Characterization of the Inhibition of Photosynthetic Electron Transport in Pea Chloroplasts by the Herbicide 4,6-Dinitro-*o*-cresol by Comparative Studies with 3-(3,4-Dichlorophenyl)-1,1-dimethylurea

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An attempt to characterize the mechanism of inhibition of photosynthetic electron transport in isolated pea chloroplasts by the herbicide 4,6-dinitro-*o*-cresol (DNOC) by a comparison with the effects of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) revealed the following:

1. The percent inhibition of oxygen evolution by a given herbicide concentration is the same at various light intensities except at very low intensities where the percent inhibition becomes larger. The same results are obtained with the herbicide DCMU.
2. The concentration of DCMU causing 50% inhibition of oxygen evolution decreases with decreasing chloroplast (and thus of chlorophyll) concentration. With DNOC, the relative decrease is much less than with DCMU. At the inhibited molecule, there appears to be a cooperative binding of DCMU with two binding sites and a noncooperative binding of DNOC with only one binding site.
3. The chlorophyll a fluorescence induction is influenced by DNOC in the same characteristic way as it is by DCMU: both herbicides cause a faster rise in fluorescence yield than in control chloroplasts, although a higher concentration of the former is required for the same effect.
4. The chlorophyll fluorescence emission spectra at 77 °K show a slight decrease in the bands at 685 and 735 nm, and no or only a very slight decrease at 695 nm upon addition of high concentrations of either DCMU or DNOC before the onset of illumination.
5. The degree of polarization of chlorophyll a fluorescence is lower after addition of DCMU or DNOC upon excitation by 460 or 660 nm light.

It is concluded that, although the chemical structure of DNOC is completely different from that of DCMU, its site and mechanism of inhibition is similar to that of DCMU. Both herbicides inhibit electron transport between the primary electron acceptor of photosystem II and the plastoquinone pool. This causes a closing of the reaction centers of photosystem II. However, the interaction with the inhibited molecule is different for the two herbicides.

The herbicide 4,6-dinitro-*o*-cresol (DNOC) is a potent inhibitor of photosynthesis. Van Rensen *et al.* [1] showed that the uncoupled Hill reaction (with ferricyanide as an electron acceptor) is inhibited 50 percent with 1 μM DNOC. This herbicide has no influence on the silicomolybdate-mediated Hill reaction in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), but inhibits the phenylenediamine-mediated photoreduction of ferricyanide in the presence of the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). Since the site of electron acceptance by silicomolybdate is at the primary electron acceptor, Q [2, 3], it was concluded that the site of action of DNOC is between Q and plastoquinone. This is also the site of action of DCMU (see *e. g.*, Duysens [4]).

All herbicidal inhibitors of photosystem II dependent electron transport share the common chemical structure $-CO-\bar{N}=$ (Trebst and Harth [5]). Since DNOC does not have this basic chemical structure, it is important to further characterize these DNOC-effects and to compare them with those of DCMU. Therefore the effects of DCMU and DNOC in isolated pea chloroplasts were studied on the Hill reaction at various light intensities and at various chloroplast concentrations, on chlorophyll a fluorescence induction, on chlorophyll a emission spectra at 77 °K and on chlorophyll a fluorescence polarization.

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNOC, 4,6-dinitro-*o*-cresol; I_{50}, a concentration, inhibiting 50 percent of the reaction; PQ, the plastoquinone "pool"; Q, the primary electron acceptor of photosystem II.

It was found that the site and mode of action of DNOC indeed is similar to that of DCMU; the interaction with the molecule to which they bind, however, appears to be different.

Methods

Peas (*Pisum sativum*, var. Laxton Progress No. 9) were grown in vermiculite in half-strength Hoagland's solution. Chloroplasts were isolated as described earlier by van Rensen *et al.* [1] for spinach, except that the chloroplasts were washed once with a 50 mM sodium phosphate buffer (pH 7.8) to obtain broken chloroplasts.

Electron transport was measured as oxygen evolution, using ferricyanide as the electron acceptor, with a Clark electrode and a Yellow Springs Oxygen monitor (Model 53). The signal was recorded on an Esterline Angus recorder (Model E 11015). The samples were illuminated with a tungsten lamp. After passing a Corning CS 3-71 glass filter the light intensity at the reaction vessel was 1000 $W \cdot m^{-2}$. Since a part of this radiation was heat, the reaction vessel was thermostated by a water jacket with streaming water connected to a thermostat, set at 25 °C.

Chlorophyll a fluorescence measurements were carried out with a laboratory-constructed spectrofluorometer [6]. Fluorescence transients were measured as described by Munday and Govindjee [7]. The photomultiplier signal was fed through a Tektronix oscilloscope (Type 502) and recorded on an Esterline Angus (Model E 11015) recorder. Chlorophyll a fluorescence was excited with broad-band blue light (Corning CS 4-96 and 3-73 filters). The light intensity was 200 $W \cdot m^{-2}$. Fluorescence was measured at 685 nm (half-band width, 6.6 nm) through a Bausch and Lomb monochromator. A Corning CS 2-59 filter, placed at the entrance slit of the analysing monochromator, eliminated stray exciting light. The transients were measured at room temperature.

The procedure for measuring fluorescence emission spectra at liquid nitrogen temperature was as described by Cho and Govindjee [8]. Fluorescence was excited at 460 nm and measured with an EMI 9558B photomultiplier through a Corning CS 2-59 glass filter and a Bausch and Lomb monochromator (half-band width, 6.6 nm). The spectra were corrected for photomultiplier sensitivity and monochromator characteristics.

Chlorophyll a fluorescence polarization was measured as described by Vacek *et al.* [9]. Fluorescence was measured at right angles to the exciting beam (460 or 660 nm) with an EMI 9558B photomultiplier through a Schott RG 665 and a 686 nm interference filter (half-band width, 6.8 nm). The degree of polarization was calculated as $(Fv - Fh)/(Fv + Fh)$, where Fv is the vertically polarized component and Fh is the horizontally polarized component after correction for systematic instrumental errors. These measurements were performed at room temperature.

Results

The results obtained earlier on effects of DNOC on electron flow in isolated spinach chloroplasts (van Rensen *et al.* [1]) were tested with pea chloroplasts. The results with peas were found to be the same as those with spinach.

Studying the effect of an inhibitor at various light intensities may yield information on its mechanism of action. If the inhibition is observed only at high light intensities, it means that a dark, prob-

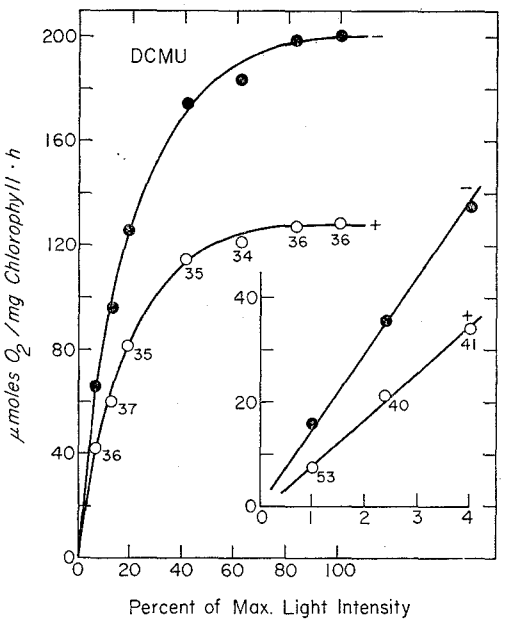

Fig. 1. Effects of 2.5×10^{-8} M DCMU on the ferricyanide Hill reaction at various light intensities. The reaction medium contained in 2 ml: 50 mM tricine-NaOH, (pH 7.6), 0.3 M sorbitol, 5 mM $MgCl_2$, 5 mM NH_4Cl, 0.5 mM ferricyanide and broken chloroplasts containing 50 μg chlorophyll. Maximum light intensity was 1000 $W \cdot m^{-2}$. Numbers below the lower line are percentages of inhibition. Inset, data for low intensities. +, with DCMU; −, without DCMU (control).

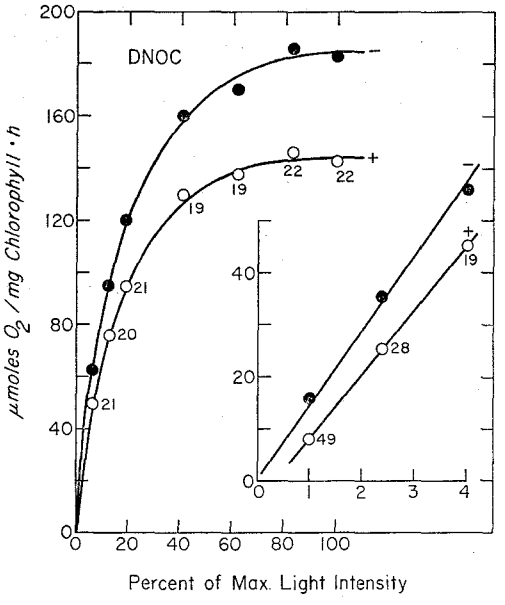

Fig. 2. Effects of 10^{-5} M DNOC on the ferricyanide Hill reaction at various light intensities. Experimental conditions were the same as in the legend of Fig. 1.

ably enzymatic, reaction is affected. If the inhibition occurs only at low light intensities, it implies that photochemical events are influenced. However, if the effect occurs at all the intensities, it may imply that two effects are involved, one in the photochemical and one in the dark events. Figs 1 and 2 show that the percent inhibition by either DCMU or DNOC on the uncoupled electron flow from water to ferricyanide is the same at almost all light intensities: 2.5×10^{-8} M DCMU inhibits 36 percent at all light intensities down to 6% of maximum light intensity and 1×10^{-5} M DNOC inhibits about 21% down to 4% of maximum intensity. This suggests an effect on the photochemistry of photosynthesis and an effect on dark reactions, probably electron flow, for both herbicides. An influence on the photochemistry is also suggested by Fig. 3, in which the $(\text{rate})^{-1}$ is plotted against $(\text{intensity})^{-1}$. In such a plot the reciprocal quantum yield is directly related to the slope of the lines and the intercept is equal to $(\text{saturation rate})^{-1}$. The decrease in both quantum yield and light-saturated rate of ferricyanide reduction indicates that both photochemical and enzymatic reactions are being affected. Also, at very low light intensities, where photosynthetic quantum yield is essentially dependent on photochemistry, the percent inhibition of either herbicide increases up to 53 percent with DCMU and up to 49 percent with DNOC at 1% of maximum light intensity (Figs 1 and 2, insets).

Tischer and Strotmann [10] recently suggested a method to estimate the inhibition constant (K_i) by measurement of the I_{50} value at various chloroplast (chlorophyll) concentrations and extrapolating to zero chlorophyll concentration. Such an experiment is illustrated in Fig. 4. The inhibition constant for DCMU in our experiment is 15 nM, which is close to the value of 40 nM, found by Tischer and Strotmann. For DNOC, however, we find an inhibition constant of a thousand-fold higher, 15 µM. The concentration of specific binding sites (x_t) can be calculated from the data in Fig. 4 by the equation, given by Tischer and Strotmann:

$$I_{50} = K_i + {}^1/_2\, x_t\,.$$

By relating the concentration of specific binding sites to the chlorophyll concentration, a value of 1

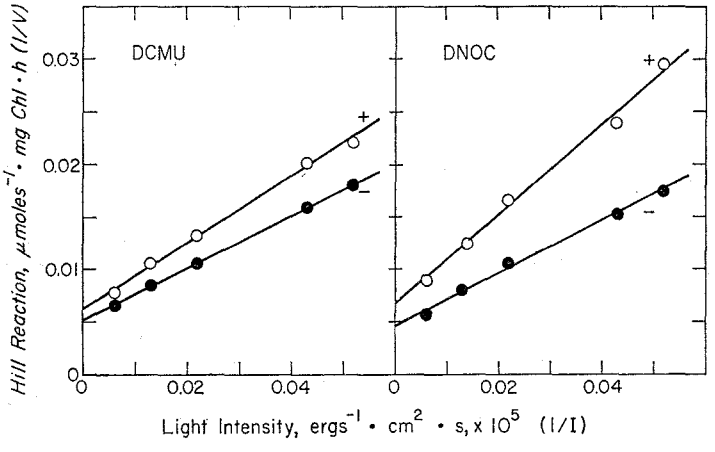

Fig. 3. Effects of 2.5×10^{-8} M DCMU and 10^{-5} M DNOC on the reciprocal quantum yield of the ferricyanide Hill reaction [$(\text{rate})^{-1}$ versus $(\text{intensity})^{-1}$]. Experimental conditions were the same as in the legend of Fig. 1.

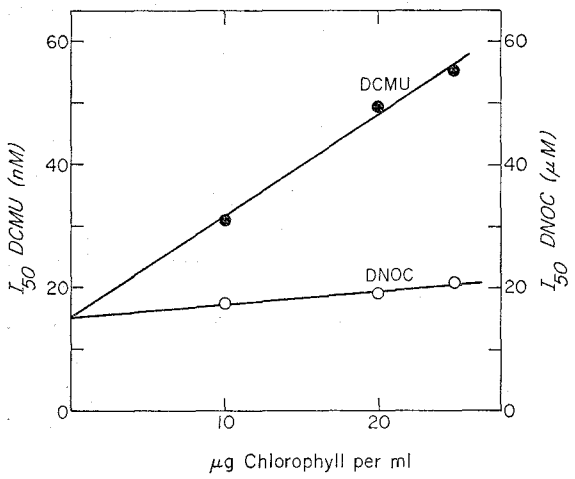

Fig. 4. I_{50} values of inhibition of the ferricyanide Hill reaction by DCMU and DNOC at various chloroplast (chlorophyll) concentrations. The reaction medium was the same as in the legend of Fig. 1.

binding site per about 300 molecules of chlorophyll is obtained for DCMU, which is close to the value found by Tischer and Strotmann. However, for DNOC a much higher value is found: 1 binding site per 2.3 chlorophyll molecules.

From the available data, Hill plots could be constructed (Fig. 5). The slope of the DCMU-curve appears to be about 2, which means that the acceptor molecule has two binding sites for DCMU, which

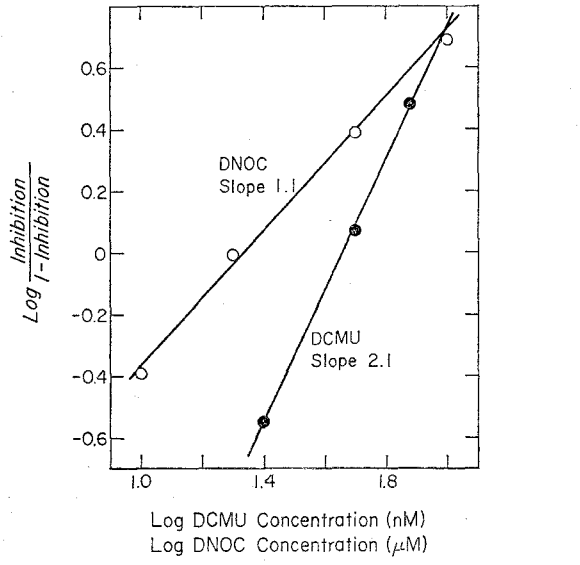

Fig. 5. Hill plots, calculated from inhibition curves; reaction medium as in Fig. 1.

are cooperative. The slope for DNOC is about 1, indicating independent binding with only one site per acceptor molecule.

The site of inhibition by DNOC was suggested to be the same as that of DCMU [1]. If this were true, the inhibitions caused by both herbicides should be additive. Table I shows the percent of inhibitions by DCMU and DNOC on the Hill reaction, separately and in combination. The inhibition by 2.5×10^{-8} M DCMU is about 20%, that by 1×10^{-5} M DNOC is about 29%. Additive inhibition of both herbicides thus should yield an inhibition percentage of about 49%. The results in Table I show indeed that combinations of the two herbicides cause 49% and about 53% inhibition.

Table I. Additions of inhibitions by DCMU and DNOC. Experimental conditions were the same as in the legend of Fig. 1. Control activity of the ferricyanide Hill reaction was about 195 μmol per mg chlorophyll per h. In the third line, first DCMU was added, then DNOC; in the fourth line this protocol was reversed.

Treatment	% Inhibition
2.5×10^{-8} M DCMU	19.6±2.0
1×10^{-5} M DNOC	29.3±1.2
2.5×10^{-8} M DCMU+1×10^{-5} M DNOC	52.7±3.0
1×10^{-5} M DNOC+2.5×10^{-8} M DCMU	49.0±1.0

The belief that DCMU inhibits electron transport between the primary electron acceptor of photosystem II (Q) and the plastoquinone pool stems from its stimulation of chlorophyll a fluorescence (Fig. 6). Duysens and Sweers [11] explained this stimulation by suggesting that DCMU blocks the reoxidation of reduced Q (see Discussion). In this way fluorescence is increased. Fig. 7 shows that DNOC exerts the same type of stimulation on chlorophyll a fluorescence as DCMU, indicating a similar mechanism of action. For comparison, the effects of the applied herbicide concentrations on the Hill reaction are given in Table II.

Table II. Effects of DCMU and DNOC on the ferricyanide Hill reaction. Experimental conditions were the same as in the legend of Fig. 1. Control activity was about 120 μmol O_2 per mg chlorophyll per h.

Concentration [M]	% of Control	Concentration [M]	% of Control
5×10^{-6} DNOC	79	2×10^{-8} DCMU	80
2×10^{-5} DNOC	43	5×10^{-8} DCMU	59
1×10^{-4} DNOC	17	1×10^{-7} DCMU	12
4×10^{-4} DNOC	0	1×10^{-6} DCMU	0

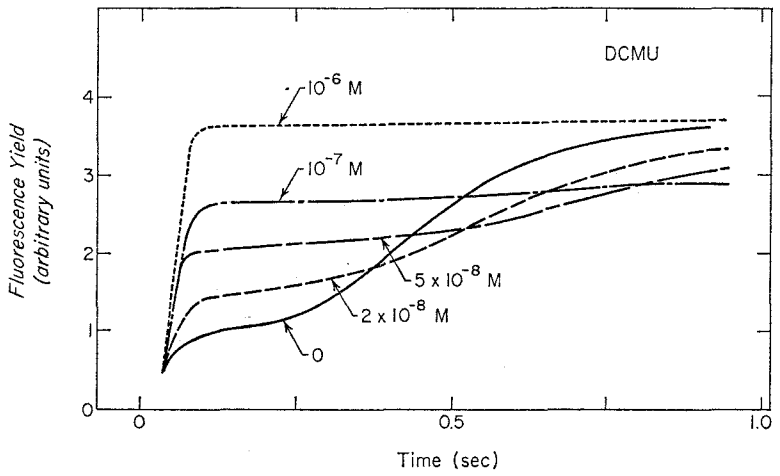

Fig. 6. Time course of chlorophyll a fluorescence in the presence of various concentrations of DCMU. Reaction medium contained in 2 ml: 50 mM tricine-NaOH (pH 7.6), 0.3 M sorbitol, 5 mM $MgCl_2$ and chloroplasts equivalent to 25 μg chlorophyll.

In a review on inhibitors of photosynthetic electron transport, Izawa [12] reported that some workers believe that DCMU interacts directly with the reaction center chlorophyll of photosystem II. Our results, illustrated in Figs 1, 2 and 3 indicate that both DCMU and DNOC affect the photochemistry of photosynthesis. Recently, Garab *et al.* [13] noted that DCMU affects the yield of the various emission bands of chlorophyll a fluorescence at low temperature differentially. Therefore, we studied effects of DCMU and DNOC on chlorophyll a fluorescence emission spectra at 77 °K (Figs 8 and 9). Our results with DCMU are comparable with those obtained by Garab *et al.* [13]. Fluorescence emitted at 685 and 735 nm is decreased; the fluorescence at 705 nm is increased; no change was observed at 695 nm. At this latter wavelength Garab *et al.* observed a relative increase. For DNOC we found almost the same result as with DCMU; however, there is a slight decrease at 695 nm, and an obvious decrease at 705 nm.

Since both DCMU and DNOC were found to affect the photochemistry of photosynthesis, we studied the degree of polarization of chlorophyll a fluorescence. It was found that DCMU, and also DNOC, decreases the degree of polarization of chlorophyll a fluorescence (Table III): 1×10^{-6} M

Table III. Degree of polarization (%) of chlorophyll a fluorescence at 686 nm as affected by DCMU or DNOC. Chloroplasts (30 μg chlorophyll) were suspended in 3 ml medium, containing 50 mM tricine-NaOH (pH 7.6), 0.3 M sorbitol, and 5 mM $MgCl_2$.

Wavelength of exciting light	460 nm	660 nm
Control	2.83 ± 0.13	3.64 ± 0.13
1×10^{-6} M DCMU	2.24 ± 0.06	2.86 ± 0.05
0.5% acetone	2.83 ± 0.04	3.79 ± 0.06
2×10^{-4} M DNOC +0.5% acetone	2.67 ± 0.01	3.30 ± 0.02

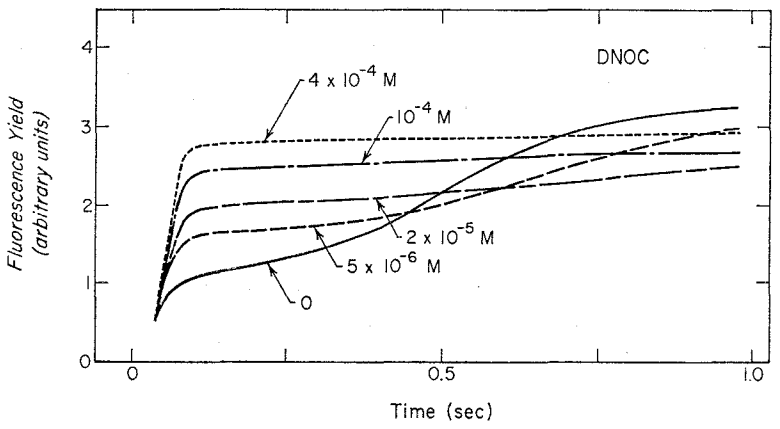

Fig. 7. Time course of chlorophyll a fluorescence in the presence of various concentrations of DNOC. The same reaction conditions as in the legend of Fig. 6.

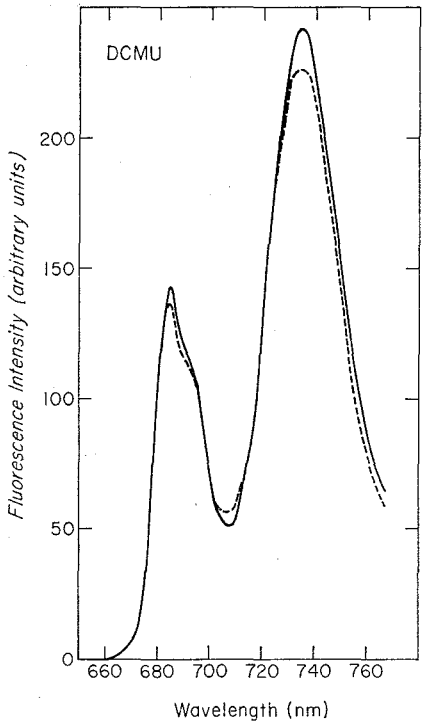

Fig. 8. Fluorescence emission spectra at 77 °K, excited at 460 nm, in the absence (——) and in the presence of 10^{-4} M DCMU (— — —). Both samples contained 1% ethanol. Chloroplasts (10 μg chlorophyll) were suspended in 1 ml of a medium containing 50 mM tricine-NaOH (pH 7.6), 0.3 M sorbitol and 5 mM $MgCl_2$. The spectra were normalized at 680 nm.

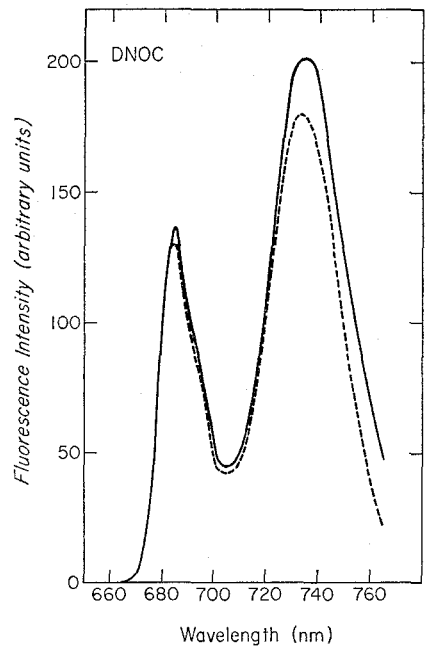

Fig. 9. Fluorescence emission spectra at 77 °K, excited at 460 nm, in the absence (——) and in the presence of 10^{-3} M DNOC (— — —). Both samples contained 1% acetone. Same conditions as in the legend of Fig. 8.

DCMU decreases the degree of polarization by about 21 percent when chloroplast suspensions are excited either at 460 nm or at 660 nm; 2×10^{-4} M DNOC decreases the degree of polarization by about 6 percent when fluorescence is excited at 460 nm and by 13 percent when excited at 660 nm.

Discussion

In a recent review on inhibitors of electron transport Izawa [12] mentioned two different effects of DCMU: (1) an effect on electron transport between the primary electron acceptor of photosystem II (Q) and the plastoquinone pool (PQ), and (2) a direct interaction with the reaction center chlorophyll of photosystem II (see Izawa [12] for references).

The effect on the electron flow between Q and PQ is consistent with the results shown in Figs 1 and 2; DCMU, and DNOC as well, inhibit the Hill reaction at light saturation. This is further supported by the effect of both DCMU and DNOC on chlorophyll a fluorescence transients (Figs 6 and 7). Duysens and Sweers [11] first proposed that Q in its oxidized state is a quencher of chlorophyll a fluorescence. In continuous light, at the start of illumination, Q is in its oxidized state and the fluorescence yield is low. With prolonged illumination Q is reduced and the fluorescence yield increases to the "P" level of fluorescence (Papageorgiou [14]). Since DCMU inhibits electron flow from Q to PQ, the reduction of Q in the light is accelerated and consequently the rise in fluorescence yield is faster (see Fig. 6). Although higher concentrations are needed, DNOC influences the fluorescence transients (Fig. 7) in the same way as DCMU does. This indicates that DNOC also inhibits electron transport between Q and PQ.

The observation that DCMU, as well as DNOC, decrease the quantum yield of the Hill reaction (Fig. 3) indicates that these herbicides also interact with photochemical events. The increase of the percent of inhibitions by DCMU at very low light intensities agrees with results obtained with unicellular algae (Gingras and Lemasson [15], using *Chlorella*; van Rensen and van Steekelenburg [16], using *Scenedesmus*). Tischer and Strotmann [10] reported that photosystem II inhibiting herbicides,

at a given concentration, have a lower effect on uncoupled electron transport in isolated chloroplasts at limiting light intensities than in saturating light. This should indicate that in their conditions, these herbicides almost only affect dark events, *i. e.*, electron transport.

The effects of both herbicides on the photochemistry of photosynthesis in continuous light could be explained by various mechanisms:

(a) a decrease in absorption cross section of chlorophyll;
(b) a decrease in absorption cross section of photosystem II with an increase in that of photosystem I (also see "Spill-over" phenomenon, Murata [17]);
(c) a decrease in exciton transfer efficiency from the antenna chlorophylls to the reaction center chlorophyll; or
(d) a slowing down of the rate of charge separation between the reaction center chlorophyll and Q by blocking of electron flow from Q to plastoquinone (closing of reaction centers).

The herbicides do not decrease the absorption cross section of chlorophyll a since they have no influence on the absorption spectrum of a chloroplast suspension. Also, possibility (b) can be ruled out, since it is shown in Figs 8 and 9 that at 77 °K the herbicides decrease the chlorophyll a fluorescence emission band at 685 nm, as well as at 735 nm. Garab *et al.* [13] also noted that DCMU decreases the yield of both photosystem II and photosystem I fluorescence emission at low temperature and Govindjee and Briantais [18] observed changes of fluorescence in algae after addition of DCMU at room temperature as well. It must be remarked that these effects are only seen at very high concentrations of the herbicides.

We prefer to suggest that mechanism (d) is the true mechanism of action of both herbicides: by blocking electron transport between Q and PQ, Q remains in its reduced state and by this the reaction centers are closed. This causes the fluorescence lifetime to increase, *i. e.*, the exciton travels longer and consequently looses some of its directional information and thus the degree of polarization of emitted fluorescence is decreased (Table III). A decrease of the degree of polarization of fluorescence after addition of DCMU to intact algae was observed by Mar and Govindjee [19] and Whitmarsh and Levine [20]. Becker *et al.* [21] measured the degree of fluorescence polarization in magnetically oriented spinach chloroplasts with and without DCMU and found that DCMU decreases the degree of polarization of fluorescence viewed at 720 nm and 740 nm upon excitation at 670 nm; the DCMU effect was much less upon excitation at 680 nm. Becker *et al.* [21] reasoned that the degree of polarization would be lost after only one exciton transfer if the mutual orientation of the various chlorophyll species were completely at random. Since the degree of polarization is not zero, there has to be a certain degree of order among the various chlorophyll species. These authors further suggested that DCMU increases the fluorescence lifetime, resulting in an increased number of energy transfer steps. Thus, relatively more fluorescence should be emitted from less oriented chlorophyll species, decreasing the degree of polarization. This could well be an explanation for our results (Table III). Since DCMU did not change the fluorescence polarization ratio, Becker *et al.* [21] concluded that it does not have any effect on the intrinsic orientation of the chlorophyll molecules. This makes mechanism (c) rather unlikely. Direct evidence against possibility (c) comes from the observation that photochemistry as measured by O_2 evolution (Figs 1 and 2) is satured at the same exciting light intensity in the presence or absence of inhibitors.

In conclusion, we suggest that the mechanism of action of both DCMU and DNOC is a blocking of electron flow from Q to PQ. Since this closes the reaction centers of photosystem II, photochemical events are also affected.

Although DNOC lacks the basic chemical structure of most photosystem II inhibiting herbicides ($-CO-\overline{N}=$, Trebst and Harth [5]), its site and mechanism of action appears to be similar to that of DCMU. However, the interaction with the inhibition site seems to be different. The inhibition constant of DNOC is a thousand-fold higher than that of DCMU. For DCMU there appears to be 1 binding site per about 300 chlorophyll molecules, which is about the same number as found by Tischer and Strotmann [10] and corresponds well to the relative concentration of electron carriers, such as Q, the primary electron acceptor of photosystem II. For DNOC, however, 1 binding site per 2.3 chlorophyll molecules was found. The reason for the lower activity of DNOC probably is that it is a weak acid. Since the inhibited molecule (probably a quinone)

is located in a lipophilic environment, the undissociated DNOC probably is the active species. Our experiments were performed at pH 7.6, at which the concentration of undissociated DNOC is much less than the total concentration of DNOC in the medium. Consequently, the binding constant for the undissociated DNOC should be much less than 15 μM (Fig. 4) and also the number of binding sites should be much less than 1 per 2.3 chlorophyll molecules, which was found for the total concentration of DNOC. The exact numbers cannot be calculated unless the dissociation constant for DNOC is known.

There appears to be 2 cooperative binding sites for DCMU at the inhibited molecule. The suggested formation of a complex between DCMU and an oxidized plastoquinone molecule (van Rensen [22, 23]) can accomodate two DCMU molecules on one acceptor molecule. For DNOC there seems to be only one independent binding site per inhibited molecule. The absence of the basic chemical structure ($-CO-\overline{N}=$, Trebst and Harth [5]) found in most photosystem II inhibiting herbicides could be the reason for this different binding characteristic.

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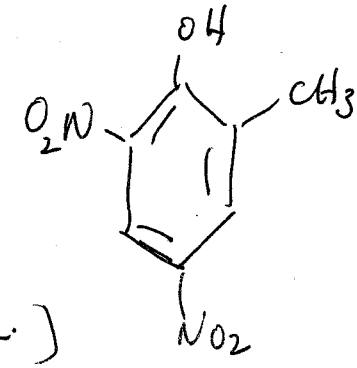
MW 198.13

alkaline aq solution
in ether
in alcohol (10%) & water (yellow)



DNOC

2-methyl-4,6-dinitrophenol.