. intricata*. This presumably reflects a greater dependence of the chloride pump in *C. corallina* on respiratory energy. The normal rates of photosynthetic $^{14}CO_2$ fixation were likewise very close to those previously reported (Smith 1968*b*), although the culture of *C. corallina* was again different.

The results obtained with the isolated chloroplasts are of considerable interest, as similar experiments have previously been carried out only with pea chloroplasts (West and Wiskich 1968). The preliminary experiments (Fig. 4) established that the chloroplasts were able to phosphorylate added ADP. The three ADP : O ratios given above (0·98, 1·0, and 1·3; Figs. 4 and 5) may be compared with values of between 1·04 and 1·36 obtained with pea chloroplasts. West and Wiskich (1968) discussed the possibility that there is more than one phosphorylation site in the

photosynthetic electron chain, as is suggested by values greater than 1. The photosynthetic control ratios measured with the *C. corallina* chloroplasts (1·95, 1·9, and 1·98; Figs. 4 and 5) were lower than those reported by West and Wiskich (2·6–5·7). However, a number of experiments (not described here) have suggested that the comparatively low values obtained with *C. corallina* are caused by a marked impermeability of the chloroplast membrane to added ADP. These results will be described in a subsequent paper; the point to be stressed at present is that the chloroplasts were in a state in which they responded to each of the three main types of photosynthetic inhibitors: those which directly inhibit non-cyclic electron flow, those which uncouple photophosphorylation from electron flow, and those which inhibit both photophosphorylation and the associated coupled electron flow.

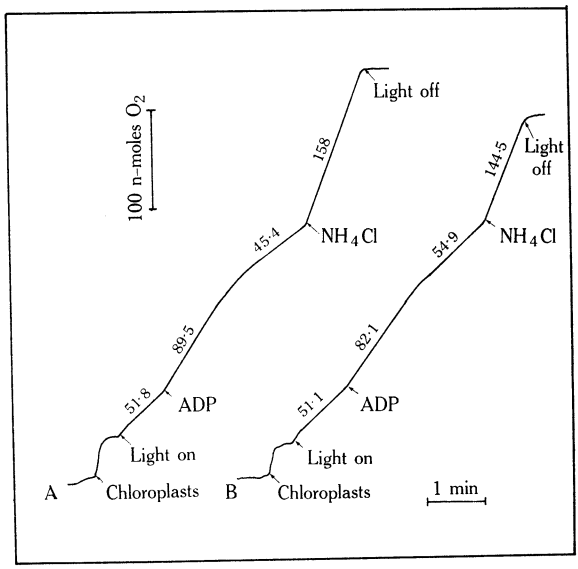


Fig. 5.—Polarographic tracing of oxygen evolution by *C. corallina* chloroplasts with ferricyanide as electron acceptor. One batch of cells was incubated in the basic solution plus 2 μM CCCP for 40 min prior to isolation (curve *B*) while another batch (curve *A*) served as control cells. The technique for isolation was exactly the same in each case. The medium (2·52 ml) for both curves contained 230 mM sucrose, 9·2 mM Tris–phosphate buffer (pH 7·2), 0·92 mM EDTA, 4·6 mM $MgCl_2$, 0·8 mM ferricyanide, and chloroplasts at a chlorophyll concentration of 0·08 (curve *A*) and 0·1 mg ml^{-1} (curve *B*). Additions (indicated by arrows) were 0·076 mM ADP and 4 mM NH_4Cl. Numbers along the trace are n-moles O_2 min^{-1}. The photosynthetic control ratio was 1·98 and 1·5 for curves *A* and *B* respectively, and the corresponding ADP : O ratios were 0·98 and 0·71.

(*b*) *Effects of Inhibitors*

The effects of the various inhibitors on chloride uptake are mostly in good agreement with those reported in previous work with Characean cells. However, it was found that phlorizin was a less effective inhibitor than was reported by Coster and Hope (1968), who found that 0·03 mM phlorizin inhibited the chloride influx by about 50%. The reason for the different results is not clear. In the present study 0·5 mM phlorizin effectively reduced coupled photosynthetic electron flow *in vitro*

(Fig. 4, curve *A*) but 1 mM phlorizin did not reduce photosynthesis *in vivo*, and it seems unlikely that the inhibition of chloride uptake was caused by a reduction in the available energy *in vivo*, as was suggested by Coster and Hope (1968). These results themselves certainly do not contradict MacRobbie's hypothesis (1965, 1966). In fact, the present work provides no evidence that phlorizin penetrates the plasmalemma of *C. corallina* cells, and it is possible that phlorizin inhibits the chloride pump mechanism in the membrane.

Essentially similar conclusions may be drawn from the experiments with Dio-9. A large inhibition of chloride uptake by similarly low concentrations of Dio-9 has been found in a number of experiments with *C. corallina* (Hope, Walker, and Smith, unpublished results), and the present work shows that the inhibition is not caused by a reduction in photosynthesis. Only the highest concentration of Dio-9 (20 $\mu g\,ml^{-1}$) caused an inhibition of photosynthesis *in vivo*, and it is interesting that 4 $\mu g\,ml^{-1}$ Dio-9 had little effect on photosynthetic electron flow *in vitro*. The concentrations of Dio-9 necessary to reduce electron flow *in vitro* were higher than those reported in the literature (McCarty, Guillory, and Racker 1965).

The stimulation of chloride uptake by 0·1 mM imidazole agrees with previous findings (MacRobbie 1966; Coster and Hope 1968) and as was also previously shown (Smith 1967*a*) this was not accompanied by any change in photosynthesis. It is significant that such a low concentration of imidazole would cause very little uncoupling of photophosphorylation *in vitro* (Table 3), and the results confirm that the stimulation of chloride uptake can not be correlated with any major increase in photosynthetic electron flow either *in vivo* or *in vitro*. The effects of imidazole *in vitro* were very similar to those reported by Hind and Whittingham (1963).

The importance of the external pH value in determining the chloride influx (i.e. in the presence of 1 mM imidazole) has not been previously reported. As it is unlikely that the external pH can affect the action of imidazole on the chloroplast, this finding adds weight to the view that, *in vivo*, imidazole affects the chloride pump mechanism at the plasmalemma.

Raven (1967) showed that the chloride influx in *H. africanum* is affected very little by 10 μM CCCP, whereas $^{14}CO_2$ fixation is inhibited more than 95% by 5 μM CCCP. In view of the doubts as to the mode of action of imidazole, Raven's work (1967) provides the best supporting evidence for MacRobbie's hypothesis (1965, 1966) that chloride uptake is linked to an electron transfer reaction which would not be inhibited by CCCP, provided that CCCP does uncouple *in vivo*, and that a suitable natural electron acceptor is present. The progressive inhibition of chloride uptake in *C. corallina* by increasing concentrations of CCCP (Fig. 3) agrees with the results of Coster and Hope (1968), whilst similar results have been obtained with *N. translucens* (Smith 1967*b*) and *T. intricata* (Smith 1968*a*). Nevertheless, in these two species it was found that chloride uptake was less sensitive to CCCP than was $^{14}CO_2$ fixation, and MacRobbie's hypothesis was still tenable. In the present experiments with *C. corallina* there was no differential effect of CCCP on the two processes, and the simplest explanation is that CCCP progressively inhibits phosphorylation, which prevents both $^{14}CO_2$ fixation and chloride uptake. In the presence of 2 μM CCCP (which partly inhibits both $^{14}CO_2$ fixation and chloride uptake) the isolated chloroplasts were partly uncoupled, but oxygen evolution still responded to the addition

of ADP. This was also found when cells were pretreated for 40 min in 2 μM CCCP, and the chloroplasts were isolated and tested (Fig. 5). This latter experiment was designed to show the state of the chloroplasts during the time period used for the measurements of chloride uptake and photosynthesis *in vivo*. There is thus good agreement between the different experiments. However, the possibility that during the latter experiment the chloroplasts become "recoupled" during isolation should be borne in mind. Finally, it should be pointed out that CCCP is much less effective as an uncoupler of photosynthetic electron flow than ammonium chloride (Fig. 5).

It must be concluded that, of the four inhibitors used in this study, CCCP is the only one with which it is possible to correlate the results *in vitro* with those obtained *in vivo*. Even this conclusion may be rash, as it has been shown that CCCP inhibits passive (down-hill) cation uptake into *N. translucens* (Smith 1967*b*) and *T. intricata* (Smith 1968*a*), whilst effects on electrical properties of *N. translucens* have been mentioned by MacRobbie (1966). It would obviously be valuable to make a detailed study of the effects on Characean cells of reputable uncouplers other than CCCP.

These conclusions may seem depressing, in view of the emphasis placed on inhibitors by many workers (including one of the present authors). Perhaps it would be more realistic to consider that *direct* effects of photosynthetic or respiratory inhibitors on ion transport indicate basic similarities between energy-transferring reactions in the chloroplast or mitochondrial membranes and those involved in the transfer of ions across the plasmalemma. It may be objected that these two types of reactions must be very different, but this is not necessarily so, since Mitchell (1966) has developed a hypothesis that charge separation reactions provide the energy for phosphorylation, whilst Robertson (1960, 1967) has repeatedly stressed the importance of similar reactions in ion transport.

At the very least, the present work shows the dangers involved in the uncritical use of metabolic inhibitors *in vivo*.

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