Lipid Peroxidation and Chlorophyll Destruction Caused by Diquat during Photosynthesis in *Scenedesmus*

By

J. J. S. VAN RENSEN

Laboratory of Plant Physiological Research, Agricultural University Wageningen, Gen. Foulkesweg 72, Wageningen, The Netherlands

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Abstract

Lipid peroxidation and decrease in chlorophyll-a and chlorophyll-b content in *Scenedesmus* cells was followed in the course of time. Addition of diquat to the algae in the light causes lipid peroxidation and a decrease in chlorophyll content. This decrease is mainly due to chlorophyll-a, the concentration of chlorophyll-b remains more or less constant during the experiment. In the presence of N'-(3,4-dichlorophenyl)-NN-dimethylurea (DCMU), of cysteine, or during nitrogen-flushing of the algal suspension, the lipid peroxidation caused by diquat is strongly suppressed. The decrease in chlorophyll-a content caused by diquat is somewhat smaller in the presence of DCMU or during nitrogen-flushing than with diquat alone, but is not influenced by cysteine. The chemical antioxidant butylated hydroxytoluene does not affect lipid peroxidation and chlorophyll destruction caused by diquat.

Introduction

The mode of action of bipyridylium herbicides has been studied with higher plants by various authors (e.g. Mees 1960, Van Oorschot 1964, Baldwin 1969) and with unicellular algae by Van Rensen (1969) and Turner et al. (1970). These chemicals are supposed to exert their herbicidal action after reduction to their free radicals during photosynthesis and, more slowly, by respiration in the dark or in the absence of chlorophyll. In the presence of oxygen, these radicals are reoxidized, giving rise to the production of hydrogen peroxide or superoxide radicals (O₂⁻; Farrington et al. 1973), which disturb the membranes of the chloroplasts. The damage then spreads further to disrupt cell membranes.

This explanation was further tested by studying lipid peroxidation and chlorophyll degradation in *Scenedesmus* cells after addition of diquat. Finding that lipid peroxidation indeed takes place, effects of additions and conditions, which interfere with diquat inhibition, were investigated. As such, addition of DCMU, cysteine, butylated hydroxytoluene, and flushing with nitrogen were studied.

Abbreviations: Diquat: 1,1'-ethylene-2,2'-bipyridylium dichloride monohydrate; DCMU: N'-(3,4-dichlorophenyl)-NN-dimethylurea; MDA: malondialdehyde; BHT: butylated hydroxytoluene.

Materials and Methods

The strain of the unicellular green algae we used, was identified by Das (1968) as *Scenedesmus obtusiusculus* Chod. The method of cultivation of the algae was described before (Van Rensen and Van Steekelenburg 1965).

The rate of oxygen evolution was measured in a Gilson respirometer, while the algae were suspended in Warburg buffer number 9 (Warburg 1919). Each vessel contained 5 ml algal suspension, with a density of 4 μl wet packed cells per ml. Temperature of the water bath was 25°C. The vessels were illuminated from below by incandescent lamps (Claude Krypton 220 V, 53 W). The light intensity of the lamps was determined with a thermopile with and without a Schott RG 8 filter to correct for infrared radiation. The incident light intensity at the bottom of the vessels was 80 W m⁻², corrected for infrared radiation. This light intensity was at a level saturating photosynthetic oxygen evolution.

Lipid peroxidation was measured with the method of Heath and Packer (1968a). To 3 ml of algal suspension, 3 ml of 0.5% thiobarbituric acid in 20% trichloroacetic acid were added. After adding a boiling grain the solution was heated at 95°C in a water bath for 25 minutes. Then the solution was cooled, clarified by centrifugation in a clinical centrifuge, and absorbance measured at 532 and 600 nm in a Beckman model DU spectrophotometer. The amount
of malondialdehyde formed, which is a product of peroxidation of unsaturated fatty acids, was calculated after subtraction of the absorbance at 600 nm from that at 532 nm, using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

After sedimentation of the algae contained in 1 ml suspension medium, chlorophyll was extracted into 96% ethanol at 80°C for 5 minutes. After cooling and clarification by centrifugation, absorbances at 649, 665 and 800 nm were measured in a Zeiss model PMQ II spectrophotometer. Chlorophyll-a and chlorophyll-b levels were calculated using the method of Wintermans (1969).

Each figure shows the results obtained from one type of experiment carried out three times. At various time intervals in a series, the algae from one single vessel were used to measure MDA formation as well as chlorophyll content. As a consequence, oxygen evolution was averaged from 6 vessels at the beginning of the experiment until one at the end.

Results

Effects of diquat alone. Diquat was added to the algal suspensions to a concentration of 4 × 10⁻⁴ M. The rate of oxygen evolution decreased with time and zero gas exchange was reached after about 60 minutes (Figure 1a). After about 60 minutes production of malondialdehyde started, and it increased gradually till 25.8 nmols per vessel at the end of the experiment (Figure 1b). No MDA formation occurred in the absence of diquat. Chlorophyll content decreased gradually from 193 till 132 µg per vessel upon addition of diquat. No detectable lag phase was apparent (Figure 1c). The decrease in total chlorophyll level was due to breakdown of chlorophyll-a (Figure 1d); the chlorophyll-b concentration remained constant for at least 250 minutes (Figure 1e).

Consequently the chlorophyll-a/b ratio decreased from 3.86 to 2.36 (Figure 1f).

In the following experiments the above effects obtained with diquat alone were compared with treatments and additions which interfere with the mode of action of diquat.

Effects of diquat in the presence of DCMU. Figure 2 shows what happened when DCMU was added to the algae simultaneously with diquat. A concentration of 10⁻⁶ M DCMU was used because it is known that this treatment inhibits photosynthetic oxygen evolution in these algae within some minutes to about 10% of the control value (Van Rensen 1971). This inhibition remains constant for hours. As shown in Figure 2a, the rate of oxygen evolution decreased within 10 minutes to zero gas exchange under the influence of 10⁻⁶ M DCMU and in the presence of 4 × 10⁻⁴ M diquat. Obviously, diquat has no visible effect on oxygen evolution in the presence of DCMU. The formation of MDA induced by diquat alone (Figure 1b) is strongly suppressed by addition of DCMU (Figure 2b). DCMU alone has no effect on MDA production. In the presence of both DCMU and diquat chlorophyll content decreased (Figure 2c). Again, this decrease could be fully ascribed to a decrease in chlorophyll-a level, resulting in a gradual lowering of the chlorophyll-a/b ratio with time (Figure 2d, e, and f). In the presence of DCMU alone, there was also some breakdown of chlorophyll, but chlorophyll-a and chlorophyll-b levels were decreased in equal proportion, resulting in an unchanged a/b ratio (Figure 2d, e, and f).

In conclusion, the effects of the combined treatment with diquat and DCMU upon oxygen evolution, MDA production and chlorophyll breakdown are strikingly different from those of a treatment with diquat alone. In the presence of
DCMU, diquat has no visible effect on oxygen evolution. MDA formation induced by diquat is strongly suppressed by DCMU. The equal destruction of chlorophyll-a and b by DCMU is changed by diquat into a preferential loss of chlorophyll-a.

Effects of diquat at low oxygen concentration. In the experiment illustrated by Figure 3, the algal suspensions were flushed during 30 minutes in the dark with nitrogen, quality "Groenband". Since this nitrogen quality contains less than 0.001 %O₂, oxygen concentration became very low. Then, at zero time, diquat was added, lights were turned on, and nitrogen-flushing was continued until the end of the experiment. In our apparatus it is not possible to measure oxygen evolution under these conditions, so only the rate of photosynthesis of a control without N₂-flushing was indicated (Figure 3a). MDA formation in the presence of air (Figure 3b) was strongly suppressed by N₂-flushing (Figure 3b). Under nitrogen-flushing conditions breakdown of chlorophyll-a by diquat (Figure 3d) was slightly less than with diquat in the presence of air (Figure 3d). N₂-flushing alone also caused substantial breakdown of chlorophyll (Figure 3e). Chlorophyll-a and chlorophyll-b levels decreased in equal proportions, so there was almost no change in a/b ratio due to nitrogen (Figure 3d, e, and f).

The effects of the combined treatment of N₂-flushing and diquat addition upon MDA formation and chlorophyll breakdown strongly resembles the effects of the combined addition of DCMU and diquat. Both DCMU addition and N₂-flushing suppressed MDA production by diquat. Equal destruction of chlorophyll-a and b by DCMU or by N₂-flushing is changed by diquat into a preferential loss of chlorophyll-a.

Effects of diquat in the presence of cysteine. Addition of cysteine (20 mM) to the algal suspensions resulted in a decrease of photosynthetic oxygen evolution, but after a period of negative gas exchange, oxygen evolution was restored (Figure 4a). The rate of gas exchange in the presence of both diquat and cysteine (Figure 4a) resembled that obtained with diquat alone (Figure 1a). As with DCMU-addition and N₂-flushing, MDA production caused by diquat was lowered by addition of cysteine (Figure 4b). Cysteine had no effect on chlorophyll destruction by diquat (Figure 4c, d, e, and f). In the presence of cysteine alone there was a slight decrease in chlorophyll level, which was due to loss of chlorophyll-a (Figure 4c, d, e, and f).

Effects of diquat in the presence of butylated hydroxytoluene. Heath and Packer (1968b) showed inhibition of MDA formation in isolated chloroplasts by the chemical antioxidant butylated hydroxytoluene (BHT). In our experiments with unicellular green algae, however, this chemical had no effect on MDA production, caused by diquat (Figure 5b). The chemical probably did penetrate into the algae, since the rate of oxygen evolution was inhibited 70-85% by BHT within two hours time (Figure 5a). BHT did not influence destruction of chlorophyll induced by diquat (Figure 5c, d, e, and f). BHT alone caused some decrease in chlorophyll level, which was mainly due to chlorophyll-a (Figure 5c, d, e, and f).

Discussion

Effects of diquat alone. The time course of the inhibition of photosynthetic oxygen evolution by diquat (Figure 1a)
electron acceptor (Heath and Packer 1968a). Such a breakdown of chlorophyll may result from overloading of the

CO₂ (Figure 3c), by chilling (Van Hasselt 1972), by adding illumination of isolated chloroplasts in the absence of an

paraquat to flax leaves (Harris and Dodge 1972) or by butylated hydroxytoluene (Figure 5c), or in the absence of e.g. photosynthesis is inhibited, by addition of diquat (1970) determined a decrease of chlorophyll content (pg Chl per vessel). Diquat and BHT were added at zero time: (○) 4 x 10⁻⁴ M diquat and 5 x 10⁻⁴ M BHT; (○) 5 x 10⁻⁴ M BHT alone; (△) 4 x 10⁻⁴ M diquat alone.

shows the same type of curve as was found by Turner et al. (1970) for Chlorella and elegantly analyzed. Malondialdehyde is a breakdown product of tri-unsaturated fatty acid hydroperoxides, which can be formed under the influence of diquat in the light (Figure 1b). The observed lag time of about one hour corresponds to the time needed to reach complete inhibition of photosynthetic oxygen evolution by diquat. Harris and Dodge (1972) also found a lag phase in the production of MDA in paraquat treated flax cotyledon leaves. The duration of this lag corresponded with the time needed to reach complete inactivation of ferricyanide reduction by chloroplasts, isolated from these leaves. In illuminated spinach chloroplasts Heath and Packer (1968a) found a lag phase of one hour for lipid peroxide formation, while Flohé and Menzel (1971) observed a lag phase of about 30 minutes.

It is not clear if there is a lag phase in the decrease in chlorophyll content (Figure 1c). The decrease amounts to 32% after 250 minutes of exposure of our Scenedesmus cells to 4 x 10⁻⁴ M diquat in the light. Under the same conditions, Stokes et al. (1970) determined a decrease of 21% in Chlorella cells with 10⁻³ M diquat. Generally, illumination causes a decrease in chlorophyll content when photosynthesis is inhibited, e.g. by addition of diquat (Figure 1c), DCMU (Figure 2c), cysteine (Figure 4c), or butylated hydroxytoluene (Figure 5c), or in the absence of CO₂ (Figure 3c), by chilling (Van Hasselt 1972), by adding paraquat to flax leaves (Harris and Dodge 1972) or by illumination of isolated chloroplasts in the absence of an electron acceptor (Heath and Packer 1968a). Such a breakdown of chlorophyll may result from overloading of the excited chlorophyll of the energy trapping system. It is remarkable that in most of these cases chlorophyll-a decreases much more than chlorophyll-b. This suggests that chlorophyll-a is located closer to the reaction centers of the photosystems than chlorophyll-b.

However, bipyridyl compounds most likely do not act by overloading the energy trapping system. These compounds act as electron acceptors of photosystem I (Van Rensen 1969), and the reaction centers are not overloaded, since electron transport is not impaired. According to its mode of action diquat is reduced to its free radical; reoxidation of this free radical gives rise to production of peroxides. Catalase being absent in chloroplasts (Gregory 1968), these peroxides attack unsaturated fatty acids and pigments, leading to the results shown in Figure 1. Arntzen et al. (1969, 1972) suggested that chloroplast lamellar membranes have a binary structure, and that photosystem I is located on the outside half of the membrane and photosystem II on the inside half. Photosystem I contains much more chlorophyll-a than chlorophyll-b; in photosystem II the a/b ratio is much lower than in photosystem I. The observed faster decrease of chlorophyll-a after addition of diquat (Figure 1d, e) could be explained by the assumption that chlorophyll destruction starts at the site of reduction of diquat (photosystem I) and then spreads further to photosystem II.

Heath and Packer (1968a), studying photoperoxidation in isolated chloroplasts, found the ratio of MDA production over chlorophyll-a loss to be 0.23 after 3.5 hours of incubation. This ratio increases in the course of time. In our Figure 1, the ratio is 0.43 after 250 minutes of incubation, while the ratio decreases with time. Heath and Packer calculated the number of unsaturated fatty acids attacked per chlorophyll destroyed to be about 7, and the efficiency of MDA-production from tri-unsaturated loss to be 0.02. The production of 26 nmoles MDA (Figure 1) then means that 1300 nmoles of fatty acid are degraded. The corresponding loss of chlorophyll is about 61 nmoles. The ratio of fatty acids destroyed over chlorophyll loss is then 21, which is much higher than the number found by Heath and Packer.

In the case of isolated chloroplasts, peroxidation starts from the reaction centers of the photosystems, while in the case of diquat addition to the algae it starts from the site of diquat reduction, thus outside the reaction centers. The difference then suggests that the reaction centers of the photosystems contain little or no lipid.

Effects of diquat in the presence of DCMU. The simultaneous addition of DCMU and diquat speeds up the decrease in rate of oxygen evolution (Figure 2a), but prevents the negative dip in the curve, obtained with diquat alone (Figure 1a). DCMU prevents reduction of diquat, because it inhibits photosynthetic electron transport at a site prior to the reduction site of diquat (Van Rensen 1971). Therefore much less peroxides are formed and lipid peroxidation is much less (Figure 2b) than with diquat alone (Figure 1b).
Effects of diquat at low oxygen concentration. MDA formation by diquat is inhibited both by simultaneous addition of DCMU (Figure 2b) and by flushing the algal suspensions with nitrogen (Figure 3b). The mechanisms of suppression, however, are quite different. In the case of DCMU addition the reduction of diquat to its free radical is prevented. In the case of N₂ flushing, the reoxidation of the diquat free radical is suppressed.

Effects of diquat in the presence of cysteine. Figure 4a shows that photosynthetic oxygen evolution is strongly inhibited by cysteine, but recovers after about two hours. As Katoh and San Pietro (1967) found that cysteine can donate electrons to photosystem II in heated Euglena chloroplasts, we suggest that also in intact algae cysteine may donate electrons to photosystem II, thus inhibiting oxygen evolution. There is indeed a stoichiometric relationship between the rate of photosynthesis and the amount of cysteine present. With a rate of 400 μl O₂ per vessel and hour, 800 μl O₂ should have been produced when photosynthesis recovery (after about two hours); this is equivalent to 35.6 μmoles O₂, representing 143 μequivalents of electrons. In each vessel there were 100 μmoles cysteine present, suggesting a 1:1 stoichiometry.

Cysteine also oxidizes the diquat free radical, since it protects lipids from peroxidation by diquat to some extent (Figure 4b).

Effects of diquat in the presence of butylated hydroxytoluene. The mechanism of BHT-inhibition of oxygen evolution probably is different from that of cysteine, since the time course of inhibition by BHT (Figure 5a) differs from that of cysteine (Figure 4a). Also BHT does not affect MDA production by diquat, as cysteine does. However, Heath and Packer (1968b) did measure inhibition of MDA formation in isolated chloroplasts by BHT. We have no explanation for this discrepancy.

Conclusion. These data support the proposed mechanism of the action of diquat. After addition of diquat to algal cells photosynthetic oxygen evolution is inhibited, followed by lipid peroxidation and chlorophyll destruction by peroxides, which result from reoxidation of the reduced diquat. Addition of DCMU, cysteine, or flushing the algal suspension with nitrogen, strongly suppresses the peroxidation of lipids, caused by diquat.

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