Effects of 3-(3,4-Dichlorophenyl)-N-N′-Dimethylurea on Oxygen Evolution and Fluorescence by Whole Filaments and Isolated Thylakoids of the Cyanobacterium *Anabaena cylindrica*

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Dedicated to Prof. Dr. Wilhelm Menke *at the Occasion of His 70th Birthday*

Oxygen-Evolving Side, Cyanobacteria, DCMU-Sensitivity, Fluorescence

The site of inhibition of DCMU against photosystem II in the cyanobacterium (Blue-green alga) *Anabaena cylindrica* was examined in electron transport and fluorescence studies. Isolated thylakoids catalyzed silicomolybdate photoreduction using H_2O as electron donor; the steady-state reaction was completely inhibited by DCMU. This reaction is insensitive to DCMU in chloroplasts, since silicomolybdate accepts electrons from the primary photosystem II acceptor, and thus before the site of action of DCMU in higher plants. DCMU did not increase the steady-state level of fluorescence by inact *A. cylindrica,* nor affect the monophasic fluorescence induction, with or without dithionite, in contrast to the DCMU-dependent stimulation of steady-state fluorescence and replacement of rapid initial transients by a monophasic rise in *Chlorella.* Fluorescence by isolated cyanobacterial thylakoids was lowered by the electron acceptors silicomolybdate, *p*-benzoquinone, ferricyanide and anthraquinone-2-sulfonate, and not restored by DCMU addition, in contrast to results obtained with tobacco chloroplasts. Together with previous findings that diphenylcarbazide-dependent photosystem II activity of *A. cylindrica* thylakoids is insensitive to DCMU. The data indicate that the principal site of action of the inhibitor lies on the O_2-evolving side of photosystem II in this cyanobacterium.

Although DCMU has been extensively used as an inhibitor of photosynthesis in the cyanobacterium (blue-green alga), *Anabaena cylindrica* [1] its site of action in this organism is equivocal. The DCMU-dependent abolishment of photoautotrophic growth [2], photosynthetic O_2-evolution [3] and non-cyclic photophosphorylation [4] attests to the efficacy of the herbicide as an inhibitor of the photolysis of water by *A. cylindrica.* The evolutionary relationships and biological similarities between cyanobacteria and chloroplasts [5], together with the overall similarity in the structural and fuctional organization of oxygenic cyanobacterial and eucaryotic photosynthesis [6], would imply that the site of action of DCMU in *A. cylindrica* may lie after the primary acceptor of photosystem II, as in chloroplasts [3]. However, recent findings that DPC-supported photosystem II activity by isolated *A. cylindrica* thylakoids is unaffected by the addition of DCMU suggest that this may not be so. The insensitivity of DPC-dependent ferricyanide and 2,6-dichlorophenolindophenol photoreduction to DCMU-treatment catalyzed by *A. cylindrica* thylakoids indicates that a site of action of the inhibitor may lie on the donor side of photosystem II, before the site of electron donation by DPC [7; 8]. Similar findings were previously reported using isolated thylakoids of the cyanobacterium *Oscillatoria chalybea* [9].

The present communication reports on additional studies of O_2 evolution and fluorescence to further elucidate the site(s) of action of DCMU on photosystem II in *A. cylindrica.*

Materials and Methods

Anabaena cylindrica Lemm. (Strain CU 1403/2a), obtained from the Culture Centre of Algae and Protozoa, Cambridge, G. B., was cultured in BG-11 medium containing nitrate [10]. *Chlorella fusca* var. Emersonii was grown in standard Knop medium containing nitrate. Both organisms were grown axenically at 25 °C in glass aspirators which were stirred magnetically and sparged with 4% CO_2 in air at a rate of about 71 per h and illuminated by white

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Abbreviations: DPC, diphenylcarbazide; DCMU, 3-(3,4-dichlorophenyl)-N-N′-dimethylurea; A-2-sulf, anthraquinone-2-sulfonate.

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fluorescent tubes which provided approximately 2000 lux at the surface of the vessels. Logphase cells were used throughout.

Preparation of A. cylindrica thylakoids

Cells were subjected to enzymic digestion by incubation in the presence of 0.05% w/v glucuronidase (Boehringer) in a reciprocating shaker water bath at 37 °C for 1 h in the light. This was followed by 1 h of incubation under similar conditions with 0.05% w/v lysozyme (Sigma) plus 0.3% w/v cellulase (Kinki Yakoult, Japan). The entire procedure was performed in the presence of 0.6 M mannitol, essentially as described by Lehmann-Kirk *et al.* [11] and resulted in the formation of protoplasts which were subsequently disrupted.

Tobacco chloroplast preparation

Chloroplasts were isolated from the Connecticut cigar variety of *Nicotiana tabacum*, John William's Broadleaf, according the Homann and Schmid [12].

Fluorescence measurements were performed as detailed previously [13]. Excitation was achieved by light filtration through a blue Schott (Mainz) BG 38 filter. Excitation energy integrated from 400 nm upwards was 85 μE sec^{-1} and emission detected via a photomultiplier, PM : EMI 9558 QB, after passing through both a Corning CS 264 and a Wratten 97 filter.

Electron transport reactions were measured as described previously [9, 14]. Rate of silicomolybdate photoreduction were calculated either in terms of O_2 evolution or using a millimolar extinction coefficient of 8 mM^{-1} for the reduction product as given by Barr *et al.* [7].

O_2 Evolution was measured polarographically in a three electrode system as described earlier [15].

Results

O_2-Evolution

Rates of steady state O_2-evolution by intact filaments of *Anabaena cylindrica* and whole cells of *Chlorella fusca* at saturating intensities of red light were almost identical when calculated on a chlorophyll basis (Table I). However, the kinetics of initial transient O_2-evolution differed considerably, a high O_2 gush being found with *C. fusca* which was not so pronounced with *A. cylindrica* (Fig. 1). The steady-state and transient O_2 evolution by both organisms were completely prevented by the presence of 4×10^{-6} M DCMU. Preparations of *A. cylindrica* thylakoids also evolved O_2 if supplied with sodium silicomolybdate. This reaction was sensitive to DCMU, which caused complete inhibition of the steady-state silicomolybdate-dependent O_2-evolution at 2×10^{-6} M (Fig. 2a). Estimations of the effect of DCMU on total silicomolybdate-dependent O_2-evolution measured during the 30 sec of illumination given revealed a 50% inhibition (Fig. 2b), the dif-

Table I. Capacity for photosynthetic O_2 evolution by whole cells of *Anabaena cylindrica* and *Chlorella fusca*.

Organism	Rate of O_2 evolution [μmol/mg chlorophyll/h]
Anabaena cylindrica	514 ± 15
Chlorella fusca	564 ± 30

O_2 evolution was measured at 25 °C and saturating intensity of red light (580 nm < λ < 700 nm).

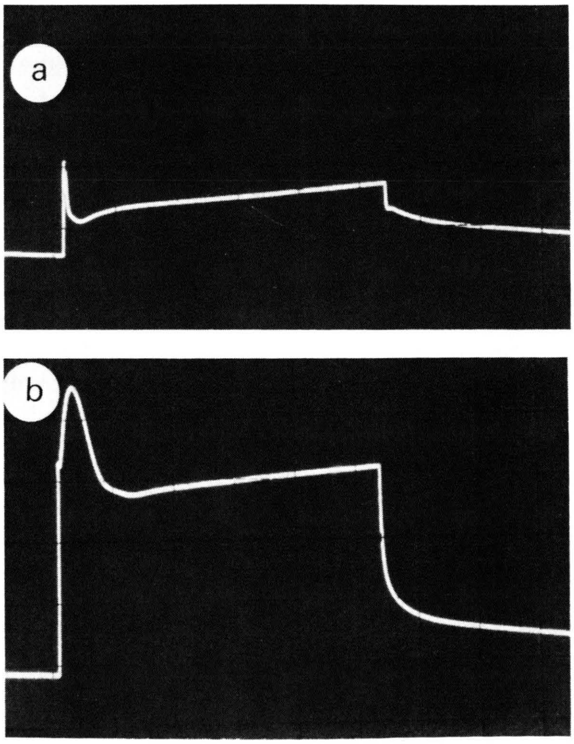

Fig. 1. O_2 gush by whole cells of *Anabaena cylindrica* (a) and *Chlorella fusca* (b). White light ($20 \cdot 10^3$ ergs/sec/cm^2) was given for 32 sec. Samples containing 25 μg total chl were used in both cases and the curves measured at the same sensitivity.

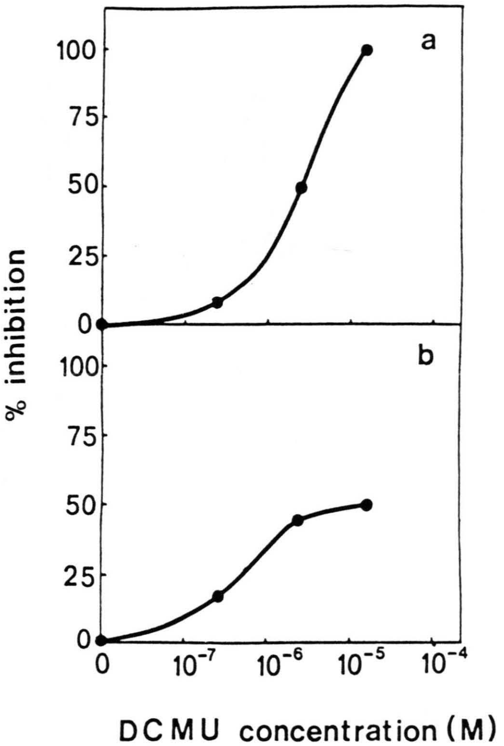

Fig. 2. Sensitivity of silicomolybdate photoreduction by *Anabaena cylindrica* thylakoids, measured as O_2 evolution, to DCMU. a: Inhibition of steady-state rate; b: inhibition of total O_2 evolution during illumination period (32 sec), including initial O_2 gush and steady-state evolution. The lower degree of inhibition in graph b is due to a time requirement (~ 5 sec) for DCMU inhibition to become fully effective.

ference being due to the fact that DCMU inhibition only becomes complete in the light [16], or that only part of the O_2-evolution observed in the first 5 to 10 sec after the onset of illumination is DCMU-sensitive, as found with *Phormidium luridum* by Piccioni and Mauzerall [17]. Since, however, a lag period before the onset of DCMU inhibition was observed at the beginning of the 30 sec illumination period in the present studies, the first interpretation is preferred.

Barr and coworkers [7] working with spinach chloroplasts demonstrated that silicomolybdate accepts electrons from Q, the primary electron acceptor of photosystem II, before the site of action of DCMU. The photoreduction of silicomolybdate by spinach chloroplasts was, accordingly, largely unaffected by DCMU. Assuming that silicomolybdate accepts electrons from Q, or its equivalent, in *A. cylindrica*, the inhibitory effect of the herbicide against the isolated thylakoids (Fig. 2) indicates that the site of action of DCMU must lie before that of electron acceptance by silicomolybdate in this cyanobacterium. These data are fully consistent with previous findings that the DPC-dependent ferricyanide Hill reaction of isolated *A. cylindrica* thylakoids is insensitive to DCMU [8] and provide further indication that a site of inhibition by DCMU lies between the water-splitting reaction and the photosystem II reaction centre in this organism.

As a complementary approach to determine whether DCMU acts before or after Q or its equivalent in *A. cylindrica* we have measured the effects of the inhibitor on fluorescence characteristics both *in vivo* and *in vitro*, using *Chlorella* cells and tobacco chloroplasts, where appropriate, for comparison.

Fluorescence studies

The excitation of whole cells of *C. fusca* produced the standard variable fluorescence pattern characteristic of the Kautsky effect (Fig. 3, curve 1). In accordance with the inhibitory action of DCMU after the photosystem II primary acceptor, the addition of the herbicide to *Chlorella* typically caused fast-rise fluorescence kinetics (Fig. 3, curve 1). In contrast, *A. cylindrica* filaments exhibited a considerably lower steady-state level of fluorescence, when compared with *C. fusca* on an equal chloro-

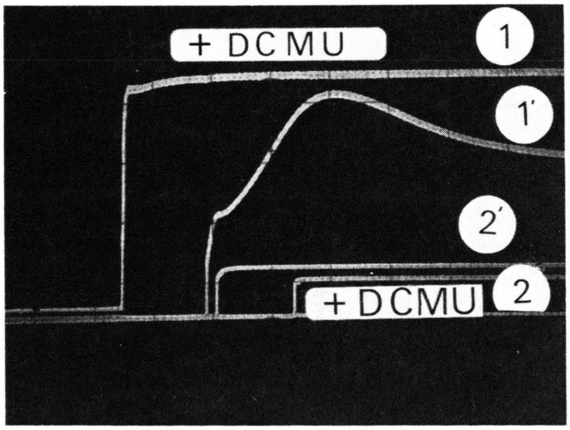

Fig. 3. Comparison of the Kautsky effect with whole cells of *Chlorella fusca* and *Anabaena cylindrica* measured on an equal chl basis (25 µg/assay) after 10 mn dark adaptation. 1: *Chlorella* plus 4×10^{-6} M DCMU; 1′ *Chlorella* minus DCMU; 2: *Anabaena* plus 4×10^{-6} M DCMU; 2′: *Anabaena* minus DCMU. Oscilloscope sensitivity 200 mV and sweep speed 100 msec/cm. Squares are 1 cm² in area.

phyll basis, with virtually a rapid monophasic rise in fluorescence yield only (Fig. 3, curve 2′). The *A. cylindrica* fluorescence kinetics were little affected by the addition of DCMU, which consistently caused a small decrease in the steady-state yield in over six experiments (*e. g.* Fig. 3, curve 2). The low steady-state level of fluorescence by *A. cylindrica* was initially considered to be possibly due to an active photosystem I, exceeding photosystem II activity and thus resulting in a preponderantly oxidized state of the primary acceptor of photosystem II, Q^+. This possibility was, however, lessened by the finding that dithionite, which chemically reduces Q in higher plants and algae to Q^-, did not significantly influence the fluorescence level with *A. cylindrica* (Fig. 4).

The fluorescence data presented so far were obtained at a sweep speed on the oscilloscope of 100 msec/sm. Increasing this to 5 msec/cm still resulted in a monophasic fast rise in fluorescence, whose kinetics were unaffected by DCMU. These findings indicates either that no variable fluorescence by *A. cylindrica* occurred, and that only dead fluorescence, F_0, was being measured, or that the exciting wavelength region was incorrectly chosen. However, fluorescence excitation was performed in different spectral regions including those of phycobilin absorption (ca. 600 nm) with the same result, namely a rapid monophasic rise in fluorescence, leading to a relatively low steady-state yield.

Isolated *A. cylindrica* thylakoids also showed the rapid monophasic rise in fluorescence, although the addition of the artificial electron acceptors silicomolybdate, *p*-benzoquinone, ferricyanide and anthraquinone-2-sulfonate clearly affected the steady state fluorescence level. The general trend observed was that the further away from Q that these compounds accept electrons, the smaller was their effect on the steady state fluorescence level. Thus, silicomolybdate exhibited the largest effect by depressing the steady state fluorescence yield by about 75 per cent (Fig. 5). The resulting low fluorescence was not increased by the addition of DCMU, but was rather lowered further. This latter decrease though small (Fig. 5 *cf.* curves b and c), provides additional evidence that the/a site of inhibition by DCMU is

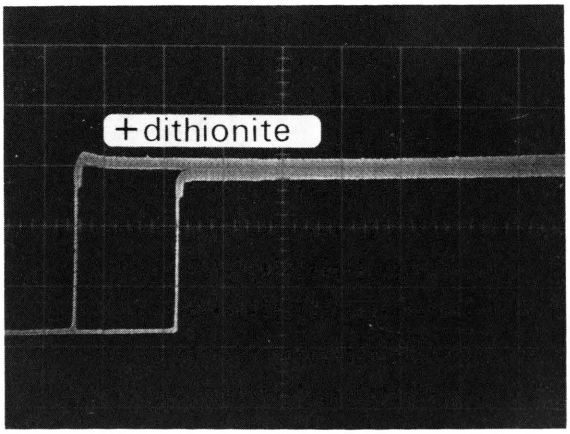

Fig. 4. Fluorescence induction in intact *Anabaena cylindrica* plus excess dithionite and minus dithionite. Oscilloscope sensitivity 50 mV and sweep speed 100 msec/cm. Other conditions as in legend to Fig. 3.

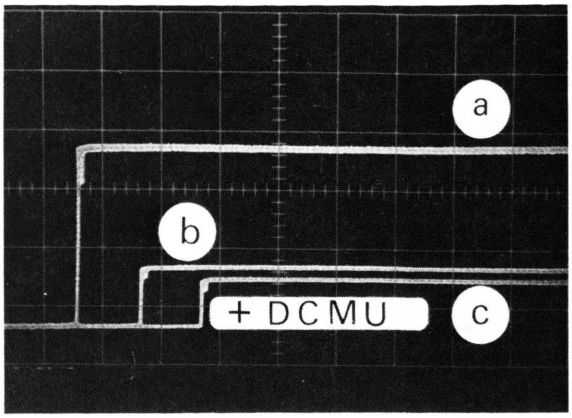

Fig. 5. Fluorescence induction with isolated *Anabaena cylindrica* thylakoids. a: no additions; b: plus silicomolybdate 3×10^{-4} M; c: plus silicomolybdate plus DCMU, 5×10^{-6} M. 63 µg chl/assay. Oscilloscope sensitivity 200 mV and sweep speed 10 msec/cm. Other conditions as in legend to Fig. 3.

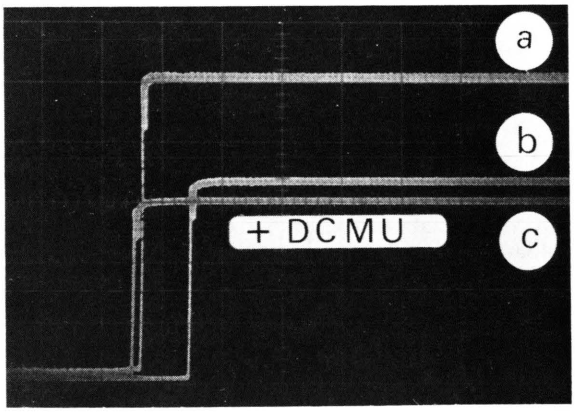

Fig. 6. Fluorescence induction with isolated *Anabaena cylindrica* thylakoids. a: no additions; b: plus *p*-benzoquinone, 10^{-3} M; c: plus *p*-benzoquinone plus DCMU, 5×10^{-6} M. 78 µg chl/assay. Conditions as in legend to Fig. 5.

on the water-splitting side of photosystem II. In higher plant chloroplasts, for example in the photosynthetic electron transport system from water to silicomolybdate (excess) in tobacco, DCMU has either no or a slight stimulatory effect on the fluorescence level (G. H. Schmid and G. A. Codd, unpublished), which is consistent with the main location of the site of photosystem II inhibition by DCMU being after the site of electron acceptance by silicomolybdate [7]. A similar effect of DCMU *i. e.* a lowering of the steady-state fluorescence level though not so pronounced as with silicomolybdate, was observed with *p*-benzoquinone as electron acceptor with *A. cylindrica* thylakoid preparations (Fig. 6). When ferricyanide or anthraquinone-2-sulphonate were used as acceptors, the steady state fluorescence level was barely affected. DCMU addition did not change the fluorescence rise kinetics observed (*e. g.* Fig. 7). In contrast, DCMU caused a considerable increased in the steady state level of fluorescence emitted by isolated tobacco chloroplasts in the presence of ferricyanide (Fig. 8a). These data provide further evidence either that the inhibition site of DCMU in *A. cylindrica* is situated elsewhere than in higher plant chloroplasts, or that preventing electron flow through photosystem I does not have the same consequences, implying that also the reduced primary acceptor of photosystem II in *A. cylindrica* is a fluorescence quencher.

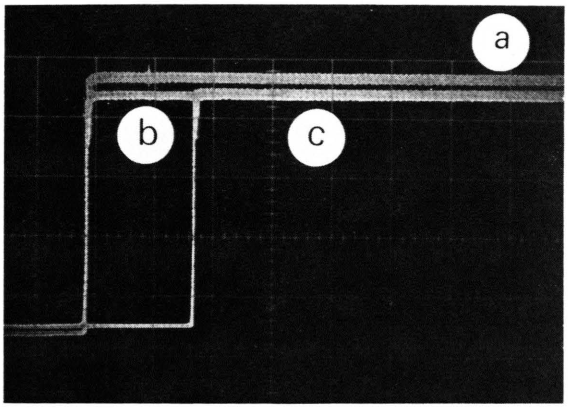

Fig. 7. Fluorescence induction with isolated *Anabaena cylindrica* thylakoids. a: no addition; b: plus ferricyanide, 10^{-3} M; c: plus ferricyanide plus DCMU, 5×10^{-6} M. 78 µg chl/assay. Conditions as in legend to Fig. 5.

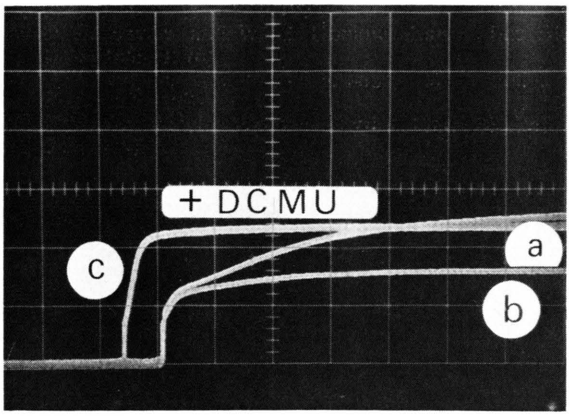

Fig. 8a. Fluorescence induction with isolated tobacco chloroplasts. A: no additions; b: plus ferricyanide, 10^{-3} M; c: plus ferricyanide plus DCMU, 5×10^{-6} M; 45 µg chl/assay. Conditions as in legend to Fig. 5.

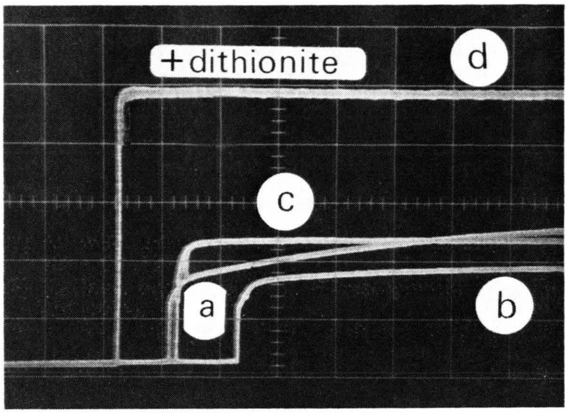

Fig. 8b. Fluorescence induction with isolated tobacco chloroplasts. a: no additions; b: plus anthraquinone-2-sulfonate 1.4×10^{-4} M; c: plus anthraquinone-2-sulfonate plus DCMU 5×10^{-6} M; d: assay in the presence of excess dithionite 45 µg chl/assay. e: sensitivity 200 mV sweep speed 100 m sec/cm (square 1 cm).

Discussion

During the characterization of the O_2-evolving side of photosystem II in higher plants and algae [11, 9, 14, 18], it was found that the DPC-dependent photoreduction of 2,6-dichlorophenol indophenol by thylakoids of the cyanobacterium *Oscillatoria chalybea* was not affected by DCMU, which completely inhibited the Hill reaction with water as electron donor. These findings were supported by work with thylakoids isolated from another filamentous cyanobacterium, *Anabaena cylindrica* in which DCMU completely inhibited the water → ferricyanide Hill reaction but had no effect if DPC was provided as an electron donor [8]. Since DPC can function as an electron donor to photosystem II [7, 19], it has been suggested that a site of inhibition by DCMU may lie on the O_2-evolving side of photosystem II in these cyanobacteria. The inhibition of the photoreduction of silicomolybdate, catalyzed by *A. cylindrica* thylakoids, by DCMU (Fig. 2) is in marked contrast to results obtained with chloroplasts [7] and provides further evidence that DCMU inhibits photosystem II on the O_2-evolving side in this cyanobacterium.

The fluorescence studies reported herein, performed with the objective of determining the effect of DCMU upon fluorescence kinetics failed to reveal a multiphasic fluorescence rise with either whole filaments or isolated thylakoids of *A. cylindrica* (Figs. 3 to 7). The fast rise kinetics strongly resemble the kinetics obtained in higher plant chloroplasts with dithionite (Fig. 8b). This was in contrast to the fluorescence transients observed with *Chlorella* cells (Fig. 3) and tobacco chloroplasts (Fig. 8a, b). The steady state level of fluorescence emitted by the *A. cylindrica* thylakoid preparations was lowered by the addition of electron acceptors (Fig. 5–7) which suggests an excess photosystem II capacity in the cultures used. The interpretations drawn necessarily assume that the primary electron acceptor of photosystem II, Q or its equivalent, in this cyanobacterium has the same influence and role in fluorescence, as in higher plants. Factors affecting the ratio of photosystem II to photosystem I activity in *A. cylindrica* are largely unknown but it is noted that nutritional conditions during cyanobacterial growth influence this ratio [20]. This possibility is currently under investigation in this laboratory.

Despite the apparent absence of early transients, the *A. cylindrica* fluorescence data provide additional evidence that the inhibition site of DCMU lies before the reaction center of photosystem II. DCMU did not cause an increase in fluorescence by the isolated cyanobacterial thylakoids supplied with artificial electron acceptors, but rather caused a slight decrease in the steady-state fluorescence level, in marked contrast to the results obtained with tobacco chloroplasts (Figs. 5–8). Although the principal site of action of DCMU in chloroplasts is thought to lie between Q and the secondary acceptor of photosystem II plastoquinone [2], Etienne has found an inhibitory effect on the S_3 state in chloroplasts which implies inhibition on the donor side of photosystem II [12].

Variation clearly exists within the cyanobacteria in relation to the inhibitory site(s) of DCMU, since fluorescence studies with the unicellular forms *Anacystis nidulans* [23, 24], *Aphanocapsa* 6714 [25] and *Aphanothece halophytica* [26] have shown that the herbicide mainly acts after Q in these organisms, whilst it is inferred to act before the photosystem II reaction center in the filamentous *A. cylindrica* and *O. chalybea*. The site(s) of action of DCMU in additional representatives of the coccoid and filamentous cyanobacterial groups must be compared before it can be ascertained whether this character is of phylogenetic significance. Finally, the implications of DCMU inhibition occurring on the O_2-evolving side, rather than on the reducing side of photosystem II, are worthy of consideration. The efficacy of the herbicide as an inhibitor of the photolysis of water during oxygenic photosynthesis is undisputed. However, though inhibiting the provision of electrons from water, the action of DCMU prior to the photosystem II reaction center would not necessarily prevent electron donation to photosystem II from other sources (*e. g.* DPC, refs. [8, 9]) and the possibility therefore remains that cyanobacterial photosystem II may continue to operate in the presence of exogenous electron donors of suitable redox potential and donor specificity even in the presence of DCMU.

Acknowledgements

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