THE EFFECTS OF THE DIPYRIDYL DIQUAT ON THE METABOLISM OF *CHLORELLA VULGARIS*

I. GAS EXCHANGE IN THE LIGHT

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Summary

The effects of diquat on gas exchange in illuminated *C. vulgaris* have been studied by manometric procedures. Estimates of dark oxygen uptake were made by transfer of treated cells to darkness. Analysis of the results indicates that on adding diquat to *C. vulgaris* there is a rapid fall in the rate of apparent photosynthesis. This is due to an immediate and large increase in dark metabolism (CO_2 and O_2 exchange) accompanied by irreversible inhibition of real photosynthesis firstly, then of respiration. At some unspecified stage a light-dependent oxygen uptake begins, rises to a maximum rate, and declines to a low value, continuing after the inhibition of respiration. These results are discussed in relation to the known effects of diquat on plastid reactions and to the hypothesis that in illuminated green tissue diquat produces a toxic substance which damages both the photosynthetic and respiratory apparatus.

I. INTRODUCTION

It has been shown that the dipyridyl quaternary salts active as herbicides are those which can be reversibly reduced, by single electron acceptance, to free radicals stable in aqueous solutions. Irreversibly reducible compounds of this series, even those known to produce free radicals on reduction, and unstable quaternary salts are not phytotoxic. As oxygen gas is also a prerequisite for toxicity, it is believed that a reoxidation of the stable free radical is concerned. This reoxidation is postulated to produce hydrogen peroxide, which acts as the toxic substance (Homer, Mees, and Tomlinson 1960; Mees 1960).

It has also been proposed by these authors that the free radicals are produced by interaction of the quaternary salts diquat (1,1′-ethylene-2,2′-bipyridylium-2A) and paraquat (1,1′-dimethyl-4,4′-bipyridylium-2A) with radicals produced during both photosynthesis and respiration. The light reaction of photosynthesis appears to be primarily implicated in toxicity.

Published work on diquat since 1960 deals mainly with its action as a herbicide of higher plants or with its biochemical effects on plastids. In this series of papers we investigate the effects of diquat, applied in light and darkness, on the physiology of *Chlorella vulgaris*. The use of a unicellular organism avoids some of the complications concerned with penetration and translocation which are unavoidable when the higher plant is used.

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II. Materials and Methods

The experimental material used throughout this work was *C. vulgaris* Beij strain 211–11h Pratt, obtained from the Cambridge collection. According to Kessler (1967) this species should be named *C. kessleri* Fott & Novakova. However, Shihira and Krauss (Mr. E. George, personal communication) classify the strain as the type of a new taxon, *C. vulgaris* var. *aprica*.

The alga was cultured on agar slopes at low light intensity and subcultured at 2-monthly intervals. However, all material used for manometry was subcultured from a liquid suspension grown under standard conditions; the culture medium (autoclaved) contained 10 mM $MgSO_4.7H_2O$, 12·4 mM KNO_3, 9·2 mM KH_2PO_4, 0·001 mM $CaCO_3$, and 0·084 mM ferric citrate (added after inoculation and sterilized by filtration). Satisfactory growth was obtained without the addition of micronutrients provided that Melbourne tap water was used as the solvent; this provided an additional 40 p.p.m. total solids, including 5·6 p.p.m. sodium and 10 p.p.m. chloride. The inoculated 250-ml flasks were gently shaken over a bank of Daylight fluorescent lamps which gave a light intensity of 800 f.c. A water-screen acted as a heat shield above the lamps and the temperature was maintained at 20±1°C. Sterile air, continuously bubbled through the cultures, was the sole carbon source. Samples were usually harvested for experiment or for use as inocula after 8–10 days growth. All *C. vulgaris* cultures used were checked microscopically for bacterial or fungal infection and any infected cultures were discarded.

Gas exchange was measured in a Braun circular Warburg apparatus. Light intensity was varied by rheostat or by covering vessels with black nylon gauze of varying mesh. Light intensities at the base of each vessel were measured by Weston photocell. Respiration measurements were made in a dark room or with appropriate vessels covered with double-thickness black cotton bags; respiratory quotients were measured by the two-vessel direct method with the cells in water or phosphate buffer; values of carbon dioxide output were corrected for retention by the method of Johnson (in Umbreit, Burris, and Stauffer 1964). Warburg buffers as modified by Pratt (1943), or 0·067M phosphate buffers (Na^+ and K^+ salts), pH 6·7, served as the suspension medium. Dry weight determinations (24 hr at 80°C) were usually made on replicate control samples of the cultures used; sometimes it was necessary to use the experimental material itself for dry weight measurements at the end of an experiment. All such samples were centrifuged twice at 500 *g* for 10 min, with an intervening rinse in distilled water. All experiments were at 25±0·02°C unless otherwise specified.

In most experiments the diquat was dissolved in carbonate–bicarbonate buffer (pH 9·1), whereas it has usually been supplied as a herbicide simply in aqueous solution (Mees 1960). However, we found that the rate of blackening of *Vicia faba* leaves was the same whether the diquat was supplied in water, in phosphate buffer, pH 5, or in carbonate–bicarbonate buffer, pH 9·1. Diquat is unstable in the alkaline solutions, slowly turning pale brown over a period of days, owing probably to the opening of the pyridyl ring (Brian *et al.* 1958). Experiments involving alkaline buffers were generally concluded at the end of 5–6 hr.

Solutions of diquat dibromide made up in distilled water were added to the buffers when necessary. The dilution of the buffer (0·4 ml diquat, 4·6 ml buffer) was shown in itself not to affect the rate of photosynthesis or respiration. Diquat dibromide (A.700 $2P_1$, 99–100% pure), 1,1′-trimethylene-2,2′-dipyridylium dibromide, and monuron [*N′*-(4-chlorophenyl)-*NN*-dimethylurea] were kindly supplied by I.C.I.A.N.Z.

Unless otherwise stated, diquat was applied to samples of *C. vulgaris* at a light intensity which was saturating for photosynthesis. Preliminary studies showed that for the densities used (3–8 mg dry wt./5 ml) light saturation was reached between 400 and 650 f.c.

In all the figures oxygen efflux is plotted as a positive value and oxygen influx as a negative value on the same scale.

III. Results

(*a*) *Effect of Diquat at Constant Light Intensity on Gas Exchange*

Diquat, at normal herbicidal concentrations (10^{-5}M–10^{-3}M) applied in carbonate–bicarbonate buffer, has a very rapid and strongly marked effect on oxygen exchange in illuminated mineral-grown *C. vulgaris*. Because the cells ultimately die we call

this the "toxic reaction". The characteristic effects are illustrated in Figure 1. In this experiment the diquat was added to the samples in the low light at the laboratory bench at the time of setting up the manometers. Light at 400 f.c. (saturating) was switched on immediately after temperature equilibration had been reached in the water-bath. In the control sample (no diquat) the net oxygen efflux in the light (apparent photosynthesis) exceeded the oxygen influx in the dark by a factor of 10. In diquat-treated samples the apparent photosynthesis rate fell rapidly to zero, the rate of inhibition being a positive function of the diquat concentration.

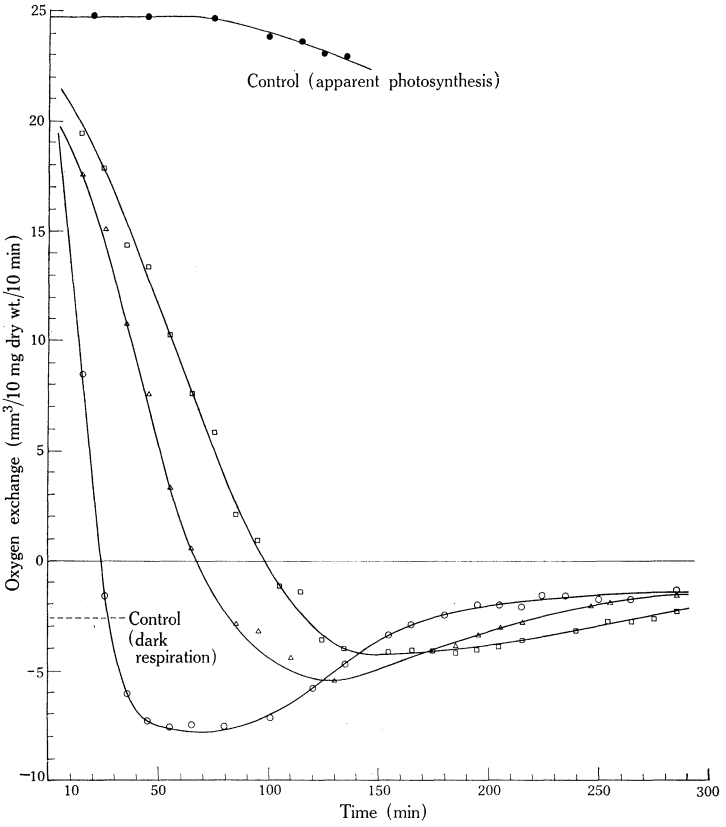


Fig. 1.—Effects of diquat at concentrations of 10^{-3}M(○), 10^{-4}M(□), and 2×10^{-4}M(△) on oxygen exchange in *C. vulgaris* in continuous light (400 f.c.). Gas phase air; 25°C; Warburg buffer No. 9, 91 μmoles/l CO_2. In Figures 1–9, positive values indicate oxygen efflux, negative values oxygen influx.

Following complete inhibition of oxygen output, the diquat-treated cells, still in the light, absorbed oxygen, and high rates of oxygen uptake were quickly reached. The rate at the peak of oxygen absorption was greatest for the highest diquat concentration, being equivalent to one-third of the apparent photosynthesis or approximately three times the control respiration; the peak was reached the more quickly the higher the diquat concentration. At all three diquat concentrations the

rates of oxygen uptake then declined, reaching at 300 min values lower than that of the control dark respiration.

This pattern of response to diquat in light is highly consistent and curves similar to those of Figure 1 have been obtained in over 25 experiments, mainly with 10^{-3}M diquat. For any one concentration of diquat, at constant and saturating light intensity, the rate of inhibition of apparent photosynthesis and the height of the subsequent peak of oxygen influx vary somewhat with the age and condition of the algal culture. For replicate samples of one culture, closely similar curves are obtained. At diquat concentrations greater than 2×10^{-4}M the apparent photosynthesis is fully inhibited within 1 hr; this is the time required for concentrations of this order to cause extensive blackening in the illuminated leaves of *V. faba*.

In all our experiments it was observed that, when the peak of oxygen influx had been passed, the cells continued to absorb oxygen *in the light* at rates somewhat below that of the dark respiration rate of control samples (e.g. Fig. 1). We shall, however, present evidence to show that such cells are dead; the relatively small persisting oxygen uptake ceases when the light is turned off—it is apparently a slow photoxidation quite separate from respiration.

Experiments with potassium bromide (10^{-3}M) showed that the bromide ion itself had no significant effects on gas exchange. Moreover, 1,1′-trimethylene-2,2′-dipyridylium dibromide, applied at concentrations ranging from 10^{-3} to 10^{-4}M, gave only slight inhibition of oxygen production in *C. vulgaris*. According to Homer, Mees, and Tomlinson (1960) this compound, with $E_0 = -548$ mV and possessing non-coplanar pyridyl rings, is not effective as a herbicide.

(*b*) *Effect of Diquat on Gas Exchange at Variable Light Intensity*

Mees (1960) showed that the rate of blackening of the leaves of *V. faba* treated with diquat was a positive function of light intensity. This is to be expected on the hypothesis that the toxic substance is a product of the light reaction. A similar effect of light intensity on the reaction of gas exchange to diquat appears to be established in the results plotted in Figure 2: the actual rate of fall of apparent photosynthesis is more rapid at the higher light intensities. However, the time taken for the complete inhibition of apparent photosynthesis was, if anything (this experiment was not replicated), slightly less for the lowest light intensity. The time taken to reach maximum oxygen influx is approximately the same (100 min) for all light intensities. It does appear, however, that the total oxygen uptake following inhibition of apparent photosynthesis increases with decreasing light intensity.

(*c*) *Effect of Diquat on Pre-illuminated Cells*

We have shown that pre-illumination has marked effects on the reaction of darkened *C. vulgaris* to diquat, probably because of the production of additional photosynthate (Stokes and Turner, unpublished data). We therefore tested the effects of pre-illumination on the effects of diquat supplied in the light. Comparable samples of *C. vulgaris* cells, taken from storage in darkness, were given 10 min (three replicates) and 170 min (two replicates) of light at an intensity of 700 f.c. Diquat at 10^{-4}M was added (without change of light intensity) to all five samples,

which were initially evolving oxygen at closely similar rates. It was found that the time taken for diquat to inhibit apparent photosynthesis completely was independent of the duration of exposure to light (10–170 min) prior to diquat addition.

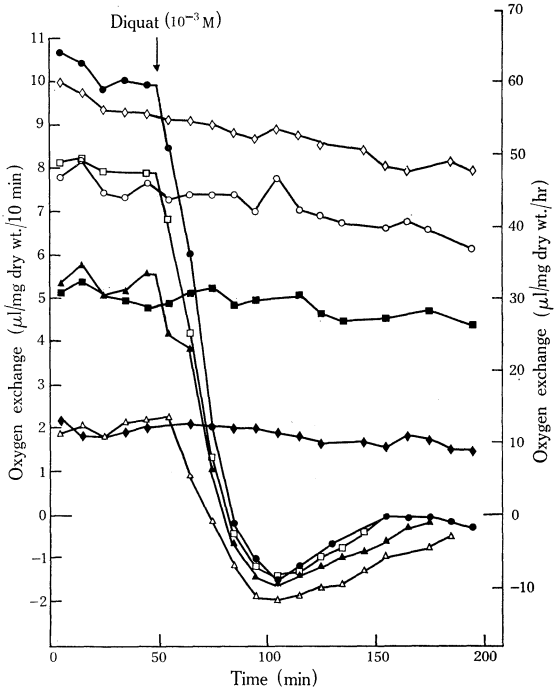


Fig. 2.—Effects of 10^{-3}M diquat on oxygen exchange in *C. vulgaris* at light intensities (f.c.) of: 60(△), 170(▲), 350(□), and 540(●). Oxygen-exchange curves in the absence of diquat are also shown for light intensities (f.c.) of 60(♦), 170(■), 350(○), and 540(◊). Gas phase air; Warburg buffer No. 9, 91 μmoles/l CO_2; 25°C.

(*d*) *Effect of Oxygen Concentration on Diquat Treatments*

Mees (1960) has shown that leaves of *V. faba* treated with diquat (10^{-3}M) in the dark do not subsequently blacken when illuminated if they are injected with water or in other ways deprived of free oxygen. Some of the evidence indicated that it was the herbicidal activity, not the polyphenolase reaction, that was inhibited by the lack of oxygen. He concluded that the free radicals formed from diquat in the light initiate chain reactions in the presence of oxygen gas which end in the degradation of the cytoplasmic components.

We find that the rate of inhibition by diquat of apparent photosynthesis of *C. vulgaris* is also a function of external oxygen concentration; high oxygen concentration not only increases the rate of fall of apparent photosynthesis, it also shortens the time required for the attainment of the peak of oxygen uptake and modifies the height of this peak. Figure 3 illustrates this for diquat at 2×10^{-4}M, all samples receiving the same light intensity. For control samples (without diquat) there was the normal "Warburg effect" of high oxygen concentration (Turner and Brittain 1962), i.e. a 20% inhibition of apparent photosynthesis. At all three oxygen concentrations, addition of diquat gave results which followed the normal toxic

curve for oxygen exchange and the final level of oxygen uptake, reached after 16 hr, was at about half the rate of the dark respiration. Generally it seems that increasing

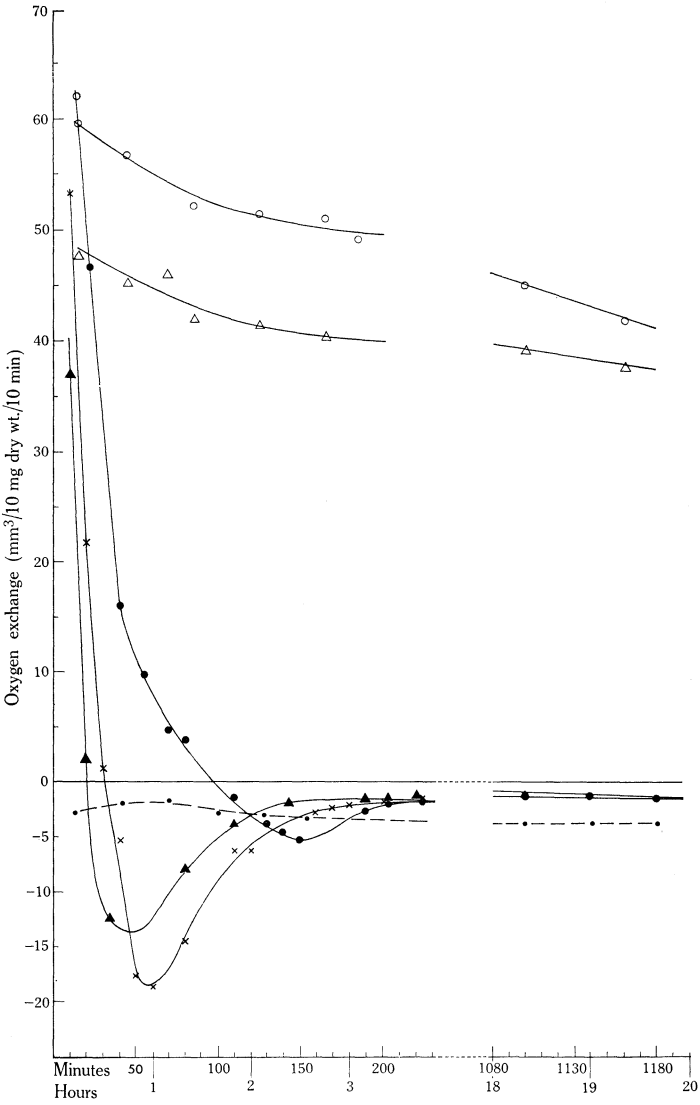


Fig. 3.—Effects of 2×10^{-4}M diquat on oxygen exchange in *C. vulgaris* at oxygen concentrations of 100%(▲), 21%(×), and 1%(●). Control curves (no diquat) at oxygen concentrations of 100%(△), and 21 and 1%(○) are also shown. Warburg buffer No. 9, 91 μmoles/l CO_2; 25°C. ● – – – ● Dark oxygen uptake.

oxygen concentration is equivalent to increasing the applied concentration of diquat in air; however, in the example illustrated the peak in oxygen uptake was smaller in oxygen than in air.

(*e*) *Effect of Monuron*

The substituted ureas diuron and monuron inhibit the oxygen-evolving system in photosynthesis without affecting the carboxylation pathway. They are believed to act by blocking the transfer of electrons from OH^- to the illuminated chlorophyll (Wessels and van der Veen 1956; Bishop 1958; van Overbeek 1962). Monuron was used by Mees (1960) and by Funderburk and Lawrence (1964) in combination with diquat as a possible means of elucidating the mode of action of diquat.

In our experiments with *C. vulgaris* the effects of monuron on gas exchange differed markedly from those caused by diquat. At 10^{-5}M monuron completely inhibited oxygen output but had no effect on the respiration surviving in the light; it did not cause a marked but temporary acceleration of oxygen uptake following inhibition of photosynthesis, as did diquat. At concentrations lower than 10^{-5}M, monuron rapidly reduced the rate of oxygen output to values depending on the monuron concentration; but the surviving apparent photosynthesis then continued at a steady rate. In contrast, diquat causes a progressive inhibition of apparent photosynthesis at all concentrations (Figs. 1 and 4).

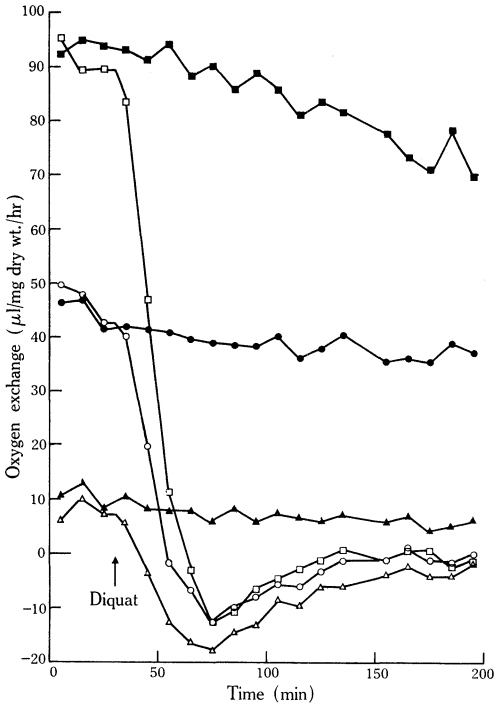


Fig. 4.—Effects of adding monuron and diquat either separately or in combination on oxygen exchange in *C. vulgaris*. Monuron added 20 min before the first reading; diquat added as indicated. ■ Control. □ 10^{-3}M diquat. ● 5×10^{-7}M monuron. ▲ 5×10^{-6}M monuron. ○ 10^{-3}M diquat $+5\times10^{-7}$M monuron. △ 10^{-3}M diquat $+5\times10^{-6}$M monuron. Gas phase air; light intensity 700 f.c.; 25°C; Warburg buffer No. 9, 91 μmoles/l. CO_2.

When diquat was added to *C. vulgaris* whose apparent photosynthesis had already been diminished by monuron, the surviving apparent photosynthesis was inhibited and a normal toxic curve resulted (Fig. 4). The effect was closely similar to that given by lowering the light intensity before adding diquat (Fig. 2). Here again, although the actual rate of fall to zero apparent photosynthesis or to maximum

oxygen influx is decreased by the addition of monuron, the time taken to reach the zero level is clearly reduced in the presence of monuron. The time taken to reach maximum oxygen influx is not influenced by the presence of monuron.

Additional experiments showed that when real photosynthesis had been completely inhibited by monuron (10^{-5}M), the addition of diquat (10^{-3}M) in the light still caused a marked increase in the oxygen uptake; this might be taken to imply that the substance involved in the toxic reaction was still produced; but this conclusion is not valid because, as we shall show in a later paper, diquat added in complete darkness to *C. vulgaris* also causes a similar rapid increase in the rate of respiration. Whether the complete inhibition of real photosynthesis by monuron prevents the toxic action of diquat on *C. vulgaris* cannot be determined by a study of the effect on chlorophyll bleaching, because, according to van Overbeek (1962), the blocking of electron flow by monuron itself leads to photoxidative bleaching.

(*f*) *Effect of Temperature*

If the diquat concentration and light intensity are kept constant, raising the temperature has an effect similar to that caused by increasing the diquat or the oxygen concentration; it increases the rate of inhibition of apparent photosynthesis, shortens the time taken to reach the peak of oxygen uptake, and increases the extent of this uptake (Fig. 5). The interpretation of some of these results is difficult because,

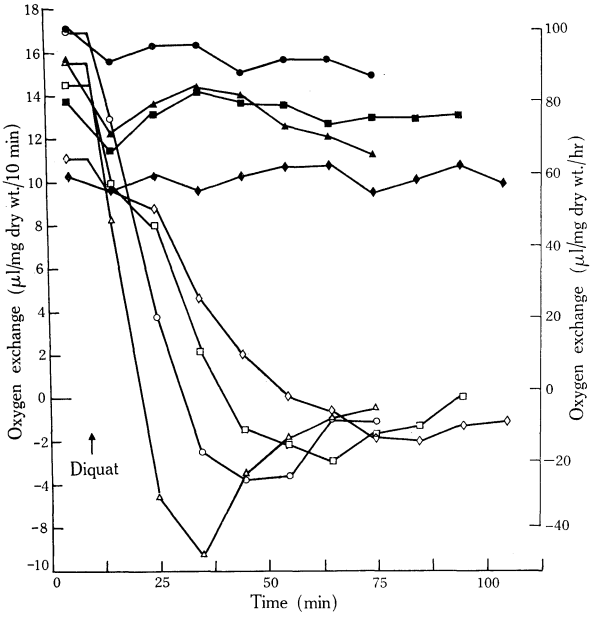


Fig. 5.—Effects of 10^{-3}M diquat on oxygen exchange in *C. vulgaris* in continuous light (260 f.c.) at temperatures of 20°C(◊), 25°C(□), 30°C(○), and 35°C(△). Corresponding control curves for temperatures of 20°C(♦), 25°C(■), 30°C(●), and 35°C(▲) are also shown. Each point is the mean of two samples. Gas phase air; Warburg buffer No. 9, 91 μmoles/l CO_2.

under the conditions used, increased temperature (from 20 to 30°C) also increased the rate of apparent photosynthesis in the controls, in spite of the choice of a non-saturating light intensity. However, at 35°C this rate in the controls was actually less than that at 30°C, yet the temperature effects on the form of the toxic curve

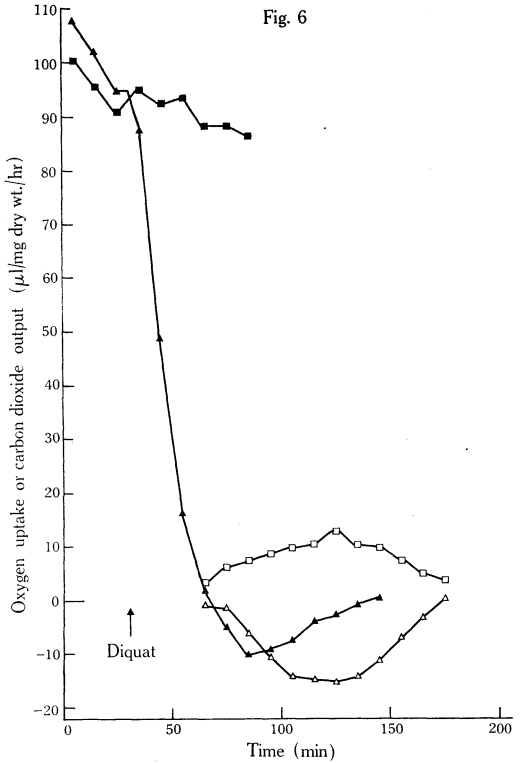
were still as described above. These results were not unexpected; even if the production of reduced diquat is purely photochemical there must be chemical reactions involved in the toxic effect—e.g. the reoxidation of the reduced diquat, the formation of hydrogen peroxide, and the effects of this on the metabolism.

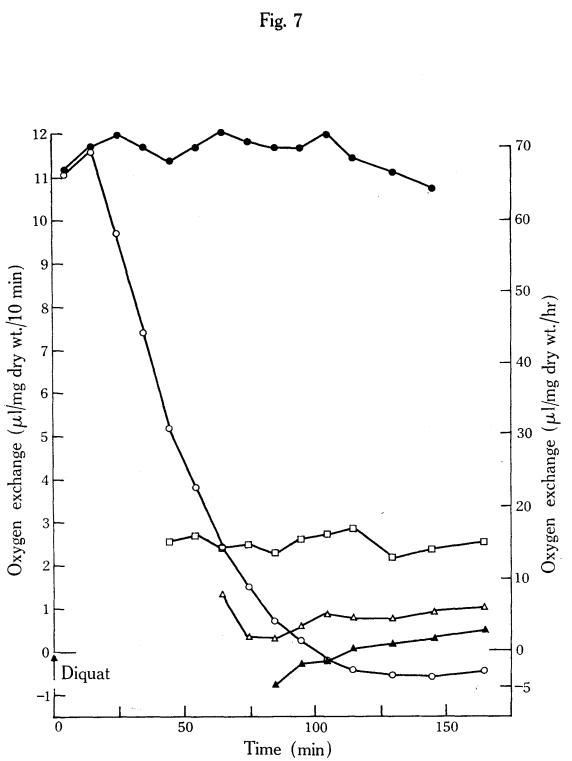
(*g*) *The Toxic Effect in Phosphate Buffer, pH 6·7*

The herbicidal effects of diquat are said not to require the presence of free carbon dioxide (Mees 1960). The following experiment was planned to check this and also to obtain a measure of the respiratory quotient of the process which succeeds the inhibition of photosynthesis (Fig. 6). Replicate samples of *C. vulgaris* were mounted in either Warburg buffer (No. 9), pH 9·1, or phosphate buffer, pH 6·7, with and without KOH in the central well. All were illuminated continuously and diquat added at 10^{-3}M, except for a control in Warburg buffer. Measurements of the gas exchange in the phosphate were made after the apparent photosynthesis had come to zero in the parallel experiment in Warburg buffer. It appears clear that the later part of the toxic curve (a temporary and large increase in oxygen uptake) is still obtained, which must mean that the lack of free carbon dioxide in the phosphate buffer has not prevented damage to the photosynthetic apparatus, although it may have delayed it (note the lateral shift of the oxygen uptake curve in phosphate). The fact that the curve for carbon dioxide output in the phosphate buffer does, to an extent, mirror the curve for oxygen exchange also indicates that some at least of this oxygen uptake is accompanied by the breakdown of a carbon substrate. The respiratory quotient of normal untreated and darkened *C. vulgaris* can vary over the range 0·8–1·5, according to the method of cultivation of the cells and their pretreatment. The respiratory quotient of the process measured in phosphate buffer in the light (after treatment with diquat) reached a minimum of 0·7 at time 125 min (Fig. 6). We have shown (Stokes and Turner, unpublished data) that diquat stimulates respiration when applied in the dark, and it now appears that the toxic curve in the light also involves some stimulation of a respiratory process.

(*h*) *Irreversibility of the Toxic Effect*

Diquat at 10^{-4}–5×10^{-5}M was applied to *C. vulgaris* samples in the light. As soon as oxygen output had ceased, the cells were centrifuged, washed quickly in diquat-free buffer, recentrifuged, and immediately resuspended in fresh buffer. There was no recovery of the apparent photosynthesis; this, however, could have been due to the failure of the washing to remove diquat (and toxin) from the inhibited cells. The following experiments were therefore carried out. Replicate samples of *C. vulgaris* were illuminated in the presence of diquat (10^{-4}M); in the treated control sample, oxygen output ceased at 100 min. Samples were removed from the illuminated water-bath at 20, 40, and 60 min from the time of addition of diquat. Each sample (5-ml suspension) was diluted to 40 ml with distilled water, centrifuged, the cells resuspended in 40 ml water, again centrifuged, and resuspended in buffer for gas exchange measurements. The total time of washing and centrifugation in each case was 20 min. As shown in Figure 7, the inhibition in these samples was not reversible; the oxygen output of partially inhibited samples remained steady in the light—there





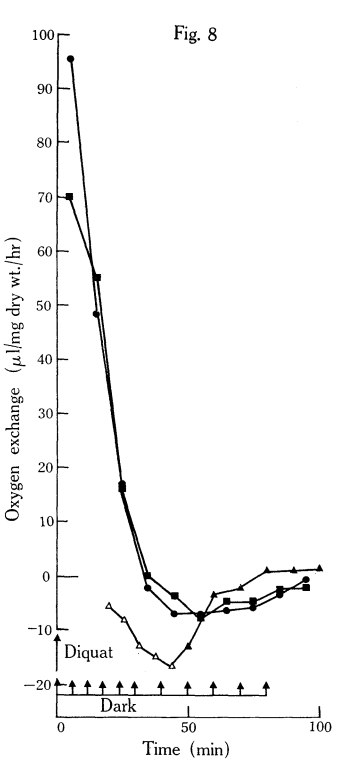


Fig. 6.—Effects of 10^{-3}M diquat on oxygen exchange (▲), oxygen uptake (△), and carbon dioxide output (□) in *C. vulgaris* in continuous light (700 f.c.). Gas phase air; 25°C. Warburg buffer No. 9, 91 μmoles/l CO_2 for oxygen-exchange experiments; phosphate buffer, pH 6·7, for oxygen uptake and carbon dioxide output experiments. ■ Control for oxygen-exchange experiments. Each point, except the controls, is the mean of two samples.

Fig. 7.—Effects of washing after diquat treatment (10^{-4}M) on oxygen exchange in *C. vulgaris* in continuous light (700 f.c.). Gas phase air; Warburg buffer No. 9, 91 μmoles/l CO_2; 25°C. ● Control (no diquat, no washing). ○ Diquat treatment (no washing). □, △, ▲ Cells removed and washed after treatment with diquat for 20, 40, and 60 min respectively.

Fig. 8.—Effects of 10^{-3}M diquat on oxygen exchange (●, ■) in *C. vulgaris* in continuous light (1400 f.c.) and of 10^{-3}M diquat on rates of oxygen uptake in darkness (△, ▲), 20 min after darkening for two successive experiments. ●, △ Experiment 1. ■, ▲ Experiment 2. The arrows in the lower horizontal row indicate times of darkening for samples given diquat in the light at time zero. Gas phase air; 25°C; Warburg buffer No. 9, 91 μmoles/l CO_2.

was neither recovery to the initial rate, nor a fall such as one would expect if active diquat remained in the washed cells.

This type of experiment was repeated with diquat at a concentration of 5×10^{-4}M; here the apparent photosynthesis was reduced to zero in only 55 min; replicates washed after only 20 min contact with diquat in the light showed zero oxygen output and no subsequent recovery of photosynthesis when replaced in the bath at 40 min.

From *C. vulgaris* cells treated with 10^{-3}M diquat in the light, and then washed (as above), we could not extract any diquat with 70% ethanol; if, however, such cells were treated with 2·5 ml 0·1 N HCl for 5 min, appreciable amounts of diquat (0·49 μmole/5-ml cell sample) could be detected in the bathing fluid by measuring the absorption of diquat at 310 nm. We suggest that this represents diquat absorbed (and immobilized) on cell material: it has been reported that diquat residues in potato tubers are strongly adsorbed on to starch (Calderbank, unpublished data).

Thus it may be that the concentration of active soluble diquat present in a cell is very small, and that removal of cells from the bathing solution, coupled with adsorption within the cell, is sufficient to prevent further inhibition. The lack of recovery of apparent photosynthesis in such washed cells supports the concept of an irreversible inhibition.

In the (non-synchronous) cultures used in most of our experiments we cannot tell whether a 50% inhibition of apparent photosynthesis represents 50% inhibition of the photosynthesis in each cell or complete inhibition in some and partial inhibition in others. For the purpose of this section it seems immaterial: those cells partially or completely inhibited do not show recovery after washing. In later experiments we found that synchronized *C. vulgaris* cultures gave a typical toxic reaction when treated in the light with diquat (Stokes, Turner, and Markus 1970).

IV. Experimental Analysis of the Toxic Reaction

It is established that, in *C. vulgaris* cells treated with diquat in the light, the inhibition of apparent photosynthesis is followed by a large, but temporary, increase in oxygen uptake; the gas quotient (CO_2/O_2) over the whole period of the toxic reaction has not yet been established, but there is some evidence that a stimulation of carbon dioxide output is also involved [see Section III(*g*)]. We have shown (Stokes and Turner, unpublished data) that treatment of *C. vulgaris* with diquat in darkness markedly accelerates respiration (but without subsequent toxic effects). We therefore suspected that the acceleration of a respiratory process might begin from the time of addition of diquat in the light, being masked at first by the surviving photosynthesis. This would imply large differences (as measured by oxygen exchange) in diquat-treated cells. Further analysis of the situation was made as follows:

A series of manometric experiments was carried out, each involving 8–12 replicate samples of *C. vulgaris* in Warburg buffer No. 9. In each vessel the sample of *C. vulgaris* was treated with diquat (10^{-3}M) in the light (1400 f.c.) and the fall in apparent photosynthesis measured. At various intervals from the start of the experiment individual vessels were darkened without removal from the water-bath. In each of these the oxygen exchange rates were continually measured in darkness.

The object was to obtain a measurement of the effect of diquat (applied in the light) on the "dark metabolism", rather than to attempt to *estimate* any light-stimulated respiration or photoxidation which would cease almost immediately on transfer to darkness. We therefore made no attempt to extrapolate the dark curves to zero time in darkness—which would, in any case, have required a different experimental technique. Instead, we used as the estimate of dark metabolism the rate of oxygen uptake measured at 20 min after transfer to darkness. This period was chosen as the minimum time to allow gas equilibration after the change in conditions, and the maximum time possible to prevent the measurement from being affected by the changes in rate occurring during the dark period. The results of two successive experiments (with one batch of *C. vulgaris*) are plotted in Figure 8.

It is clear that when diquat is applied in the light there is a marked stimulation of the dark metabolism (oxygen uptake) in the early stages of the effect; that the extent of this stimulation varies with time and that the oxygen uptake measured *in the light* during the later stages of the toxic reaction is in part due to a form of photoxidation.

The results of several such experiments are summarized in the generalized curves of Figure 9 and an interpretation is now attempted.

(*a*) *Scheme A*

The continuous line ABXCD (Fig. 9) represents the actual oxygen exchange rate with time for *C. vulgaris* treated with diquat under continuous illumination (i.e. it is the typical curve of the toxic reaction). Line MM′ represents the dark respiration of comparable untreated samples.

Line MNXPQ is the curve for stimulated dark metabolism (dark oxygen uptake) in the presence of diquat, estimated by transferring samples to darkness at various times and measuring oxygen uptake at 20 min from zero time of darkness.

The curve so obtained takes an unexpected course. In the first place it is different from the form of the curve for dark respiration stimulated by diquat in darkness (e.g. MN′). Secondly, it does not follow the course MNCRD. Instead it crosses the "toxic curve" at X and reaches zero at P. From this point of time onwards, gas exchange is no longer measurable *in the dark* (line PQ) and we assume that the cells are killed at time P.

Because, at point X, the same rate of oxygen uptake is given in light and darkness, on scheme A we make two assumptions:

(1) That real photosynthesis is represented by the line A′B′Q; it falls to zero at B′. Points on this curve are given by:

Real photosynthesis = apparent photosynthesis+dark oxygen uptake.

Apparent photosynthesis falls as soon as the diquat is applied, and there is hardly any lag phase in the inhibition of real photosynthesis.

(2) That a light-dependent oxygen uptake, called for convenience "photoxidation", begins at the point of time B′. On the axis 00 as baseline the time course of this process is given by the line B′RD. The rate of this photoxidation at any time is also represented on the graph as the distance between the lines XPQ and XCRD.

Therefore, the slow but continuous oxygen uptake in the last phase of the toxic reaction is (on any hypothesis) not a surviving dark respiration but a light-stimulated oxygen uptake. If scheme A is followed, this process begins as soon as,

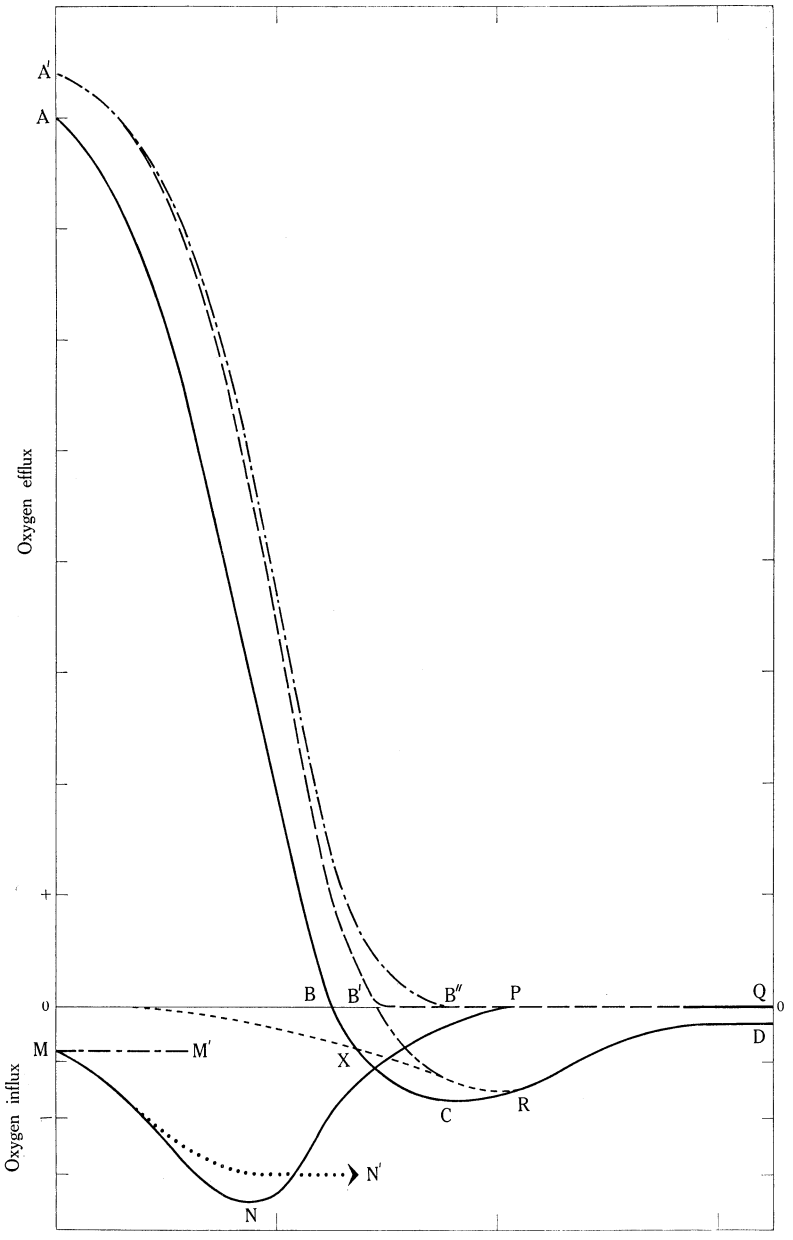


Fig. 9.—Analysis of the toxic curve. Measured and estimated rates of oxygen exchange in light and darkness, after the addition of diquat, plotted against time. For explanation, see text.

doxin requirement for the reduction of diquat and that when diquat was reduced by the photosynthetic mechanism there was no reduction of $NADP^+$. This is consistent with the evidence of Black (1966) that diquat is reduced prior to the natural oxidants of the Hill reaction and probably at the same site as ferredoxin.

When reduced ferredoxin is oxidized by molecular oxygen [equation (2)], water is formed, but the reoxidation of reduced diquat resembles the Mehler reaction, and a product is hydrogen peroxide. Davenport (1963) observed the spectrum of a metmyoglobin–peroxide compound on the illumination of plastids provided with diquat and metmyoglobin.

It therefore appears that the following equations may represent the reactions of diquat in illuminated *C. vulgaris* cells:

$$\text{Diquat} + \epsilon \xrightarrow{h\nu} \text{reduced diquat} \tag{4}$$

$$\text{Reduced diquat} + O_2 \rightarrow O_2^- + \text{diquat} \tag{5}$$

$$O_2^- + H^+ \rightarrow HO_2 \tag{6}$$

$$HO_2 + \text{reduced diquat} \rightarrow HO_2^- + \text{diquat} \tag{7}$$

$$HO_2^- + H^+ \rightarrow H_2O_2 \tag{8}$$

The sum of equations (5)–(8) is:

$$2 \text{ reduced diquat} + O_2 + 2H^+ \rightarrow 2 \text{ diquat} + H_2O_2 \tag{9}$$

In equation (9) one molecule of oxygen is consumed for each molecule of hydrogen peroxide produced. If the $2H^+$ are supplied by the photochemical process, i.e. from water, and if the hydrogen peroxide is not broken down by catalase, then the result is the net uptake of a half oxygen molecule for every two diquat molecules reduced. If the hydrogen peroxide is destroyed by catalase, the net result would be zero oxygen exchange. From the work on plastids it would also follow that if diquat substituted for ferredoxin in the electron acceptance, the reduction of $NADP^+$ would not occur and carbon fixation would be inhibited. Diquat then should inhibit the gas exchange associated with photosynthesis and, provided that hydrogen peroxide is not broken down in subsequent reactions, it could mean that the diquat-treated cells should absorb some oxygen in addition to that used in respiration. If oxygen-linked non-cyclic photophosphorylation continued, this process (oxygen uptake) would continue to accompany the production of hydrogen peroxide.

Black and Myers (1966) argue that because catalase is widely distributed in green cells, then hydrogen peroxide is not the herbicidal substance; they suggested that dipyridyls deprive the plant of reduced pyridine nucleotides and thus cause death. However, it seems unlikely to us that this could explain the very rapid blackening, wilting, and bleaching of *V. faba* plants treated with diquat (Homer, Mees, and Tomlinson 1960). Moreover, we still know little about the localization and role of catalase in plastids. According to Trebst and Eck (1961) catalase destroys hydrogen peroxide produced from the oxidation of vitamin K in broken plastids, but Davenport's (1963) work, referred to above, points to a different conclusion.

Therefore, the rapid inhibition of apparent photosynthesis by diquat in *C. vulgaris* may, in the first place, be due to a by-pass of the electron chain when diquat substitutes for ferredoxin. This process, however, should be reversible and we have shown in Section III(*h*) that the inhibition is irreversible. The greatly increased oxygen uptake that follows (and accompanies) inhibition of apparent photosynthesis could, in part, be due to the continuous oxidation of reduced diquat, but the evidence [see Section III(*g*)] is in favour of its being accompanied by increased carbon dioxide production.

If hydrogen peroxide or some other toxic substance is produced in the chloroplast this toxin could then destroy cell membranes and cause the death of the cell. The final inhibition of respiration might well be preceded by a temporary increase of gas exchange as shown in Figure 9.

While the results described in Sections III(*f*), III(*g*), and III(*h*) are not at variance with the hypothesis of Homer, Mees, and Tomlinson (1960), they offer no direct support for it. As regards the results described in Section III(*d*), the form of the toxic curve is compounded of real photosynthesis, dark oxygen uptake, and photoxidation (see Section IV), and all three processes may be affected by oxygen concentration. A reasonable explanation of the effect of oxygen upon the net oxygen exchange is that proposed by Mees—that high oxygen concentration accelerates the oxidation of reduced diquat, leading to greater production of the toxic hydrogen peroxide. A check of this hypothesis would require full analysis of the toxic curve at various oxygen concentrations, and this we have not attempted in view of the experimental difficulties.

According to the hypothesis of Mees (1960), the reduction of diquat and the production of the toxin is dependent on the activity of the photochemical process. Thus the rate of inhibition of apparent photosynthesis at high light intensity should be greater than that at low light intensity or in the presence of monuron. This is shown to be so, in terms of the actual rates of apparent photosynthesis. To express the results in another way, the time required to bring apparent photosynthesis to zero is approximately the same at high and low light (and in the presence of monuron), yet there are more photosynthetic sites to inhibit at high light, and hence more toxin must be produced under these conditions. The results are not, however, a a sensitive test of the Mees hypothesis because we do not know the extent to which the earliest stages of inhibition are due to a by-pass of the electron chain rather than to the destruction of the photosynthetic apparatus by a toxin such as hydrogen peroxide.

We conclude that the effects of diquat, applied to *C. vulgaris* in the light, are:

(1) to cause irreversible inhibition of real photosynthesis and later of respiration. We favour the view that the inhibition and the death of the cells are caused by a toxic substance (such as hydrogen peroxide) which destroys cell membranes. Evidence for this is given in Part II of this series (Stokes, Turner, and Markus 1970);

(2) to cause, during illumination, a marked but temporary increase in oxygen uptake which reaches a maximum when real photosynthesis has been inhibited by less than 20%;

(3) to stimulate some form of photoxidation. Much of the oxygen uptake measurable after the extinction of apparent photosynthesis is due to this, and the process continues for many hours at a low rate during the bleaching of the chlorophyll. The possibility exists that what we have called photoxidation is partly the oxidation of reduced diquat; if this does not accumulate in the cell its production from diquat itself (and hence its subsequent oxidation) would be light-dependent. While, however, some of the oxygen uptake of the accelerated respiration and of the photoxidation may possibly be accounted for in this way, there is evidence that a part of the enhanced oxygen uptake of the toxic reaction is associated with a release of carbon dioxide.

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