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Inhibitor Effects on Photosynthesis, Respiration and Active Ion Transport in *Hydrodictyon africanum*

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Summary. The effects of various inhibitors on photosynthesis, respiration, and active influx of K and Cl in light and dark in *Hydrodictyon africanum* is reported. The inhibitors used were arsenate (uncouples electron-transport phosphorylations), dicyclohexylcarbodiimide (energy-transfer inhibitor in electron-transport phosphorylation), quinacrine (uncouples photophosphorylation and inhibits oxidative phosphorylation), and ethionine (traps adenylates as S-adenosyl ethionine). The action of these inhibitors, and of those previously used on *Hydrodictyon africanum*, suggests that K influx requires ATP, while Cl influx requires some earlier manifestation of the ATP synthesizing process. Possible reasons for the greater sensitivity of K influx than of CO_2 fixation to treatments which nterfere with photophosphorylation are discussed.

Previous attempts to determine the energy source for the active influxes of K and Cl in *Hydrodictyon africanum* have suggested that the active K influx (linked to Na efflux) is powered by ATP, whereas the active Cl influx (linked to K and Na influx) is powered by some other manifestation of electron transport (references in Raven, 1971*a*). These conclusions have been based largely on experiments with inhibitors of various phases of ATP synthesis in chloroplasts and mitochondria.

These inhibitors include (1) compounds which inhibit electron transport, (2) compounds which uncouple electron transport and phosphorylation, (3) compounds which inhibit the terminal steps of phosphorylation (energy-transfer inhibitors), and (4) compounds which inhibit the use of ATP in bringing about active ion transport at the plasmalemma. Many of the compounds in class (3) also inhibit as those in class (4) (Raven, MacRobbie & Newmann, 1969). Compounds in groups (3) and (4) should inhibit processes which need ATP, but not those which only require the participation of the "high-energy intermediate" or electron transport. Insensitivity to

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compounds in groups (2), (3), and (4) suggests coupling to electron transport, with no requirement for ATP or the high-energy intermediate. In general, Cl influx is inhibited only by group (1), whereas K influx is inhibited by all four groups.

All of the uncouplers so far used in experiments on *H. africanum* are known to increase the rate of transport of H^+ across biological membranes (e.g., CCCP[1], DNP), or to be related in their action to such uncouplers (e.g., desaspidin) (Hind, 1966). This ability to promote H^+ transport is related to their uncoupling activity (Mitchell, 1966; Ting, Wilson & Chance, 1970). It has been suggested that uncouplers can interfere directly (i.e., not through ATP synthesis) with some solute transport processes which require the presence of pH gradient across the membrane (e.g., Smith, 1970 and MacRobbie, 1970). The direct investigation of uncoupler effects on membrane-transport processes is best carried out in cells which lack electron-transport phosphorylations, since even when fermentation is the source of ATP in other cells the presence of uncouplers can lead to ATP consumption by mitochondria (Galeotti, Kovac & Hess, 1968). Experimental material in which uncoupler effects can be tested in the absence of complications from the presence of an oxidative phosphorylation system includes certain bacteria (e.g., *Streptococcus faecilis*), anaerobically grown yeasts, and mammalian erythrocytes.

In *S. faecilis* (Harold & Baarda, 1968; Harold, Pavolosova & Baarda, 1970), proton-translocating uncouplers inhibit ATP use in ion transport at concentrations which abolish the pH gradient across the membrane, but which do not alter the rate of glycolysis, the ATP level in the cell or the use of ATP in macromolecular synthesis. Here a specific effect of the uncouplers on membrane transport appears likely.

In anaerobically grown yeast, it has been shown that proton-translocating uncouplers inhibit energy-requiring processes without altering the ATP level in the cell; the concentrations required are larger than those needed to uncouple oxidative phosphorylation in air-grown yeast. These inhibitions are not specific to active transport processes (Galeotti *et al.*, 1968).

In mammalian erythrocytes, DNP at relatively high concentrations (2.5 mM) did not inhibit active K and Na transport (Maizels, 1951; Hoffman, 1962).

This suggests that a transmembrane pH gradient is not essential for the ouabain-sensitive K-Na pump such as often occurs in *Hydrodictyon.*

1 Abbreviations used: CCCP = carbonyl cyanide *m*-chlorophenyl hydrazone; DCCD = dicyclohexylcarbodi-imide; DCMU = 3′(3,4 dichlorophenyl), 1′,1′-dimethyl urea; DNP = 2,4-dinitrophenol; HOQNO = 4(*n*-heptyl)hydroxy-quinoline-N-oxide.

Nonetheless, it was thought advisable to carry out further experiments on the energy sources for the K-Na and Cl-cation pumps in *Hydrodictyon.*

The experiments reported in this paper include: (1) *The effect of four inhibitors on photosynthesis and ion transport.* These are arsenate and quinacrine, which uncouple by mechanisms different from those uncouplers previously used; ethionine, which acts as a trap for adneylate compounds; and DCCD, an energy-transfer inhibitor in mitochondria and chloroplasts. (2) *The effects of these inhibitors, and those used in previous investigations on Hydrodictyon, on respiration.* An effect on respiration suggests that the inhibitor must be entering the cell; the nature of the effect can be used to check if the assumed mode of action is correct.

Materials and Methods

Culture of *H. africanum*, and measurements of ^{42}K and ^{36}Cl influxes, and of photosynthetic $^{14}CO_2$ fixation in the presence and absence of inhibitors, were as described previously (Raven, 1967*a*, *b*, 1969*b*). Respiration was measured in the dark as O_2 uptake, using a Gilson differential respirometer with 1 N KOH in the center well. In experiments on the effect of cyanide on O_2 uptake, the procedure of Robbie (1946) was followed. Respiration measurements involved comparison of O_2 uptake rates in cells pretreated for 2 hr in inhibitor with those pretreated in the absence of the inhibitor.

Pressure changes were recorded at 90 and 180 min after the end of pretreatment in each of three replicates of each treatment, and the O_2 uptake was computed as the mean of these six measurements $\pm$ the standard error of the mean. Even with large numbers of cells in each flask (up to 10 cm^2 surface area), measurements at shorter intervals were not practicable.

Sodium dihydrogen arsenate, dicyclohexylcarbodi-imide and quinacrine (atebrin) were purchased from B.D.H. Ltd. (Poole, England). L-Ethionine was purchased from Kock-Light Laboratories, Ltd. (Colnbrook, Bucks, England). ^{74}As-sodium dihydrogen arsenate (containing ^{77}As at 10% of the original specific activity of ^{74}As) was purchased from the Radiochemical Centre (Amersham, England).

In all cases the K influx was checked for its sensitivity to ouabain. Only cells with an inhibition of the K influx by ouabain of greater than 50% were used in the experiments reported here. Inhibitions of K influx reported in this paper largely represent inhibitions of the ouabain-sensitive K influx.

Results

Arsenate

Arsenate competes with phosphate in biological phosphorylation reactions; this competition appears to be more effective in inhibiting substrate-level phosphorylations than in uncoupling oxidative or photosynthetic phosphorylations (Good, Izawa & Hind, 1966; Heldt, Jacobs & Klingenberg, 1964; Slater, 1966). At arsenate-phosphate ratios of 3 to 4, 50%

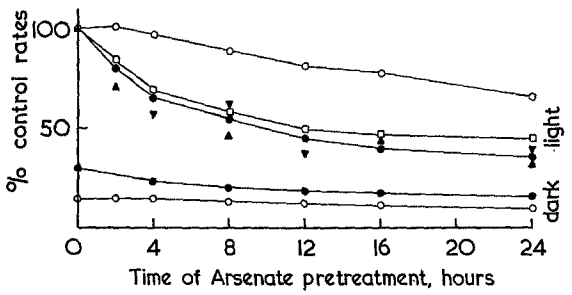

Fig. 1. Time course of arsenate effects on photosynthetic $^{14}CO_2$ fixation, and on tracer K and Cl influx in light and dark. Cells pretreated in artificial pond water +1 mM arsenate for the time indicated in light or dark, then 2 hr in active solution in the absence of arsenate. Control rates (=100%). $^{14}CO_2$ fixation in light (□)=32.6±3.9 pmole cm^2 sec^{-1}. ^{42}K influx in light, air (•)=1.19±0.13 pmole cm^{-2} sec^{-1}. ^{36}Cl influx in light (○)=1.94±0.15 pmole cm^{-2} sec^{-1}. ^{42}K influx in light N_2+0.1 μM DCMU (▲)=1.01± 0.12 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, N_2+1 mM CO_2 (▼)=1.14±0.13 pmole cm^{-2} sec^{-1}

inhibition of electron-transport phosphorylation occurs. Arsenate does not inhibit the K, Na-ATPase in animal cells (Jarnefelt, 1962; Hoffman, 1962).

When compared with proton-conducting uncouplers, arsenate has a smaller relative effect on the high-energy intermediate of photophosphorylation than on ATP synthesis. This is possibly because the rate at which it can dissipate the intermediate is limited by the enzymic capacity of the terminal steps of ATP synthesis (Dilley, 1970). It would thus be expected *in vivo* to inhibit processes which require ATP synthesis more than those which need only the high-energy intermediate, and to inhibit processes which only require electron flow to an even smaller extent. *In vivo*, the effects of arsenate are consistent with action as an uncoupler of oxidative and photosynthetic phosphorylations in plant cells; thus it inhibits photosynthesis, stimulates respiration, and lowers the ATP level in both light and dark in green cells (Syrett, 1958; Kessler & Bucher, 1960; Kandler & Liesenkotter, 1961).

Fig. 1 shows the effects of arsenate on photosynthesis and on K and Cl influx in the light and dark in *Hydrodictyon.*

The slow development of the arsenate inhibition is related to the rate of entry into the cells (*see* below). It will be seen that the most sensitive process in both light and dark is active K influx; after 24 hr the ouabain-sensitive influx of K in both light and dark is inhibited by about 80%. Photosynthesis is rather less sensitive, with 60% inhibition after 24 hr. Cl influx is least sensitive, with more than 60% of the control flux remaining after 24 hr in both light and dark. These results suggest that Cl influx is the process which

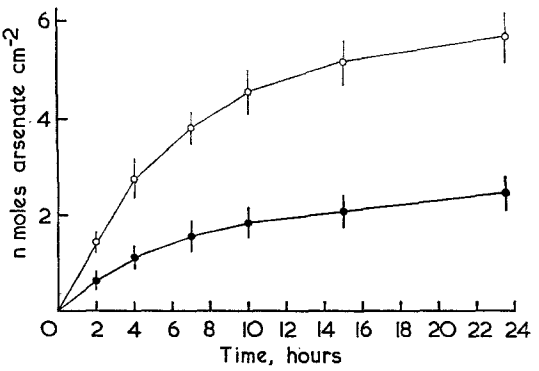

Fig. 2. Time course of arsenate uptake into the whole cell (○) and the cytoplasm alone (●) in the light in *Hydrodictyon*

is least sensitive to inhibition of the terminal stages of ATP synthesis, and that K influx is rather more sensitive than is photosynthesis.

Fig. 2 shows the time course of arsenate uptake into *Hydrodictyon* cells in the light. The time course of uptake into the cytoplasm is similar to that of the development of arsenate inhibition of photosynthesis and active K influx (Fig. 1). Assuming a cytoplasmic thickness of 10 μm (Raven, 1967*a*), the cytoplasmic arsenate concentration after 12 to 24 hr of arsenate treatment is about 2 mM. The inorganic phosphate concentration in various non-vacuolate algal cells is 1 to 5 mM (Kanai & Simonis, 1968; Bassham & Krause, 1969); if phosphate concentration in the cytoplasm of *Hydrodictyon* is similar, then the 2 mM arsenate in the cytoplasm could cause the observed inhibitions *via* inhibition of electron-transport phosphorylation with the affinity noted earlier in this section.

Reversal of arsenate inhibition is slow after arsenate is removed from the external solution, and appears to be due to transport of arsenate from the cytoplasm into the vacuole rather than loss to the external solution (Table 1). The non-linearity with time of arsenate uptake into *Hydrodictyon* is probably a result of inhibition by the accumulation of arsenate in the cell; both phosphate and arsenate are actively transported into *Hydrodictyon* by an uncoupler-sensitive mechanism (Raven, *unpublished experiments*).

Quinacrine

Quinacrine inhibits oxidative phosphorylation and uncouples photosynthetic phosphorylation (Good *et al.*, 1966). The action of quinacrine on chloroplasts is different from that of proton-translocating uncouplers such as CCCP, DNP, and desaspidin; it does not abolish all manifestations of

Table 1. *Effects of arsenate treatment on photosynthesis and arsenate content of Hydrodictyon*

Effect	Time after addition of arsenate (hr)			
	0	12	24	96
Photosynthetic $^{14}CO_2$ fixation (pmole cm^{-2} sec^{-1})	26.3 ± 2.8	12.6 ± 1.5	16.6 ± 1.5	21.5 ± 2.1
Arsenate in cytoplasm (nmole cm^{-2})	–	2.1 ± 0.3	1.5 ± 0.2	0.7 ± 0.1
Arsenate in vacuole (nmole cm^{-2})	–	3.8 ± 0.3	4.2 ± 0.4	5.0 ± 0.4
Arsenate in external solution (nmole cm^{-2})	–	–	0.05 ± 0.001	0.08 ± 0.002

^{74}As arsenate was supplied at 1 mM for 12 hr, and then removed. Subsequent treatment was in arsenate-free artificial pond water.

the high-energy state at concentrations which completely uncouple. (Good *et al.*, 1966; Avron & Neumann, 1968).

Thus it is possible that a quinacrine-inhibited system could still energize reactions which require the high-energy state of photophosphorylation rather than ATP. Quinacrine inhibits ATP synthesis in isolated chloroplasts more than it inhibits CO_2 fixation (Vose & Spencer, 1967, 1968). However, the kinases of the CO_2 fixation cycle require a high-energy phosphate, if not ATP then some phosphorylated intermediate of photophosphorylation. However, phosphate is not required for quinacrine uncoupling (references in Good *et al.*, 1966); this suggests that quinacrine uncoupling occurs at a stage prior to the formation of a high-energy phosphorylated intermediate. The work of Wu, Myers and Forrest (1970) supports this view. Further, Simonis (1967) has shown that quinacrine inhibition of photosynthetic $^{14}CO_2$ fixation in *Ankistrodesmus* is closely paralleled by inhibition of phosphate incorporation in photophosphorylation. Thus the evidence for a quinacrine-insensitive formation of the assimilatory power needed for CO_2 fixation is equivocal.

The effects of quinacrine on photosynthesis, and on tracer K and Cl influx in light and dark in *Hydrodictyon* are shown in Fig. 3.

The process most sensitive to quinacrine is K influx in the light: the dark K influx and photosynthesis are less sensitive; Cl influx in light or dark is even less inhibited than is photosynthesis. Thus, in the light, Cl influx is less inhibited by this uncoupler than is K influx or photosynthesis; this supports the relative independence of Cl influx from ATP synthesis.

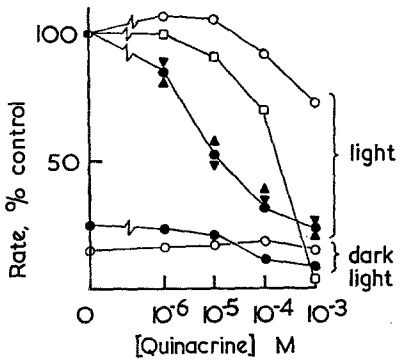

Fig. 3. Effects of various concentrations of quinacrine on photosynthetic $^{14}CO_2$ fixation, and on tracer K and Cl influx in light and dark. Control rates (=100%): $^{14}CO_2$ fixation (○), 28.2±2.9 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, air, (●), 1.03±0.12 pmole cm^{-2} sec^{-1}. ^{36}Cl influx in light (○), 1.81±0.21 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, N_2+0.1 μM DCMU (▲)=0.89±0.09 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, N_2+1 mM CO_2 (▼)= 1.09±0.12 pmole cm^{-2} sec^{-1}. Quinacrine effect on K influx reversible to 60% control; effect on photosynthesis reversible to 70% control rate

A possible explanation of the greater sensitivity of K influx than of CO_2 fixation to quinacrine could be that there is a direct inhibitory effect of quinacrine on the K pump (Jarnefelt, 1962; Jeschke, 1970). The onset of quinacrine inhibition of both K influx and CO_2 fixation in *Hydrodictyon* is too rapid to permit precise measurement, but there is no evidence that K influx is inhibited sooner than is CO_2 fixation (as would be expected if it acted at the cell membrane).

DCCD

DCCD acts as an energy-transfer inhibitor in isolated mitochondria (Beechey, Robertson, Holloway & Knight, 1967) and in isolated chloroplasts (references in Dilley, 1970). Similarly to Dio-9, DCCD acts as an uncoupler in chloroplasts at high concentrations (Trebst & Pistorius, 1969). It also inhibits the K, Na-activated ATPase of animal cell membranes (Palladin, Lishko & Smetna, 1969; Schoner & Schmidt, 1969; *see* the introduction).

DCCD also inhibits a membrane ATPase in *S. faecilis* which seems to have a role in maintaining a pH gradient across the cell membrane; this pH gradient appears to be involved in active K, phosphate, and amino-acid transport into the cell (Harold, Baarda, Baron & Abrams, 1969; Harold *et al.*, 1970). Kovac, Galeotti and Hess (1968) showed that DCCD inhibited respiration and, to a lesser extent, fermentation in *Saccharomyces*; the respiratory inhibition was reversed by CCCP, as would be expected of an energy-transfer inhibitor.

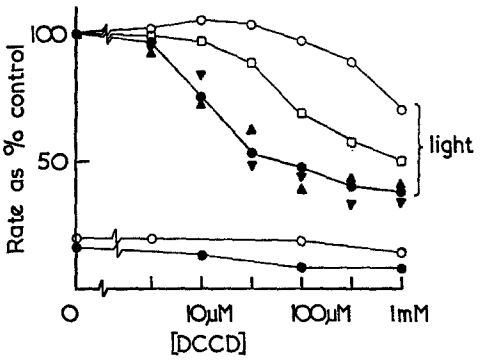

Fig. 4. Effects of various concentrations of DCCD on photosynthetic $^{14}CO_2$ fixation, and on tracer K and Cl influx in light and dark. Control rates (=100%): $^{14}CO_2$ fixation (□) 26.6±2.9 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, air (•)=0.93±0.11 pmole cm^{-2} sec^{-1}. ^{36}Cl influx in light (○)=2.12±0.25 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, N_2+0.1 μM DCMU (▲)=0.87±0.08 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, N_2+1 mM CO_2 (▼)= 0.99±0.09 pmole cm^{-2} sec^{-1}. DCCD (1 mM) effect on K influx reversible to 65% of control values; effect on CO_2 reversible to 75%

The effects of DCCD on active K and Cl influxes in light and dark, and on photosynthesis in *Hydrodictyon* are shown in Fig. 4. It will be seen that K influx is the process most sensitive to DCCD in either light or dark; photosynthesis is considerably less sensitive, and Cl influx is even less sensitive. The greater inhibition of K influx than of CO_2 fixation by energy-transfer inhibitors is found in *Hydrodictyon* for both phlorizin (Raven, 1968) and Dio-9 (Raven *et al.*, 1969). These results support the independence of Cl influx from at least the terminal steps of ATP synthesis.

Since the inhibition of CO_2 fixation and of K influx both develop with a half-time of about 30 min, it is likely that both of these effects are mediated by DCCD which has entered the cell; thus any effect of DCCD on the membrane ATPase would have to be exerted from the inside rather than the outside of the membrane. It is unlikely that DCCD takes no longer to reach the inside of the cell membrane than it does to reach the sites of electron-transport phosphorylation. Thus it is likely that the inhibitory effect of DCCD on K influx is exercised at the level of ATP synthesis rather than ATP breakdown. The greater effect of DCCD on K influx than on photosynthesis is considered further in the discussion.

Ethionine

Ethionine reduces the ATP level in cells by the formation of S-adenosyl ethionine. This compound apparently undergoes transalkylation reactions less readily than its methionine analogue (references in Vogt & Farber,

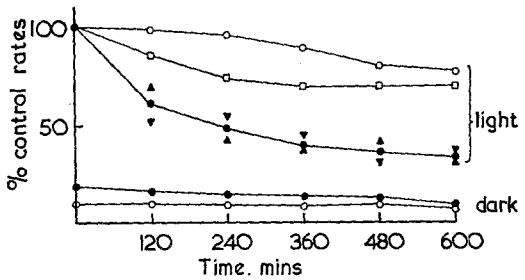

Fig. 5. Time course of ethionine effects on photosynthetic $^{14}CO_2$ fixation, and on tracer K and Cl influx in light and dark. Cells pretreated in artificial pond water +5 mM ethionine for the time indicated in light or dark, then 2 hr in active solution in the absence of ethionine. Control rates (=100%): $^{14}CO_2$ fixation in light (○)=31.7±3.6 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, air (●)=1.17±0.09 pmole cm^{-2} sec^{-1}. ^{36}Cl influx in light (○)=1.95±0.21 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, N_2+0.1 μM DCMU (▲)= 1.07±0.10 pmole cm^{-2} sec^{-1}. ^{42}K influx in light, N_2+1 mM CO_2 (▼)=1.21±0.13 pmole cm^{-2} sec^{-1}. Ethionine effects are essentially irreversible unless adenylates are added. (Table 2)

1970; Atkinson & Polya, 1968). Adenine and its derivatives overcome this inhibition, probably by supplementing the slow synthesis of adenine to replace that sequestered as S-adenosyl ethionine (references in Norris & Margum, 1970). Inhibition of protein synthesis, respiration, and photosynthesis can all be adequately explained in terms of reduced ATP level and turnover (references in Vogt & Farber, 1970; Atkinson & Polya, 1968).

The effects of ethionine on photosynthetic $^{14}CO_2$ fixation, and on tracer K and Cl influxes in the light and in the dark, are shown in Fig. 5. Inhibition of $^{14}CO_2$ fixation and of ^{42}K influx in the light develops with a half-time of 2 to 3 hr. The final level of inhibition of active K influx which is reached is some 60 to 70%, whereas the inhibition of CO_2 fixation reaches about 25%. Inhibition of active K influx in the dark proceeds more slowly than does inhibition in the light. This may be because of a slower uptake of ethionine in the dark than in the light, or a slower rate of incorporation into S-adenosyl ethionine. Cells pretreated with ethionine in the dark, and then tested for inhibition of photosynthesis or K influx in the light, show less inhibition than those in the light throughout. The effect of ethionine on Cl influx in light and dark is smaller in extent and rate of development than are the effects on K influx and on CO_2 fixation. This supports the view that the whole sequence of reactions of ATP synthesis are required for K influx and possibly for CO_2 fixation, but not for Cl influx. The independence of Cl influx from the complete reaction sequence of ATP synthesis is in agreement with the conclusions of Atkinson and Polya (1968) and Polya and Atkinson (1969) on the active influx of Cl into carrot xylem tissue;

Table 2. *Reversal of ethionine inhibition of photosynthesis by AMP or ADP in Hydrodictyon africanum*

Conditions	Fixation rate in the absence of ethionine (pmole cm^{-2} sec^{-1})	Fixation rate in the presence of 5 mM ethionine (pmole cm^{-2} sec^{-1})
Control, no adenylates	25.3 ± 1.5	18.3 ± 1.1
AMP (1 mM)	29.7 ± 2.2	27.7 ± 2.8
ADP (1 mM)	25.7 ± 0.9	25.5 ± 1.4

12-hr pretreatment in light in artificial pond water with or without the specified additions of ethionine, ADP or AMP; then 2 hr $^{14}CO_2$ fixation in the light in the absence of ethionine, ADP or AMP.

they also used ethionine. The experimental periods used by them were too long for the observed effects to be purely on Cl transport at the plasmalemma (Cram, 1969*b*). Other evidence suggests that Cl influx at the tonoplast in carrot requires a high-energy intermediate of oxidative phosphorylation, whereas that at the plasmalemma is more closely related to mitochondrial electron transport (Cram, 1969*a*).

The results shown in Table 2 support the view that ethionine inhibition of photosynthesis involves trapping of adenylates at a rate faster than the cell can synthesize them. It will be seen that ethionine inhibition is substantially relieved if either AMP or ADP is supplied at the same time.

Respiration

Table 3 shows the results of experiments on the effect of various inhibitors of ATP synthesis and ATP use in ion uptake on the rate of O_2 uptake in *Hydrodictyon*. The concentrations of inhibitors used were those which gave maximal inhibition of tracer K influx in the dark (Raven, 1967*a*, *b*, 1968, 1969; Raven *et al.*, 1969; results presented in this paper).

It will be seen that all of the uncouplers of oxidative phosphorylation tested gave a significant stimulation of O_2 uptake at the concentration which inhibits K influx (CCCP, DNP, desaspidin, arsenate). This is consistent with uncoupling *in vivo*. The electron-transfer inhibitors inhibited O_2 uptake (cyanide, HOQNO, antimycin, quinacrine); this inhibition was not relieved by uncouplers, which is consistent with the mode of action of these compounds in isolated mitochondria (references in Raven, 1969).

The energy-transfer inhibitors (phlorizin, Dio-9 and DCCD) also inhibit respiration. This inhibition can be substantially relieved by CCCP or by

Table 3. *Effects of compounds which interfere with respiratory reactions on O_2 uptake by Hydrodictyon*

Inhibitor	O_2 uptake (pmoles cm^{-2} sec^{-1})				
	Control	+Inhibitor	+Inhibitor +CCCP 3 μM	+Inhibitor +DNP 100 μM	+Inhibitor +arsenate 1 mM
None	2.6±0.5 (100)[a]	2.6±0.5 (100)	3.9±0.7 (150)	4.3±0.7 (165)	3.5±0.5 (134)
Phlorizin (1 mM)	2.1±0.3 (100)	1.2±0.3 (57)	2.3±0.4 (105)	2.6±0.5 (124)	1.0±0.3 (48)
Dio-9 (10 μg ml^{-1})	3.2±0.5 (100)	1.7±0.4 (53)	3.7±0.6 (116)	3.9±0.4 (122)	1.5±0.4 (47)
DCCD (1 mM)	2.4±0.3 (100)	1.0±0.3 (42)	2.7±0.4 (113)	2.9±0.5 (121)	1.2±0.3 (50)
Ethionine (5 mM)	3.1±0.4 (100)	2.2±0.4 (71)	3.7±0.4 (120)	3.9±0.6 (126)	2.7±0.2 (87)
NaCN (1 mM)	3.7±0.4 (100)	1.7±0.4 (46)	1.9±0.3 (51)	–	–
HOQNO (3 μM)	2.9±0.3 (100)	1.3±0.2 (45)	–	1.1±0.2 (38)	–
Antimycin (100 μM)	3.3±0.4 (100)	1.8±0.3 (55)	1.6±0.2 (49)	–	–
Quinacrine (100 μM)	2.7±0.4 (100)	1.6±0.3 (59)	–	1.8±0.3 (67)	–
Desaspidin (10 μM)	3.3±0.4 (100)	4.7±0.5 (143)	–	–	–
DCMU (10 μM)	4.1±0.5 (100)	4.4±0.6 (107)	–	–	–
Ouabain (1 mM)	3.4±0.4 (100)	3.2±0.3 (94)	–	–	–
Absence of salts	3.5±0.4 (100)	3.2±0.3 (92)	–	–	–

[a] Figures in brackets = treated as % controls.

DNP, but not by arsenate. This indicates that these compounds are acting at a point between the site of action of CCCP and DNP and that of arsenate. If their predominant effect were to inhibit ATP-consuming reactions and hence inhibit electron flow *via* an effect on the ATP/ADP+P_i ratio, then the inhibition should be relieved by arsenate, which allows re-cycling of ADP without ATP synthesis. So the energy-transfer inhibitors must be acting primarily on the terminal steps of mitochondrial ATP synthesis. That the effect of the energy-transfer inhibitors cannot be exerted solely through

respiratory control, following inhibition of K-Na transport at the level of the membrane ATPase is supported by the finding that inhibition of K transport by other means (presence of ouabain, absence of salts from the medium) does not significantly inhibit respiration. This is to be expected, since the work done on each K ion (including that on the coupled Na efflux) is only some 1.5 kcal $mole^{-1}$ (Raven, 1967*a*). This means that some four K ions could be moved per ATP hydrolyzed at 60% efficiency. Even if the K/ATP ratio were only 1, the O_2 uptake associated with the active dark influx of less than 0.2 pmole cm^{-2} sec^{-1} would, assuming a P/O ratio of 3, be only about 0.03 pmole O_2 cm^{-2} sec^{-1}. This is negligible in comparison with the normal respiration rate of *Hydrodictyon* of 2 to 4 pmole O_2 cm^{-2} sec^{-1}.

Removal of external ions would not necessarily inhibit a pump which extrudes H^+, an ion which is present in the medium (1 μM), and which can be produced metabolically inside the cell.

Assuming a H^+/ATP ratio of 1, then the 50% inhibition of respiration noted with the energy-transfer inhibitors would correspond (P/O = 3) to an active H^+ extrusion of some 12 pmole cm^{-2} sec^{-1}. If the H^+/ATP ratio were higher than 1, then even more H^+ would be lost. Fluxes of H^+ of this order have been reported by Kitsato (1969) for *Nitella clavata*, and inhibition of H^+ efflux by DCCD has been reported for *S. faecilis* (Harold, Pavolosova & Baarda, 1970). However, Walker and Hope (1969) and Spanswick (1970) have placed an upper limit on H^+ influx in *Chara corallina* and *Nitella translucens* which is much lower than this. The situation in *Hydrodictyon* seems to be more similar to that in *C. corallina* and *N. translucens* than that in *N. clavata*.

Thus it is unlikely that the observed inhibition of respiration by the energy-transfer inhibitors can be accounted for in terms of inhibition of ion transport processes at the plasmalemma, and some direct action on the terminal steps of oxidative phosphorylation is required.

Ethionine inhibits respiration (Atkinson & Polya, 1968). The inhibition is partially reversed by all three of the uncouplers tested, which is consistent with an action in trapping ATP rather than directly inhibiting its formation. CCCP and DNP are more effective in reversing the ethionine inhibition than is arsenate, possibly because the respiratory stimulation caused by CCCP and DNP are independent of ADP, whereas that due to arsenate is enhanced by ADP (Ernster, Lee & Jarda, 1967); ethionine probably lowers the level of ADP.

Thus the results presented here are consistent with the view that the inhibition of K influx by the compounds tested here is associated with their

entry into the cell and action on mitochondria (or on cytoplasmic ATP in the case of ethionine). This does not, of course, prove that this is their sole site of action.

These experiments in which respiration was measured manometrically involved more stirring of the medium than is normal in experiments on *Hydrodictyon* other than those in which the effects of various gas mixtures had been used. In these cases (Raven, 1969, 1970) it was found in control experiments on the K influx and CO_2 fixation that stirring of the medium *per se* had no effect on control rates or the effects of inhibitors or other experimental treatments. Control experiments run in parallel with those on respiration showed that, in the case of Cl influx, stirring of the medium did alter the influence of some inhibitors (Raven, 1971*b*). Thus, the effect of 3 μM CCCP on Cl influx in the light was an inhibition of less than 15% in unstirred controls, whereas in stirred treatments the inhibition is 50 to 85%. In both stirred and unstirred experiments, the CO_2 fixation in the presence of 3 μM CCCP was inhibited more than 95%. A similar increase in inhibition of Cl influx due to stirring was found in experiments with 100 μM DNP, but not with 1 mM arsenate, 1 mM phlorizin, 0.3 mM DCCD, 5 mM ethionine, or 0.3 μM or 1 μM DCMU. It would appear that the increased sensitivity of Cl influx due to stirring is confined to proton-translocating uncouplers. The effect is only slowly reversible; cells which have been subjected to shaking or stirring never fully regain their original insensitivity to these inhibitors.

The effects of these uncouplers on Cl influx in *Hydrodictyon* cells which have been subjected to shaking or stirring thus resemble that found by Smith (1968) for the differential effect of CCCP on Cl influx and CO_2 fixation in *Tolypella intricata*; the complete absence of a differential effect reported for *C. corallina* (Smith & West, 1969; Smith, 1970) is not found even in stirred *Hydrodictyon*.

Discussion and Conclusions

K Influx

The measurements of respiration show that, at the concentrations which inhibit active K influx in the dark, the electron-transport and energy-transfer inhibitors and the uncouplers are acting in the predicted fashion on O_2 uptake in *H. africanum* cells. This means that they are indeed acting on the mitochondria, and can thus potentially inhibit an ATP-requiring process by inhibiting ATP synthesis in oxidative phosphorylation. This does not, of course, prove that they are not also having a direct effect on the plasmalemma. However, the finding that there is a considerable lag before the

inhibitory effects of arsenate, ethionine, DCCD, and phlorizin are manifested suggests that the effect of these compounds on active K influx is exerted from inside rather than outside the cell. By contrast, the inhibition of K influx by ouabain only shows a lag of a few minutes (Raven, *unpublished*); ouabain appears to inhibit by binding to the outside of the cell outer membrane (Baker & Manil, 1968).

Thus the respiration measurements reported in this paper, together with the effects of arsenate, quinacrine, DCCD, ethionine, and the inhibitors used in the papers cited in the introduction are all consistent with a requirement for ATP for the ouabain-sensitive K influx.

CO_2 Fixation

The reason for the greater sensitivity of K influx than of CO_2 fixation to a number of uncouplers and all energy-transfer inhibitors as well as ethionine is not easy to explain if both processes need ATP. The greater inhibition of K influx than of CO_2 fixation by DNP and desaspidin, may be explained by a small contribution of the DNP and desaspidin-sensitive cyclic photophosphorylation to the ATP supply to K influx, but not to CO_2 fixation, under conditions in which open-chain photophosphorylation can occur (Raven, 1969, 1970, 1971). The results shown in Figs. 1, 3, 4 and 5 of this paper indicate that the inhibition of K influx in *Hydrodictyon* by arsenate, quinacrine, DCCD, and ethionine is not influenced by whether open-chain as well as cyclic photophosphorylation can (air, N_2+CO_2) or cannot (N_2 + DCMU) occur. Thus the hypothesis of differential contribution of cyclic photophosphorylation to K influx in comparison with CO_2 fixation cannot account for the differential inhibition of these processes by quinacrine, ethionine, or DCCD, or (Raven, *unpublished results*) by phlorizin or Dio-9.

Other workers have also found that quinacrine and phlorizin do not have a specific effect on cyclic compared with non-cyclic photophosphorylation *in vivo* (Simonis, 1967; Urbach & Gimmler, 1969). There are three main hypotheses which can be used to explain these differential effects (Raven, 1968, 1971*a*; Raven *et al.*, 1969; Urbach & Gimmler, 1969).

Alternative (1) is the preferential use of a limited supply of ATP for CO_2 fixation rather than for other ATP-requiring processes; Raven (1971*a*) gives references and discusses the application of this hypothesis to limitation of ATP supply at low-light intensities. This effect could be explained in terms of the kinetic characteristics of the various ATP-consuming enzymes, or, for processes occuring outside the chloroplasts, in terms of the kinetics of transfer of "high-energy" phosphate out of the chloroplasts. A difficulty

with this hypothesis [shared by alternative (3)] is that it does not readily explain the greater discrimination between CO_2 fixation and K influx found for energy-transfer inhibitors when compared with the proton-translocating uncouplers. This effect is fairly readily explained by hypothesis (2).

Alternative (2), the use of some high-energy intermediate of ATP synthesis, rather than ATP itself, in the kinase steps of CO_2 fixation, has been suggested by Kylin and Tillberg (1967), Vose and Spencer (1967, 1968, 1969), and Urbach and Gimmler (1969). Vose and Spencer found that photosynthesis by isolated chloroplasts was relatively insensitive to phlorizin, but that ATP stimulation of photosynthesis was more sensitive to phlorizin. This is consistent with the use in CO_2 fixation of some high-energy intermediate on the electron transport side of the site sensitive to phlorizin. This intermediate would presumably be phosphorylated; some evidence for the phlorizin-insensitive formation of such an intermediate has recently been presented by Wu, Myers, and Forrest (1970). However, there are a number of results which are not consistent with this scheme. One is the finding by Vose and Spencer (1969) that the ADP stimulation of photosynthesis by chloroplast preparations is at least as phlorizin-sensitive as is the ATP stimulation. Another difficulty is that quinacrine has similar effects to phlorizin on photosynthesis in chloroplasts (Vose & Spencer, 1967, 1968), yet the evidence discussed earlier (quinacrine section of Results) suggests that quinacrine-treated chloroplasts cannot make a phosphorylated intermediate of photophosphorylation.

Alternative (3) proposes that an alternative ATP source, stored polyphosphate, is used when ATP synthesis from photophosphorylation is blocked by energy-transfer inhibitors (Urbach & Gimmler, 1969; Gimmler, Urbach & Simonis, 1969). Despite the short experimental times used by these workers, the amount of polyphosphate in the *Ankistrodesmus* cell (Kanai & Simonis, 1968) would be almost entirely used up in their experiments with phlorizin or Dio-9 if the photosynthetic rate was that measured by Jeschke, Gimmler, and Simonis (1967), with an ATP/CO_2 ratio of 3. Such a large predicted decrease in polyphosphate level suggests a further experimental test for this alternative. Experiments on the interaction of uncouplers and energy-transfer inhibitors in *H. africanum* (Raven, *unpublished experiments*) are not entirely in accord with the predictions of this hypothesis.

Thus none of the three alternative explanations presented here can explain all of the phenomena associated with differential inhibitor effects on K influx and photosynthesis in *H. africanum*.

Cl Influx

The uncouplers and energy-transfer inhibitors used in this work, and in previous work on *Hydrodictyon* (for references, *see* introduction), and also ethionine, inhibit Cl influx less than they inhibit either CO_2 fixation or K influx. This is consistent with the previous suggestion that Cl influx in *Hydrodictyon* is more or less independent of the partial reactions of photophosphorylation and oxidative phosphorylation, and depends more directly on electron transport. Other cases in which Cl influx in illuminated green tissues is less sensitive to uncouplers than is either K influx or photosynthesis are discussed by Raven (1969) and MacRobbie (1970). Uncoupler stimulation in non-green tissue, allied with inhibition by cyanide, has been reported for active Cl transport through isolated cortical "sleeves" of *Zea* roots, (Ginzburg & Ginzburg, 1970).

In *Hydrodictyon* cells which have been subjected to mechanical disturbance, the inhibition of Cl influx in the light by CCCP and DNP is greater than that which is normally found (*see* respiration section of Results); in these cells, therefore, the Cl influx is relatively sensitive to those uncouplers which act near the electron-transport chain and which abolish the proton gradient, but is less sensitive to those uncouplers (quinacrine, arsenate) which do not abolish all manifestations of the high-energy intermediate, or to energy-transfer inhibitors, or to the ATP trap ethionine. Under these conditions the Cl influx has characteristics which suggest the involvement of a non-phosphorylated high-energy intermediate of photophosphorylation as well as of electron transport, but still appears to be independent of ATP synthesis. The data of Polya and Atkinson (1969) and of Atkinson and Polya (1968) on Cl influx in carrot discs also implicates a high-energy intermediate of phosphorylation, but the Cl pump looked at in this work is probably that at the tonoplast rather than at the plasmalemma (Cram, 1969*a*, *b*).

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