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ALGAL NITROGENASE, REDUCTANT POOLS AND PHOTOSYSTEM I ACTIVITY

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SUMMARY

(3′-(3,4-Dichlorophenyl)-1′,1′-dimethylurea) (DCMU) ($3 \cdot 10^{-5}$ M) inhibits nitrogenase activity by *Anabaena cylindrica* to a greater extent in cells previously incubated in air than in those preincubated in the absence of added oxygen, and to a lesser extent under argon than N_2. Low O_2 pretreatment relieves the DCMU sensitivity of acetylene reduction to a greater extent than it relieves the sensitivity of carbon fixation. The results support the view that photosynthesis makes available a pool of reductant which can be used to reduce nitrogenase in the light and in the dark.

Monofluoroacetate ($1 \cdot 10^{-3}$ M), an inhibitor of aconitate hydratase activity in the Krebs Cycle, inhibits acetylene reduction to a greater extent in the dark than in the light and DCMU and monofluoroacetate together may completely inhibit acetylene reduction in the light. The stimulation of nitrogenase activity by light in the presence of DCMU is eliminated by monofluoroacetate. The results suggest that Photosystem I may mediate a flow of electrons from organic substrates to nitrogenase.

INTRODUCTION

The sources of reductant for nitrogenase activity by the blue-green alga *Anabaena cylindrica in vivo* are controversial. The suggestion of a direct photosynthetic reduction of N_2, proposed by Fogg and Than-Tun[1], although challenged (Cox[2]), has been supported recently by Smith *et al.*[3] using possible physiological electron donors in an *in vitro* system. Inhibitor studies by Cox and Fay[1] and Bothe[5] on the other hand showed that 3′-(3,4-dichlorophenyl)-1′,1′-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport from Photosystem II, did not inhibit acetylene reduction in short term studies although it has an effect in longer term studies (Stewart and Pearson[6]). The action spectrum for acetylene reduction (Fay[7]) and the lack of Emerson enhancement of nitrogenase activity in *A. cylindrica* (Lyne and Stewart[8]) suggest that Photosystem II is not involved directly in the supply of reductant to nitrogenase. Cox and Fay[1] have proposed that reductant is supplied solely by dark processes and that light acts only by providing ATP from cyclic photophosphoryla-

Abbrevations: DCMU, 3′-(3,4-dichlorophenyl)-1′,1′-dimethylurea.

tion. Results presented below indicate that the size of the reductant pool (Lex *et al.*[9]) may be important in regulating nitrogenase activity and further that one route of electron transport to nitrogenase may involve both dark generated reductant and Photosystem I.

MATERIALS AND METHODS

Algae

Fogg's strain of *Anabaena cylindrica* Lemm., which was obtained from the Cambridge Culture Collection, was used in pure culture.

Culture conditions

The algae were grown routinely in axenic culture in nitrogen-free medium (Allen and Arnon[10]) at 25 °C under a light intensity of 2000 lux obtained from "warm white" fluorescent tubes. Stock cultures were maintained in 1-l Buchner flasks fitted with magnetic stirrers and constantly bubbled with filtered air. A constant volume was removed each evening and fresh culture medium was added to maintain uniform experimental material. All experimental cultures were incubated at 25 °C at 2000 lux supplied by "Grolux" tubes.

Nitrogenase activity assays

Nitrogenase activity was assayed by the reduction of acetylene to ethylene. The acetylene reduction assay used is detailed by Stewart *et al.*[11]. 5-ml aliquots of algae were incubated with shaking under the desired gas phase which contained 10% (v/v) acetylene in 30-ml medical flats fitted with serum liners. All gases except acetylene (British Oxygen Company) were obtained from Air Products Ltd. The gas phase was analysed for ethylene by gas chromatography using a Varian-Aerograph Model 1200-1 gas chromatograph fitted with a hydrogen-flame ionization detector and a column of Porapak T, and operating at 80 °C.

^{14}C-*fixation experiments*

$NaH^{14}CO_3$ was obtained from the Radiochemical Centre, Amersham, Bucks., and was added to the algae to give a final activity of 0.5 μCi/ml. At the end of the exposure period each sample was filtered on to a Whatman glass fibre filter. The algal residue was washed with 0.1 M HCl followed by distilled water, and then dried and counted using a Nuclear Chicago Model 4342 low background gas flow counter.

Chemicals

DCMU which was made up in ethanol was obtained from the Du Pont Company (U.K.). An equivalent amount of ethanol was added to the control series without DCMU. Monofluoroacetate was obtained from British Drug Houses as the sodium salt and made up in water. All other reagents also were obtained from British Drug Houses, "Analar" grades being used when available.

RESULTS

DCMU sensitivity of nitrogenase activity

As noted above varying results on the effects of DCMU on algal nitrogenase activity have been reported by different workers. The data in Table I show that the

TABLE I

THE EFFECT OF DCMU ON ACETYLENE REDUCTION BY ALGAE PRETREATED UNDER DIFFERENT GAS PHASES

Results are expressed in nmoles ethylene $\cdot h^{-1} \cdot 5\ ml^{-1}$. The algae were bubbled with the various gas mixtures for 12 h prior to the addition of 10% acetylene and DCMU ($3 \cdot 10^{-5}$ M) to duplicate samples. Incubation was for 1 h under the various gas mixtures.

	Pretreatments:			
	Air	N_2/CO_2	Ar/CO_2	$Ar/O_2/CO_2$
−DCMU	188	300	358	197
+DCMU	30	125	364	128
Inhibition (%)	84	58	−2	35

sensitivity of nitrogenase activity to DCMU varies depending on the gas phase under which the algae are incubated prior to and during the DCMU treatment. It may also be noted that maximum rates of acetylene reduction occur in the absence of added oxygen and nitrogen. Greatest inhibition by DCMU occurs in cultures incubated previously in air and there is less in cultures incubated in the absence of added oxygen in the presence of nitrogen. There is less inhibition of acetylene reduction in cells previously incubated in the absence of nitrogen than in its presence and in cells pre-incubated in the absence of nitrogen and oxygen there is no inhibition of subsequent nitrogenase activity in the presence of DCMU in short-term (60 min) experiments. Table II shows that conditions of low oxygen tension (Ar/CO_2) which reduce the DCMU sensitivity of nitrogenase compared with $Ar/O_2/CO_2$-pretreated cultures, do not reduce the DCMU sensitivity of carbon fixation to the same extent.

TABLE II

THE EFFECT OF DCMU ON ^{14}C FIXATION AND ACETYLENE REDUCTION IN THE PRESENCE AND ABSENCE OF OXYGEN

The algae were bubbled with Ar/CO_2 or $Ar/O_2/CO_2$ for 12 h prior to the addition of 10% acetylene or $NaH^{14}CO_3$ (0.5 μCi/ml). Results are the mean of triplicate samples incubated for 1 h in the presence or absence of DCMU ($3 \cdot 10^{-5}$ M).

		^{14}C fixation (cps)	*Inhibition (%)*	*Acetylene reduction (nmoles ethylene $\cdot h^{-1} \cdot 5\ ml^{-1}$)*	*Inhibition (%)*
Ar/CO_2	−DCMU	1740		285	
	+DCMU	350	80	278	2
$Ar/O_2/CO_2$	−DCMU	1650		184	
	+DCMU	120	93	114	38

A reductant pool for nitrogenase activity

The above results suggest that the sources of reductant for acetylene reduction and carbon fixation may be different, a suggestion first made by Cox[2] and for which additional support has come from the studies of Cox and Fay[1] and Lyne and Stewart[8]. They also support the view (Lex *et al.*[9]) that under certain conditions a pool of reductant is available for acetylene reduction which removes the dependence of nitrogenase activity on Photosystem II.

The results suggest further that the inhibition of acetylene reduction by DCMU is most marked under conditions when the carbon reserves of the cell would be depleted by the utilization of carbon skeletons, for example in ammonia assimilation (Cox and Fay[4]) and/or by photorespiration (Lex *et al.*[9]). If this is so it should be possible to alter the sensitivity of nitrogenase activity to DCMU by regulating the size of this pool of carbon compounds that supplies reductant. Table III shows that if algae are placed in the dark for a period to diminish carbon fixation and deplete carbohydrate reserves and are then placed in the light the percentage inhibition by DCMU decreases with increase in length of the period in the light. That is, the longer the exposure to conditions conducive to a build-up of carbon reserves, the less the dependence of nitrogenase activity on Photosystem II.

TABLE III

THE EFFECT OF DCMU ON ACETYLENE REDUCTION AFTER INCREASING PERIODS IN THE LIGHT BY CELLS PRETREATED IN THE DARK

Results are expressed in nmoles ethylene $\cdot h^{-1} \cdot 5\ ml^{-1}$. The algae were shaken in the dark in air for 2 h prior to the light incubation under Ar/CO_2 to maximise carbon accumulation. Triplicate 1-ml samples were withdrawn and DCMU ($3 \cdot 10^{-5}$ M) and 10% acetylene added at times shown for a 30-min incubation in the light.

Time in light (min)	*− DCMU*	*+ DCMU*	*Inhibition (%)*
0	126	106	16
30	122	108	11
60	117	110	6
120	113	112	1

Availability of carbon substrates for nitrogenase activity in the dark

If Photosystem II plays a role by making available carbon reserves which provide a source of reductant and/or ATP for nitrogenase activity it might be possible to regulate the ability to reduce acetylene in the dark by varying the pretreatment conditions in the light. Fig. 1 shows that during a 70-min incubation period in the dark, cells previously incubated in the light under Ar/CO_2 show a significantly higher rate of nitrogenase activity than cells incubated previously under $Ar/O_2/CO_2$ in the light. That is, under $Ar/O_2/CO_2$ where, perhaps as a result of photorespiration less substrate would accumulate in the light than under Ar/CO_2, the rate of subsequent dark fixation is lower. Acetylene reduction in the dark is strongly dependent on the presence of oxygen (Donze *et al.*[12]) and thus all incubations in the dark were carried out under air.

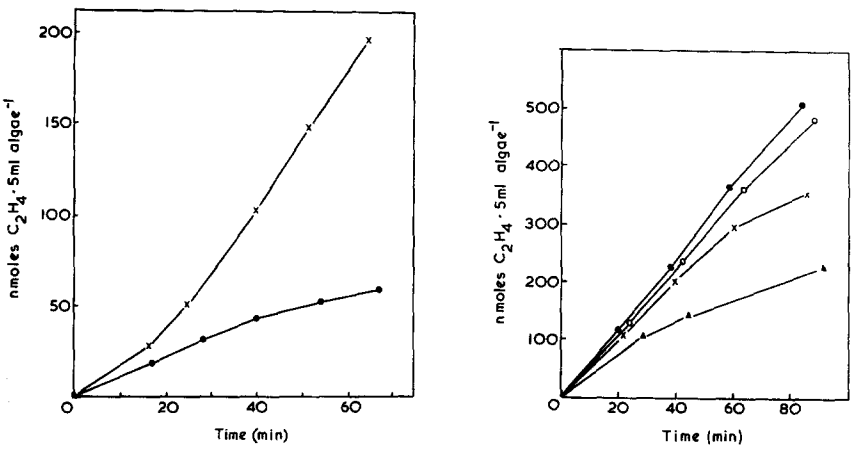


Fig. 1. Acetylene reduction in the dark under air by cultures bubbled with $Ar/O_2/CO_2$ (77.96: 22.00:0.04, v/v/v) (●—●) and Ar/CO_2 (99.96:0.04, v/v) (× — ×) in the light for 12 h prior to the dark incubation. Each point is the mean of triplicates.

Fig. 2. The rates of acetylene reduction under Ar/CO_2 (99.96:0.04, v/v) in the presence of: $1 \cdot 10^{-3}$ M monofluoroacetate (○—○), $3 \cdot 10^{-5}$ M DCMU (× — ×) and DCMU+monofluoroacetate (▲—▲). The control (●—●) was incubated alone under Ar/CO_2. Inhibitors were added 15 min after the start of the incubation.

Monofluoroacetate sensitivity of nitrogenase activity

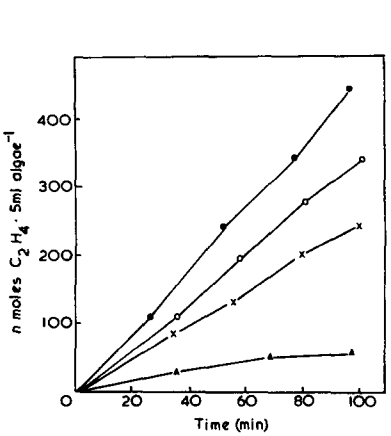
The action spectrum of acetylene reduction (Fay[7]), Emerson enhancement studies (Lyne and Stewart[8]) and the lack of inhibition by DCMU indicate a primary involvement of Photosystem I in nitrogen fixation which has been suggested by Cox and Fay[4] as being due entirely to the production of ATP *via* cyclic photophosphorylation. Table IV shows the effect of monofluoroacetate, an inhibitor of aconitate hydratase in the Krebs cycle, on acetylene reduction in the light and in the dark. It is seen that there is less inhibition of nitrogenase activity in the light than in the dark suggesting that light alleviates the inhibition by monofluoroacetate. The results in Fig. 2 which show that under Ar/CO_2 the inhibition by DCMU and monofluoro-

TABLE IV

THE EFFECT OF MONOFLUOROACETATE ON ACETYLENE REDUCTION IN THE LIGHT AND IN THE DARK

Results expressed in nmoles ethylene · h^{-1} · 5 ml^{-1}. Monofluoroacetate was added to give a concentration of $1 \cdot 10^{-3}$ M. The incubation was for 30 min under a gas phase of $Ar/O_2/CO_2$. Each value is the mean of triplicates.

	Light	*Dark*
−monofluoroacetate	560	112
+monofluoroacetate	445	52
Inhibition (%)	21	54



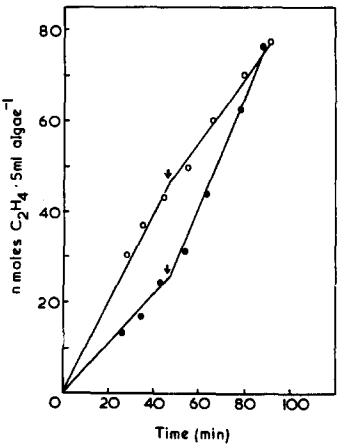


Fig. 3. The rates of acetylene reduction under $Ar/O_2/CO_2$ (77.96:22.00:0.04, v/v/v) in the presence of: $1 \cdot 10^{-3}$ M monofluoroacetate (○—○), $3 \cdot 10^{-5}$M DCMU (×—×) and DCMU+monofluoroacetate (▲—▲). The control (●—●) was incubated alone under $Ar/O_2/CO_2$.

Fig. 4. DCMU-treated cells were incubated under $Ar/O_2/CO_2$ (●—●) and DCMU+monofluoroacetate-treated cells under Ar/CO_2 (○—○) so that the rates of acetylene reduction were approximately the same in both treatments (see Figs 2 and 3). The light intensity was increased from 600–4000 lux from Grolux tubes at the times shown by arrows.

acetate together is greater than the sum of their separate inhibitions suggest that in the absence of Photosystem II there is a source of reductant to nitrogenase which is sensitive to monofluoroacetate. In oxygen grown cells where the endogenous pools of reductant are depleted both inhibitors have greater effects (Fig. 3) than under Ar/CO_2 (Fig. 2). Fig. 4 shows that there is a stimulation by light of nitrogenase activity in the presence of DCMU which appears to be *via* Photosystem I. There is no stimulation by light in the presence of DCMU and monofluoroacetate together and indeed there may be an inhibition.

DISCUSSION

The results obtained using the inhibitor of photosynthetic electron transport, DCMU, indicate that under certain conditions a pool of carbon compounds supplies reductant for nitrogenase activity. When the pool is high under conditions of low photorespiration and the absence of ammonia assimilation, acetylene reduction can proceed in the presence of DCMU and the elimination of Photosystem II has no or little effect during the period of our experiments. When the pool is low under aerobic conditions acetylene reduction is inhibited by DCMU and is thus more closely dependent on the rate of electron transport from Photosystem II. The enhanced rates of acetylene reduction in the dark when a pool of carbon reserves has accumulated in the preceding light period is consistent with the finding of Padan *et al.*[13] that dark respiration in blue-green algae is enhanced after a period of carbon fixation in the light.

The results obtained using monofluoroacetate suggest that one route of electron transport from this pool of carbon compounds to nitrogenase is *via* the Krebs cycle.

There is good evidence that monofluoroacetate specifically inhibits the aconitate hydratase enzyme of the Krebs cycle by formation of fluorocitrate (Elliott and Kalnitsky[11]; Morrison and Peters[15]). Indeed, the inhibitor has been used as a fairly unequivocal indicator of a functional cycle in various groups of organisms (Peters[16], Kelly[17]). The fact that light alleviates the inhibition by monofluoroacetate (Table IV) and that monofluoroacetate eliminates the stimulation of nitrogenase activity by light in the presence of DCMU when only Photosystem I operates (Fig. 4) implies that the monofluoroacetate-sensitive process is stimulated by Photosystem I light. These results suggest further that the stimulation by light in the absence of monofluoroacetate (Fig. 4) may not be solely *via* cyclic photophosphorylation as monofluoroacetate does not effect $^{14}CO_2$ uptake, or cyclic photophosphorylation as measured by ^{32}P uptake by DCMU-treated cells under anaerobic conditions (Lex, M. and Stewart, W.D.P., unpublished). An inhibition by monofluoroacetate of Photosystem I-dependent H_2 evolution in *Chlamydomonas* has been observed by Healey[18] and interpreted as indicating a flow of electrons from organic substrates to Photosystem I. As monofluoroacetate similarly affects Photosystem I-dependent nitrogenase activity in *A. cylindrica* it appears that a similar pathway operates in this organism but in this case supplying reductant for nitrogenase. The results do not argue against Photosystem I also supplying ATP from cyclic photophosphorylation. Such a diversion of respiratory intermediates from the usual oxygen linked pathway to Photosystem I has also been used to explain the Kok effect in blue-green algae (Healey and Myers[19]). An inhibition by light of respiration in various blue-green algae (responsible for the Kok effect) has been shown by a number of workers (Brown and Webster[20], Hoch *et al.*[21] and Carr and Hallaway[22]) and stimulation of oxygen uptake by light in *A. cylindrica* has been shown to be a different process from dark respiration (Lex *et al.*[9]). Stuart and Gaffron[23] have shown that reductant may be supplied to Photosystem I from respiratory intermediates in the evolution of hydrogen from *Scenedesmus* which is stimulated by light. In the case of blue-green algae which lack a complete Krebs cycle, (Smith *et al.*[24], Hoare *et al.*[25] and Pearce *et al.*[26]) the amount of reductant available from Krebs cycle intermediates to Photosystem I would depend on the rate of utilization of carbon skeletons and the extent to which the glyoxylate shunt pathway is in operation to complete the cycle and hence remove these.

There is good evidence (Stewart *et al.*[27], Van Gorkom and Donze[28], and Wolk and Wojciuch[29]) that in *A. cylindrica* the heterocyst is a site of nitrogen fixation. The cell has a functional Photosystem I (Wolk and Simon[30], Donze *et al.*[31], and Bradley and Carr[32]), cannot fix carbon (Wolk[33], Stewart *et al.*[27]) but receives fixed carbon from vegetative cells (Wolk[33]). Our results suggest that electrons may pass from this fixed carbon *via* the Krebs cycle to Photosystem I and probably to reduced ferredoxin (Bothe[5]), which in the absence of carbon fixation is directly available for nitrogenase. This does not exclude alternative dark routes of reductant to nitrogenase in blue-green algae.

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