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EFFECTS OF DIQUAT ON PHOTOSYNTHESIS IN *SCENEDESMUS*

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INTRODUCTION

The effect of dipyridylium compounds on photosynthesis has been studied by various authors, mainly with higher plants (1). The two best known compounds are diquat and paraquat which both cause a similar response (2).

MEES (3) showed that light increased the rate of killing of bean leaf discs by diquat. Etiolated wheat seedlings, treated in the light with diquat, behaved like light-grown seedlings, treated in the dark, indicating that chlorophyll was needed to obtain the maximum rate of killing. Upon pretreatment with monuron, or in the absence of oxygen, no stimulating effect of light on the rate of killing was observed. The herbicide sometimes caused initial stimulation of respiration, and inhibition lateron. The stimulation was dependent on continuous supply of diquat to the tissues, while the inhibition was not.

BRIAN (4) reported that the reduction of NADP in chloroplasts is competitively inhibited by diquat. The inhibition results from competition for electrons. Reduction of diquat is preferent, due to the redox properties of the two systems. In the dark, respiration supplies energy for the reduction of diquat, leading to the production of free radicals. Secondarily, in the presence of oxygen, peroxyde radicals are formed. It has been suggested that these radicals disrupt cell membranes, ultimately causing the death of the plants.

LANG and SEAMAN (5) found rapid chlorosis and death of *Lemna* and *Azolla* by low concentrations of diquat or paraquat ions. In both cases, chlorosis was directly related to concentration, light intensity, and duration of treatment. Electron microscope observations indicated major differences in the ultrastructure of chloroplasts in treated plants as compared with the controls; breakdown in the regular pattern of chloroplast lamellae and grana was frequently observed.

Anaerobiosis protected the pigment system of beans, exposed to light, from bleaching by paraquat (6). Light, also in the absence of oxygen, brought about

changes in membrane permeability by paraquat in mesquite, honeysuckle, and broadleaf bean. Changes in permeability were also temperature dependent.

VAN OORSCHOT (7, 8) showed that diquat strongly decreased the CO_2-uptake of bean leaves. The relative inhibition was almost equal at different light intensities. The reduction of CO_2-uptake was followed by a gradual development of chlorotic and necrotic spots on the leaves. Simultaneous application of diquat and simeton had a more or less additive effect on CO_2-uptake, but the development of symptoms was suppressed.

All the above results are consistent with the hypothesis (3, 4) that the reduction of diquat to a free radical is an essential step in the sequence of toxic reactions. In the light, diquat is reduced in the photosynthetic process; in the dark in the frame of respiration. Through interaction of the diquat free radical, water and O_2, toxic peroxide radicals may be formed, which are involved in the degradation of proteins and other large molecules in the protoplasm.

Most of these results are obtained from experiments with higher plants. We have tried to contribute further evidence for this hypothesis by studying the effect of diquat on O_2-evolution in a unicellular green alga, *Scenedesmus* spec.

MATERIAL AND METHODS

The herbicides used in our experiments were diquat (1,1'-ethylene-2,2'-bipyridylium dichloride monohydrate) and simeton (2-methoxy-4,6-bis[ethylamino]-1,3,5-triazine). The algae (*Scenedesmus* spec.) were cultured as described before (9).

The measurements of photosynthesis were carried out in a WARBURG-apparatus with six manometers. Temperature was 25 °C, unless stated otherwise. For an experiment, the harvested cells were centrifuged, washed once, and suspended in WARBURG-buffer no. 9 (15 ml 0.1 M Na_2CO_3 + 85 ml 0,1 M $NaHCO_3$). Cell density was determined as wet cell volume at dense packing, with TROMMSDORFF-tubes after 10 minutes centrifugation at 3000 rpm. Cell density used in the WARBURG measurements was 4 mm^3 cells/ml, unless stated otherwise.

The vessels in the WARBURG-apparatus were illuminated from below with incandescent lamps (PHILIPS Attralux S-24V, 150 W). Light intensity mostly was 4×10^5 ergs/cm^2 sec., measured at the bottom of the vessels with a thermopile, corrected by subtraction of the energy of wavelengths above 700 nm as determined with a SCHOTT RG 8 filter.

EXPERIMENTAL RESULTS

a. Inhibition of photosynthesis at different concentrations of diquat

First, oxygen evolution was measured, then the herbicide was added to the suspensions, and after 30, 60 and 90 minutes, O_2-evolution was measured again (fig. 1).

The inhibition increases with time; the photosynthetic apparatus obviously is disturbed in the course of time. In most of our further experiments, we have

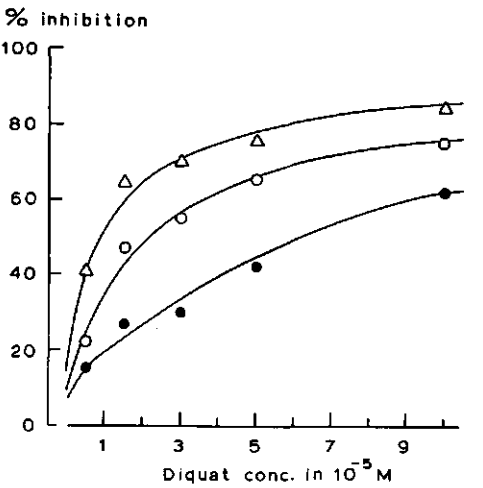

FIG. 1. Inhibition of O_2-evolution by different concentrations of diquat after 30 (●), 60 (○) and 90 minutes (△); 3 mm³ cells/ml.

measured the effect of 2×10^{-5} M diquat, one hour after addition. This treatment gives an inhibition of about 50%.

b. Inhibition at different suspension densities

O_2-evolution was measured at five different suspension densities and one hour after addition of the herbicide, measurement of oxygen evolution was again started. Photosynthesis was light-saturated, also at the highest suspension density. The percentage of inhibition becomes lower at higher suspension densities (fig. 2).

c. Washing experiments

After the estimation of the influence of the herbicide upon O_2- production, the cells were centrifuged and washed twice with tap water. Hereafter, they were suspended in a fresh amount of buffer solution to the same suspension density as before, and the O_2-evolution was measured again. Fig. 3 shows that by washing it is impossible to remove the inhibition caused by diquat. This result can be explained in two ways: either the herbicide cannot be washed out of the cells, or it has irreversibly damaged the photosynthetic apparatus. In order to discriminate between these two possibilities, we have tried to prevent damage to the photosynthetic apparatus by flushing the algal suspension with pure nitrogen, thus performing the treatment with diquat in the absence of oxygen. In figure 4, at arrow 1, the lights were turned on. Between 2 and 3, oxygen evolution was measured. At 3, the lights were turned off, and N_2-flushing was started. After ten minutes, at arrow 4, the lights were turned on again, and 2×10^{-5} M diquat was added. Between 5 and 6, oxygen evolution is shown. At 6, the algae were

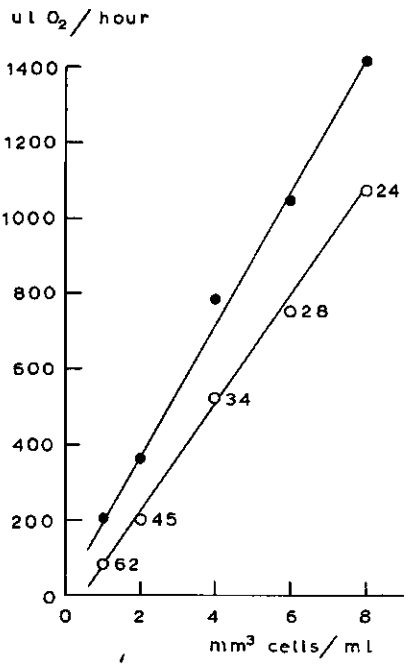

FIG. 2. Effect of 2×10^{-5} M diquat on O_2-evolution at different suspension densities; ● = before addition of the herbicide; ○ = one hour after addition. Numbers in lower line: % inhibition.

centrifuged, washed twice with tap water, and resuspended in fresh buffer at the same suspension density as before. At 7, the lights were turned on, and at 8, O_2-evolution was measured again. As is shown in fig. 4, under these conditions, the inhibition after washing is only about 1/3 of that in fig. 3.

Another way to protect the photosynthetic apparatus from damage is to prevent the reduction of diquat by blocking the electron transport chain at a site before the place of reduction of diquat. This can be done by adding the herbicide simeton which affects the photosynthetic System II. As has been shown by VAN RENSEN and VAN STEEKELENBURG (9), the inhibition caused by simeton concentrations up to 10^{-5} M can be completely removed by washing. In the experiment shown in fig. 5 and Table 1, the lights were turned on at arrow 1, and the rate of O_2-evolution measured between 2 and 3. At 3, two different concentrations of simeton were added to separate vessels; between 4 and 5, the effect of simeton was measured. At 5, diquat was added (in the presence of air in the light), and the combined effect of both herbicides is shown between 6 and 7. At 7, the algae were centrifuged, washed twice with tap water, and resuspended in fresh buffer at the same suspension density as before. At 8, the lights were turned on and at 9, measurements of oxygen evolution were started again.

Fig. 5 and Table 1 show that 2×10^{-6} M and 10^{-5} M of simeton inhibit pho-

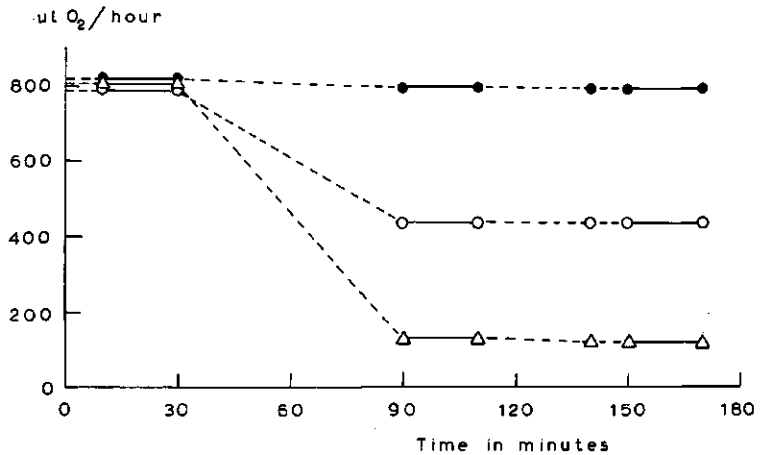

FIG. 3. Washing experiment; ● = control; ○ = 2×10^{-5} M diquat; △ = 10^{-4} M diquat. Explanation see text.

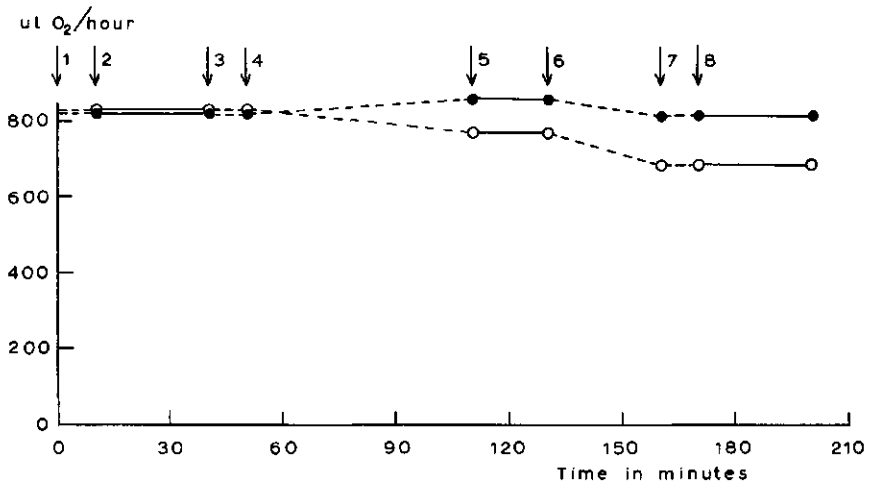

FIG. 4. Washing experiment; 2×10^{-5} M diquat is added in the absence of oxygen. Explanation see text.

tosynthesis for 45 and 86 per cent, respectively. The effect of simeton and diquat on photosynthesis is additive: 2×10^{-6} M simeton + 2×10^{-5} M diquat inhibit for 84% and 10^{-5} M simeton + 2×10^{-5} M diquat cause complete inhibition. After washing, it turns out that 2×10^{-6} M simeton has failed to prevent damaging of the photosynthetic apparatus caused by diquat, because the remaining inhibition is 41%, which is about the same as in fig. 3. In the case of the higher simeton-concentration, the inhibition left after washing is 31%, which is less than that found in fig. 3. Thus, 10^{-5} M simeton was found effective

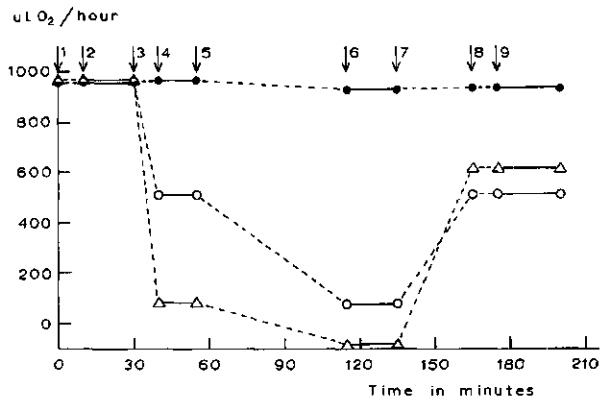

FIG. 5. Washing experiment; 2×10^{-5} M diquat is added in the presence of simeton; ● = control; ○ = 2×10^{-6} M simeton; △ = 10^{-5} M simeton. Explanation see text.

in protecting the photosynthetic apparatus from damage by diquat to some extent.

We had the hope to be able to apply such a high simeton concentration that the electron transport of photosynthesis was completely inhibited; no diquat would be reduced and no inhibition of O_2-evolution would be left after washing. Unfortunately, it is impossible to apply higher simeton concentrations, because it was observed that the inhibition of photosynthesis, caused by still higher concentrations, cannot completely be removed by washing. Therefore, a complete demonstration of the aim in view could not be achieved.

d. Inhibition of O_2-evolution by diquat in relation to light intensity and temperature

Studying the effect of light intensity on the inhibition of O_2-evolution by diquat at different temperatures, we found that dark respiration is stimulated by diquat at all three temperatures applied. The percent inhibition of photosynthesis is almost the same at all light intensities (fig. 6a, b, and c). However, when the

TABLE 1. Washing experiment with diquat in the presence of simeton; experimental procedure same as that for Figure 5; data in µl O_2 per hour, corrected for respiration. Numbers in parentheses: percentages of inhibition.

	before addition of herbicides	after addition of simeton	after addition of both herbicides	after washing
control	1019	1029 (0)	987 (0)	994 (0)
2×10^{-6} M simeton 2×10^{-5} M diquat	1023	561 (45)	158 (84)	580 (41)
10^{-5} M simeton 2×10^{-5} M diquat	1023	142 (86)	0 (100)	685 (31)

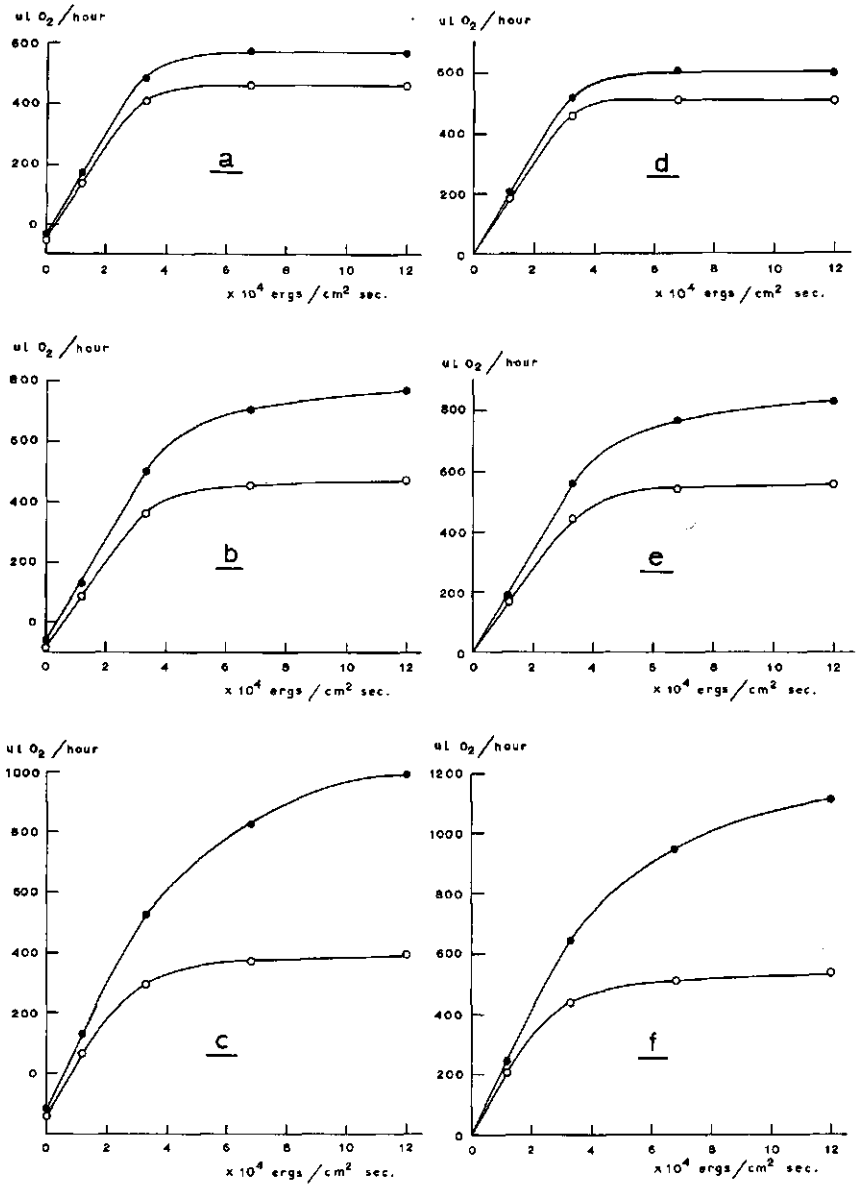

FIG. 6. Effect of 2×10^{-5} M diquat on O_2-evolution at various light intensities and different temperatures; ● = before addition; ○ = one hour after addition of the herbicide; a = 20 °C; b = 25 °C; c = 30 °C; d, e, and f are the same experiments as a, b, and c respectively, data corrected for dark O_2-uptake.

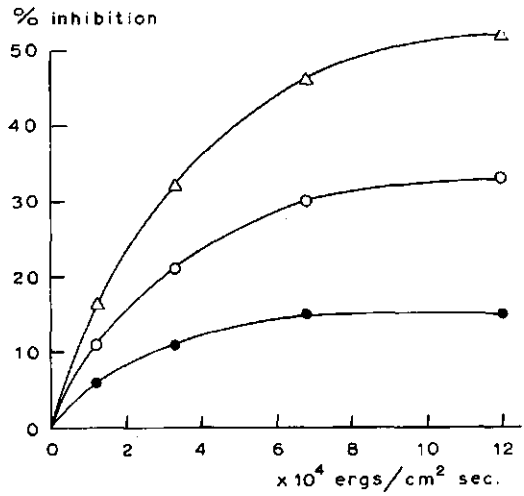

FIG. 7. Effect of temperature on inhibition percentages at different light intensities; calculated from the data of figures 6d, e, and f; ● = 20°C; ○ = 25°C and △ = 30°C.

curves are corrected for O_2-uptake in the dark, the percentages inhibition increase with light intensity and temperature until light saturation is reached (fig. 6d, e, f, and fig. 7).

e. Effect on the photosynthetic quotient

Additionally, we have studied the effect of diquat on the photosynthetic quotient during a short period after addition of the herbicide. O_2-evolution and CO_2-uptake were measured simultaneously by the two vessel method. The algal suspensions were flushed with a mixture of 95% air and 5% CO_2 during 15 minutes. Then, the algae were pipetted into the vessels, identical samples of algae being suspended in either 5 or 10 ml tap water, then the suspensions and the gas spaces in the vessels were flushed with air + 5% CO_2 for 30 minutes in the dark. During this period, the vessels were shaken in the apparatus in order to attain equilibrium between gas phase and liquid. Five minutes after the lights had been turned on, manometer readings were started. From these readings O_2-evolution and CO_2-uptake were calculated (Table 2). This table shows that CO_2-uptake is relatively more inhibited, leading to an increase of the photosynthetic quotient.

TABLE 2: Effect of diquat on the photosynthetic quotient during the first 45 minutes after addition of the herbicide.

	μl O_2-evolution	μl CO_2-uptake	*quotient*
control	1271	1207	1.06 ± 0.02
10^{-4} M diquat	278	219	1.28 ± 0.05

Discussion

As expounded in the introduction, in this paper, we have tried to collect some more evidence for the hypothesis on the mode of action of diquat (3, 4), according to which the direct effect of diquat on photosynthesis is the interaction with the electron transport chain (10), diquat being reduced to a free radical. This leads to the disturbance of the photosynthetic apparatus (5) by secondary peroxide radicals, formed by the interaction of the diquat free radical with water and oxygen.

In our experiments, one hour after addition of 2×10^{-5} M diquat, photosynthesis is inhibited for about 50%; the inhibition increases with time. This means that either diquat enters slowly into the algal cells, or that the photosynthetic apparatus is damaged by diquat. The latter is in accordance with the hypothesis on the mode of action of diquat and is supported by further experiments.

The observation that the inhibition percentages are lower at higher suspension densities, points to accumulation of diquat by the algal cells.

The fact that the inhibiting effect of diquat on oxygen evolution cannot be removed by washing could be due to irreversible binding of diquat by the cells, or to irreversible damage of the photosynthetic apparatus by the supposed toxic peroxide radicals. Flushing the algal suspension with nitrogen before and during the treatment with diquat was found to protect the photosynthetic apparatus from damage, the inhibiton left after washing being only about 1/3 of that observed in a similar experiment in the presence of oxygen. In this respect it is important that, one hour after the addition, there is more diquat in the algal cells under nitrogen flushing conditions than in the presence of air (11). The inhibition still left after washing in the oxygen-free case could be due to some oxygen, left in the suspension or to some diquat left after washing. Anyhow, this experiment shows that diquat can be removed by washing to a high extent. Baldwin *et al.* (12) reported 90% loss of the other bipyridylium herbicide paraquat from an isolated chloroplast suspension in three washings.

The effect of simeton and diquat on photosynthesis is additive on simultaneous supply as was also found by van Oorschot (7). On the other hand, 10^{-5} M simeton supplied separately, was found to have a protecting effect on the photosynthetic apparatus, with regard to the effect of diquat, supplied later. Van Oorschot (8) reported that a still higher simeton concentration prevented the development of symptoms, caused by diquat in bean leaves.

In summary, our washing experiments have shown that diquat can be washed out of the algal cells and that the reduction of diquat and the presence of oxygen are essential for the inhibiting effect on photosynthesis.

A more close study of the effect of light intensity and temperature on the inhibition of O_2-evolution by diquat revealed that respiration is stimulated at the three temperatures used, and that the percent inhibition of O_2-evolution is almost equal at different light intensities which was also found by van Oorschot (8) for leaves. However, after correction of the curves for O_2-uptake in the dark, the percentages

of inhibition increase with light intensity until light saturation is reached. Diquat was found to inhibit both in the light-limited and the light-saturated part of the photosynthesis-light curves. Moreover, the percentages of inhibition were found to increase with temperature.

The light reaction of photosynthesis leads to the production of O_2 and ATP, and the reduction of NADP. The dark reactions lead to the reduction of CO_2. In the light-limited part of a photosynthesis/light curve, the rate of photosynthesis is determined by the amounts of ATP and reduced NADP. In the light-saturated part of the curve, the amounts of ATP and reduced NADP are in excess, and the rate of photosynthesis is determined by temperature, and incidentally by CO_2-concentration. The fact that diquat affects both parts of the curve indicates that it inhibits both light and dark reactions, which may be due to its indirect effect: the disturbance of the photosynthetic apparatus by peroxide radicals (5). Another indication is that the inhibition is temperature-dependent; an inhibition of the light reaction only should be temperature-independent.

It is clear that, in addition to oxygen, light and increase in temperature increase the inhibition of photosynthesis by diquat, which is in accordance with the results reported by MEES (3) and MERKLE *et al.* (6).

It is supposed that, in the photosynthetic electron transport chain, diquat is reduced instead of NADP, which is to be considered as the direct effect of diquat on photosynthesis. Thus, during a short time after the addition of diquat, it acts as a HILL-oxidant for O_2-evolution, while CO_2-uptake is decreased by lack of reduced NADP. This implies that the photosynthetic quotient should be increased during the first time after addition of diquat. It was indeed observed that, at adequate diquat concentrations, CO_2-uptake initially is somewhat more inhibited than O_2-evolution, so that the photosynthetic quotient is increased during the first 45 minutes after addition of diquat.

SUMMARY

The effect of the herbicide diquat on photosynthesis is studied on suspensions of the unicellular green alga *Scenedesmus* spec. The inhibition of O_2-evolution by diquat increases with time; 2×10^{-5} M diquat gives an inhibition of about 50%, one hour after addition. The percentages of inhibition become lower at higher suspension densities which points to an accumulation of diquat by the cells.

The inhibiting effect cannot be washed out, but when diquat is added in the absence of oxygen or in the presence of 10^{-5} M simeton, the inhibition after washing is less. It is concluded that diquat can be removed to a large extent by washing, and that oxygen is needed for the inhibiting effect.

Respiration is stimulated by diquat. The percent of inhibition of photosynthesis increases with light-intensity, until light-saturation is reached. This shows that the light-limited and the light-saturated part of the photosynthesis-light curves are both inhibited. The percent of inhibition increases with temperature.

During the first 45 minutes after addition of diquat, the photosynthetic quo-

tient is increased, which shows that during this time diquat acts as a HILL-oxidant for O_2 evolution, while CO_2-uptake is decreased by lack of reduced NADP.

The results are consistent with the hypothesis that diquat is reduced to a free radical in the photosynthetic process. The interaction of this diquat free radical with water and oxygen leads to the formation of toxic peroxide radicals or of hydrogen peroxide. These peroxides completely disrupt cellular organization, structure and function.

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