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

III.* DARK METABOLISM: EFFECTS ON RESPIRATION RATE AND THE PATH OF CARBON

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Abstract

When *C. vulgaris* is treated in complete darkness with diquat at concentrations which are toxic in the light, there is a rapid and large stimulation of oxygen uptake with no significant alteration in the R.Q. If the *C. vulgaris* has been darkened for some hours before the addition of diquat there is a two- to threefold stimulation of the rate of respiration, which subsequently remains constant or falls only slowly for up to 500 min. With pre-illuminated or glucose-treated *C. vulgaris*, diquat causes a much greater stimulation of dark respiration, rapidly declining to near the control rate. Tracer studies with $^{14}CO_2$ and [^{14}C]glucose show that diquat when added in darkness to *C. vulgaris* accelerates the breakdown of starch and the loss of ^{14}C from most intermediate substances of carbohydrate metabolism. The exception to this is the rapid accumulation of ^{14}C counts in citric acid. These results are discussed and a mechanism for the action of diquat in darkness is proposed.

I. INTRODUCTION

The physiological effects of diquat (1,1′-ethylene-2,2′-bipyridylium dibromide) applied to *Chlorella vulgaris* (211–11h) in the light have been reported in the first two papers of this series (Turner, Stokes, and Gilmore 1970; Stokes, Turner, and Markus 1970). In this paper we show that diquat also has marked physiological effects when applied to this organism in complete darkness.

II. MATERIALS AND METHODS

(*a*) *Culture Methods and Manometric Techniques*

Unless otherwise stated the *C. vulgaris* strain was grown autotrophically by the methods described by Turner, Stokes, and Gilmore (1970). In some specified experiments the same culture solution was used but with the addition of 2% (w/v) glucose. These cultures were plugged with cotton wool stoppers and shaken but not otherwise aerated: growth was allowed to proceed for 6–7 days at 25°C under a bank of Daylight fluorescent tubes at a light intensity of 100 f.c.

(*b*) *Radiochemical Procedures*

Radiochemicals were obtained from the Radiochemical Centre, England, through the Australian Atomic Energy Commission. Glucose was supplied as the uniformly labelled D-isomer of specific activity 3·9 mCi/mmole. The other radiocarbon compound used, sodium carbonate, had a specific activity of 26·8 mCi/mmole.

All chemicals used in the extraction procedure and subsequent chromatography were supplied as the Analar reagent grade.

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(*c*) *Experiment A*

C. vulgaris, 6·9 mg dry weight per 5 ml, was pre-illuminated at 400 f.c. at 23±1°C for 50 min. During this pre-illumination period the *C. vulgaris* was suspended in a dilute Warburg buffer (9 ml 0·004M K_2CO_3 and 51 ml 0·02M $KHCO_3$) in a flat Perspex chamber, which was placed horizontally over the light source (Daylight fluorescent tubes). A water-bath between the light and the chamber acted as a heat shield. After 20 min of pre-illumination, 60 μCi of labelled sodium carbonate was injected into the *C. vulgaris* suspension and labelling was allowed to proceed for the remaining 30 min of the pre-illumination period. During this time the *C. vulgaris* was maintained in an even suspension by gently agitating the chamber. The *C. vulgaris* was then centrifuged at 500 *g* for 10 min, the supernatant discarded, and the cells resuspended in 60 ml of $6·7 \times 10^{-5}$M phosphate buffer, pH 6·7. This suspension was divided into two batches, each of 30 ml, which were darkened and agitated gently on a wrist-shaking machine. At "zero" time (20 min after removal from the light) 5 ml of *C. vulgaris* was removed from one batch, the control treatment, for extraction, and at the same time diquat (final concentration 3×10^{-4}M) was added to the other batch of *C. vulgaris*. Further samples of 5 ml were removed from each treatment at 50, 100, 150, and 200 min. Gas-exchange measurements were carried out in conjunction with these experiments, using the Warburg technique.

(*d*) *Experiment B*

C. vulgaris (9·7 mg dry weight per 5 ml) was first pre-illuminated for 30 min at 950 f.c. at 25°C in Warburg buffer No. 9. The *C. vulgaris* was then centrifuged at 500 *g* for 10 min, rinsed in distilled water, and resuspended in 80 ml of $6·7 \times 10^{-5}$M phosphate buffer. This suspension was divided into two batches each of 40 ml, darkened, and shaken at 25±1°C. Diquat (final concentration 3×10^{-4}M) was then added to one batch, the other remaining untreated as a control. After a further 10 min 4 μCi of [^{14}C]glucose was added to both treatments. At 10, 25, 50, 100, 150, and 200 min after adding [^{14}C]glucose, a 5-ml sample was taken from each treatment for extraction and analysis.

(*e*) *Experiment C*

C. vulgaris was not pre-illuminated but equilibrated in the dark at 20°C in $6·7 \times 10^{-5}$M phosphate buffer, pH 6·7. The path of carbon was then followed, in the dark, in the presence and absence of diquat. *C. vulgaris*, 10·5 mg dry weight per 5 ml, was divided into two 40-ml batches and shaken in the dark. One batch remained untreated as a control, and diquat (final concentration 3×10^{-4}M) was added to the other. After 10 min 4 μCi of [^{14}C]glucose was added to both treatments and at 10, 25, 50, 100, 150, and 200 min after adding [^{14}C]glucose a 5-ml sample was removed from each treatment for extraction and analysis.

(*f*) *Extraction Procedure*

The extraction procedure was a modification of that used by Graham and Walker (1962). Each sample of *C. vulgaris* was extracted for 3 min in boiling 80% (v/v) ethanol and allowed to stand for 5 min. After centrifuging at 1300 *g* for 10 min the cells were again extracted in 50 ml of boiling 20% (v/v) ethanol for 1 min and then recentrifuged at 1300 *g* for 10 min. The extraction in 20% ethanol was then repeated. The three volumes of ethanol were bulked and dried *in vacuo* at less than 35°C.

(*g*) *Chromatography*

The dried samples were dissolved in a small volume of 20% (v/v) ethanol. The samples were then chromatographed on Whatman No. 1 paper in the two solvents described by Benson *et al.* (1950)—solvent 1: phenol–water (100 : 39·5 w/v) for 20 hr; solvent 2: n-butanol–glacial acetic acid–water (74:19:50, v/v/v) for 20 hr. The chromatograms were autoradiographed on Ilfex X-ray film for 30 days.

(*h*) *Identification of Radioactive Compounds*

A tentative identification of the radioactive compounds was made by comparing their positions with those in the chromatographic maps given by Benson *et al.* (1950), Bassham and Calvin (1960), and Merrett and Goulding (1967). Amino acids were located by spraying one of the

duplicate chromatograms with ninhydrin [0·15% ninhydrin in 95% ethanol w/v (Lynch and Gillmour 1966)]. Acidic spots were located by spraying with 0·04% bromcresol green in 95% ethanol w/v, adjusted to blue-green with NaOH (Lynch and Gillmour 1966). Free sugars were located by spraying with a benzidine–trichloroacetic acid spray (Bacon and Edelman 1951). Individual sugar phosphates were not measured.

For co-chromatography, radioactive areas were located with a Geiger tube (type EW2C) coupled to a rate-meter. Authentic amino acids were located with ninhydrin; citric acid with the ammonium venadate spray of Buch, Montgomery, and Porter (1952). Co-chromatography of several compounds in solvent 2 (alanine, serine, aspartate, and glutamate) and citric acid in both solvents 1 and 2 showed that the distribution of the compounds on the chromatograms was consistent with the chromatographic maps in the literature.

(*i*) *Analysis of Acid-hydrolysable Polysaccharide*

Acid-hydrolysable polysaccharide was analysed by a method suggested by Graham (personal communication). The cells, after extraction of the alcohol-soluble compounds, were incubated for 2 hr *in vacuo* in 2N HCl at 100°C. The hydrolysate was then dried down *in vacuo* at less than 40°C until the pH of the hydrolysate was near 7. Sugars were purified by ion-exchange (Zeo-Carb 225, H^+ form), dried down at less than 40°C, and made up to 1 ml in water.

(*j*) *Counting Procedure*

(1) *Acid-hydrolysable Polysaccharide.*—The acid-hydrolysable polysaccharide was counted by spotting 0·1 ml on Whatman No. 1 filter paper or Whatman No. 1 GF/A 2·1 cm diameter glass-fibre disks (Davies and Cocking 1966). The paper or the glass disk was then placed flat on the bottom of a vial which contained 5 ml of scintillator—0·4% (w/v) 2,5-diphenyloxazole and 0·1% (w/v) 1,4-bis(5-phenyloxazol-2-yl)benzene in toluene. The vials were then counted in a Packard liquid scintillation spectrometer, series 314A.

(2) *Chromatograms.*—Radioactive areas on the chromatograms were cut into pieces 17 mm square and fitted flat on to the bottom of a vial. This allowed the use of 5 ml of the scintillator fluid and minimized errors due to the geometry of the system (Davies and Cocking 1966). Total radioactivity in each area was obtained by adding values obtained for squares making up the area.

III. Results

(*a*) *Effect of Diquat Treatment in Darkness on Oxygen Uptake*

When mineral-grown *C. vulgaris* samples are darkened for some hours (such cells being usually starch-free) and then treated with diquat in complete darkness, the rate of respiration (as measured by oxygen uptake) is raised to higher levels. The extent of the stimulation is positively correlated with the concentration of diquat and the effects are measurable within the first 10 min of the treatment. The form of the stimulated respiration *v.* time curve is somewhat variable and it will be shown that it is affected by the length of the dark period preceding the start of the experiment. Replicates from one culture, with similar pretreatment, give consistent results.

Representative results for treatments with 10^{-4} and 10^{-3}M diquat are given in Figures 1(*a*) and 2. For these concentrations the stimulation is maintained for at least 500 min, but its extent is generally reduced after 150–250 min. Maximum stimulation, which is two to three times R_D (the control rate of dark respiration), is usually reached within 120 min, and at 500 min rates are still often double those of the control. Similar results are given by 5×10^{-3}M diquat, but here the maximum stimulation is of the order of $5R_D$.

Mees (1960) and Funderburk and Lawrence (1964) also reported stimulation of respiration of bean roots and *Lemna* fronds respectively by diquat in darkness.

Illumination of samples of *C. vulgaris* treated with diquat for 260 min in the dark showed that only those samples receiving low concentrations of diquat ($\sim 10^{-4}$M) retained some limited photosynthetic capacity. Illumination caused a rapid large but

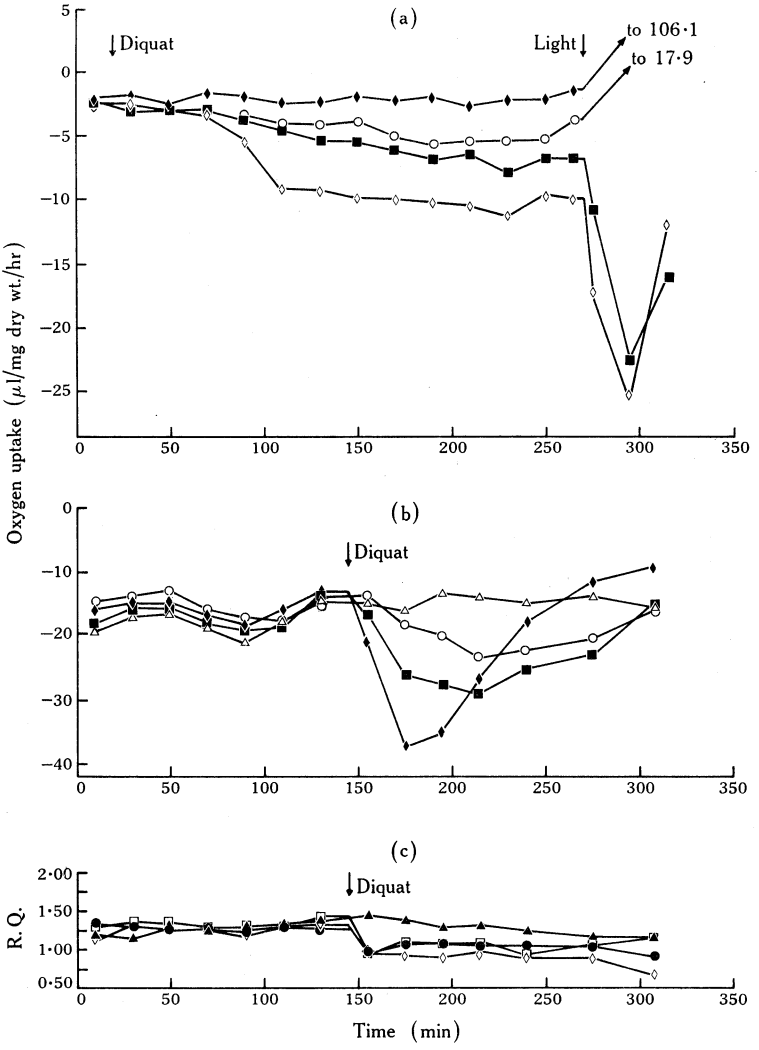

Fig. 1.—(*a*) Effects of 10^{-4}M (○), 10^{-3}M (■), and 5×10^{-3}M (◊) diquat on oxygen uptake by *C. vulgaris* in darkness and subsequent effects of illumination on gas exchange. ♦ Oxygen exchange of control. Temperature 25°C; Warburg No. 9 buffer; light intensity 400 f.c. (*b*) Effect of 10^{-4}M (○), 2×10^{-4}M (■), and 10^{-3}M (♦) diquat in the dark on the oxygen uptake of *C. vulgaris* grown in 2% (w/v) glucose. △ Control. Temperature 25°C; phosphate buffer, pH 6·7. (*c*) Respiratory quotients (R.Q.) for control (▲), 10^{-4}M (●), 2×10^{-4}M (□), and 10^{-3}M (◊) diquat treatments.

temporary increase in oxygen uptake for diquat concentrations of 10^{-3}M or higher [Fig. 1(*a*)]. In longer experiments than that illustrated in this figure the gas exchange in the light did not reach positive values after the temporary acceleration of oxygen

uptake. There is the possibility that diquat at this concentration can damage the photosynthetic apparatus directly even when applied in darkness. We think it more likely that diquat accumulates in the cells during the dark period, and that its reduction in the light is then so rapid that the photosynthetic system is damaged almost instantaneously. The enhanced oxygen uptake in the light could then be, in part, associated with the oxidation of reduced diquat and in part due to photoxidation. Brian (1967) has shown increased uptake of diquat in darkness and Homer, Mees, and Tomlinson (1960) stated that in darkness a "super-lethal" dose of diquat was accumulated and activated by subsequent illumination.

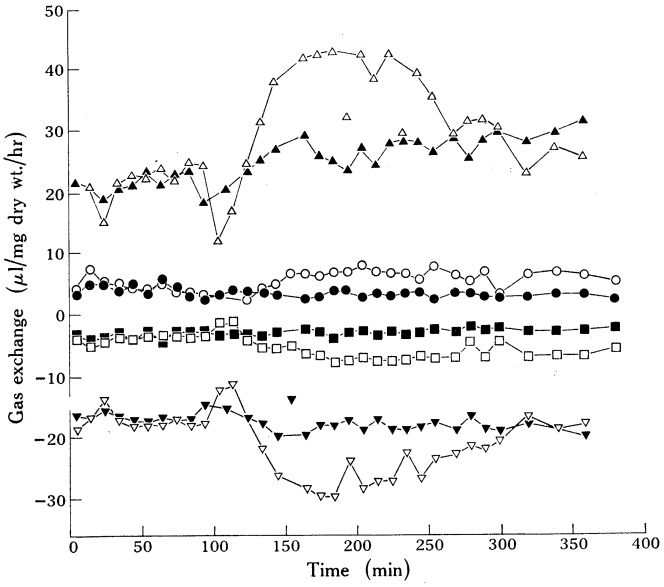

Fig. 2.—Effect of 10^{-3}M diquat on the gas exchange of *C. vulgaris* in the dark. Temperature 25°C; phosphate buffer, pH 6·7. Diquat added at 100 min. ■ Control rate of oxygen uptake. ● Control rate of carbon dioxide output. □ Oxygen uptake with diquat. ○ Carbon dioxide output with diquat. ▼ Oxygen uptake with added 2% glucose. ▲ Carbon dioxide output with added 2% glucose. ▽ Oxygen uptake with glucose and diquat. △ Carbon dioxide output with glucose and diquat.

(*b*) *Effect of Diquat on the Respiratory Quotient of* C. vulgaris

Sorokin and Myers (1956) gave R.Q. values for the endogenous respiration of *C. pyrenoidosa* of between 1·2 and 1·5, irrespective of pH between 4·5 and 6·8. Kandler and Sironval (1959), for the same species, found R.Q. values of 0·89–1·00.

The R.Q. of *C. vulgaris* was also found to vary from 0·8 to 1·4 at pH 6·7 [Fig. 1(*c*)] but the variation could, in part at least, be explained in terms of substrate. At the start of an experiment the R.Q. varied between 1·2 and 1·4, but over the next 400 min fell to values near 1·00. In *C. vulgaris* stored at 2°C in darkness for up to 1 week the R.Q. was near 0·8. It appears, therefore, that as the endogenous substrate is used up the R.Q. falls.

When *C. vulgaris* was suspended in phosphate buffer at pH 6·7 without added glucose, it was possible to show that the stimulation of oxygen uptake by diquat was

accompanied by a stimulation of carbon dioxide output (Fig. 2). The R.Q. values determined for the diquat-stimulated respiration were only slightly lower than the R.Q. values determined for the control respiration (Fig. 2). This is in agreement with the work done by Mees (1960) on bean root tips.

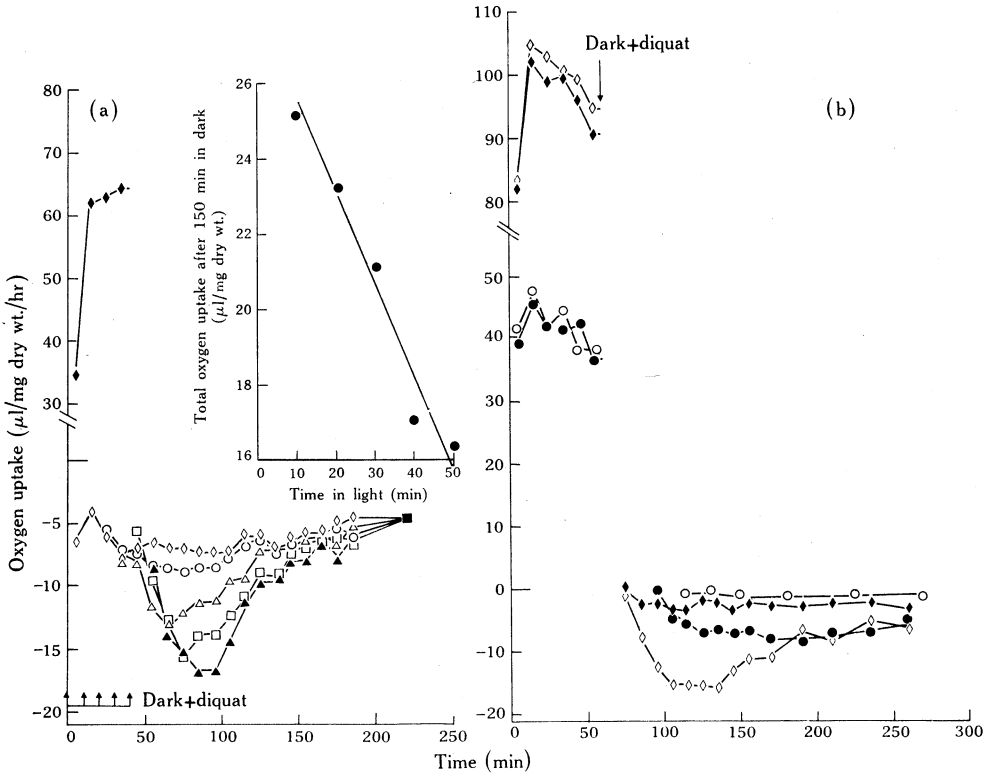

Fig. 3.—(*a*) Effect of pre-illumination periods of 0 (◊), 10 (○), 20 (△), 30 (□), and 40 (▲) min at a light intensity of 880 f.c. on the subsequent oxygen uptake in darkness of mineral-grown *C. vulgaris*. Temperature 25°C; Warburg buffer No. 9. ♦ Rate of apparent photosynthesis at 880 f.c. (control). For all other curves 10^{-3}M diquat was added (arrows) after the light was extinguished. *Inset*: Relation between the period of pre-illumination and the total oxygen uptake after 150 min of darkness in presence of diquat. (*b*) Effects of pre-illumination at light intensities of 880 and 80 f.c. on the subsequent oxygen uptake in darkness of *C. vulgaris* grown at 25°C in Warburg buffer No. 9. ♦, ○ Controls at 880 (♦) and 80 (○) f.c.: apparent photosynthesis for 60 min, followed by respiration (oxygen uptake) in darkness. ◊, ● As for controls but 10^{-3}M diquat added immediately after light (◊ 880 f.c.; ● 80 f.c.) was extinguished. Each point is the mean of two samples.

(*c*) *Effect of Glucose on the Diquat Reaction*

Emerson (1926) showed that glucose, fructose, and mannose at a concentration of 1% (w/v) caused a fourfold increase in the rate of oxygen uptake in *Chlorella*, and that glucose induced a fourfold stimulation of the respiration of *Chlorella* whether it was added at 0·4% (w/v) or 4% (w/v). It has been shown that the glucose-stimulated respiration in *Chlorella* is cyanide sensitive (Warburg 1919) and sensitive to hydrogen

sulphide (Negelein 1925), whereas the endogenous respiration is insensitive to cyanide (Warburg 1919). Emerson also found that 10^{-4}M KCN reduced the glucose-stimulation respiration of *Chlorella* by more than 50%, and that the inhibition by KCN, carbon monoxide, and hydrogen sulphide was fully reversible. Syrett (1951) found that cyanide stimulated the endogenous respiration of *C. vulgaris* and reversibly inhibited the stimulated respiration induced by adding glucose.

Our experiments with glucose were made with cells suspended in phosphate buffer at pH 6·7, as preliminary work showed that glucose penetration into cells suspended in Warburg buffer No. 9 (pH 9·1) was very slow.

The effect of 2% (w/v) glucose on the respiration rate of *C. vulgaris* when suspended in phosphate buffer, pH 6·7, is shown in Figure 2. The stimulation of respiration by glucose was near to sixfold, and the R.Q. of the glucose-stimulated respiration was near to 1·4. This is in good agreement with the work of Kandler and Sironval (1959), who found that for *C. pyrenoidosa* the R.Q. with added glucose varied from 1·21 to 1·49. They suggested that this high R.Q. was due to the synthesis of lipids and proteins. In contrast, Myers (1946) found that the R.Q. of *C. pyrenoidosa* with added glucose was close to 1·00.

Figure 2 also shows that when diquat was added to *C. vulgaris* to which glucose had already been added, there was an additional stimulation of respiration. However, this high rate of respiration quickly declined to a rate near to the control rate of respiration with added glucose. In the presence of glucose diquat did not significantly alter the R.Q. from that of the control rate of respiration.

Figures 1(*b*) and 1(*c*) show the effects of diquat on *C. vulgaris* grown on 2% (w/v) glucose. Three concentrations of diquat caused a marked, but short-lived, stimulation of oxygen uptake and carbon dioxide output. Diquat, particularly at 10^{-3}M, did slightly lower the R.Q. of respiration.

The conclusions drawn from the experiments described above are best illustrated by the curves drawn in the lower part of Figure 2:

(1) When 10^{-3}M diquat is added in darkness to autotrophically grown *C. vulgaris* (after previous darkening) the low endogenous respiration rate is increased (at least doubled) and the enhanced rate continues steadily for at least 400 min.

(2) When the *C. vulgaris* is grown with glucose or when glucose is supplied to mineral-grown *C. vulgaris* the respiration rate is greatly increased. The addition of 10^{-3}M diquat now causes a much larger but temporary stimulation of the respiration, which subsides to the control rate (with glucose) in approximately 200 min. This effect is presumably superimposed on that which raises the endogenous respiration.

Curves intermediate in form between these two extremes were sometimes given with mineral-grown cells, but we suspect that these were due to inadequate darkening and destarching of the cells prior to the addition of the diquat (see below).

(*d*) *Effects of Pre-illumination on the Effects of Diquat in the Dark*

Pre-illumination was used in these experiments as an additional means of investigating the effect of substrate on the diquat reaction in the dark.

The effect of pre-illumination [Fig. 3(*a*)] was, as expected, to alter the form of the diquat curve to that obtained when the diquat was added with glucose to autotrophically grown *C. vulgaris* (Fig. 2) or to *C. vulgaris* grown in glucose [Fig. 1(*b*)].

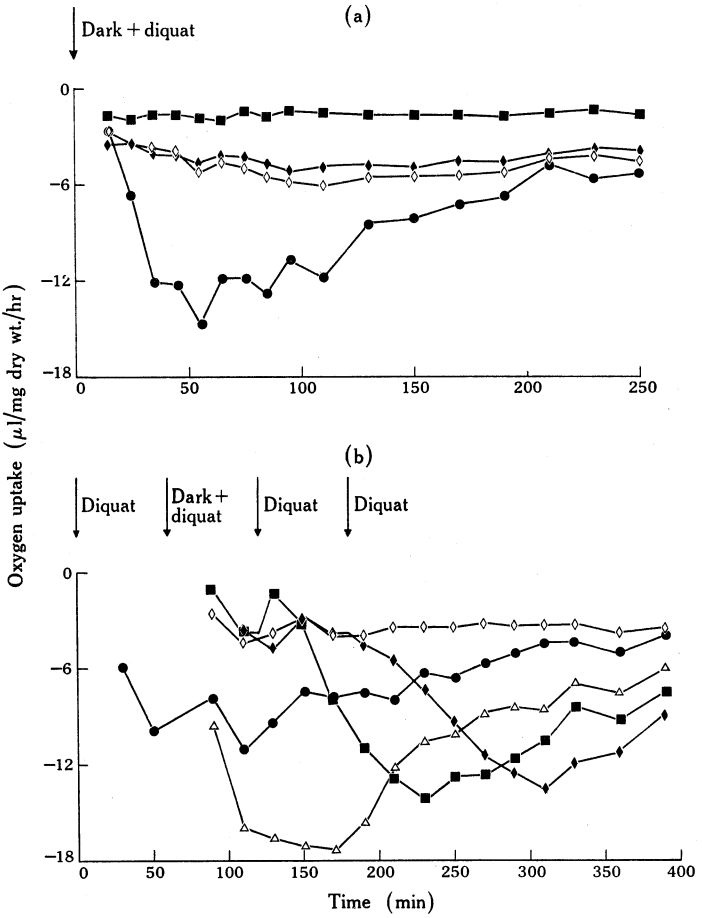

Fig. 4.—(*a*) Effect of pre-illumination for 60 min at 900 f.c. with and without a carbon source, on the subsequent oxygen uptake in darkness of *C. vulgaris* grown at 25°C. ■ Control: oxygen uptake in darkness in phosphate buffer after pre-illumination in phosphate buffer, KOH in centre well. ♦ As for ■, but with 10^{-3}M diquat added immediately on darkening. ◊ As for ♦, but with no pre-illumination. ● Oxygen uptake in darkness, with 10^{-3}M diquat added immediately after darkening, after pre-illumination in Warburg buffer No. 9. Each point is the mean of two readings. (*b*) Survival of the pre-illumination effect after darkening on oxygen uptake of *C. vulgaris* grown at 25°C in Warburg buffer No. 9. Diquat concentration 10^{-3}M. Pre-illumination time 60 min at 800 f.c. ◊ Control, no diquat, darkened at 60 min. ● Diquat added at zero time, no pre-illumination. △ Pre-illuminated, darkened at 60 min, diquat added immediately. ■ As for △, diquat added at 120 min. ♦ As for △, diquat added at 180 min. Each point (except ●) is the mean of two samples.

Figure 3(*a*) shows that the longer the time of pre-illumination the greater was the resulting oxygen uptake caused by diquat in the dark. This figure also shows the regression line for the relationship between the length of the pre-illumination period

and the total oxygen uptake following the addition of diquat. *C. vulgaris* samples were also pre-illuminated at different light intensities, darkened, and the diquat added immediately. After a high pre-illumination light intensity diquat markedly stimulated oxygen uptake in the dark. However, this rapid stimulation of oxygen uptake was transitory and the rate of oxygen uptake soon fell to a reduced rate [Fig. 3(*b*)].

To confirm that this marked stimulation of oxygen uptake with diquat after pre-illumination was related to some photosynthetic product rather than to the illumination itself *C. vulgaris* was pre-illuminated for 1 hr in Warburg buffer No. 9, and also in phosphate buffer, pH 6·7, with KOH in the centre well. Diquat was added immediately after the vessels were darkened. In the samples which were pre-illuminated in the absence of an added carbon source (phosphate buffer with KOH present) there was no stimulation of oxygen uptake by diquat above the rate of the samples which had not been pre-illuminated [Fig. 4(*a*)]. When a carbon source (Warburg buffer No. 9) was present during pre-illumination, the addition of diquat in the dark caused a marked stimulation of oxygen uptake [Fig. 4(*a*)]. The form of this curve was similar to the curve obtained when diquat was added to autotrophically grown *C. vulgaris* in the presence of added glucose or to *C. vulgaris* grown in glucose.

In the experiments presented so far in this section the diquat was added to the *C. vulgaris* samples within seconds of turning off the light. The following experiment was designed to determine whether the photosynthetic product causing the stimulation of oxygen uptake with diquat in the dark was a stable product or one which was rapidly metabolized. Samples of *C. vulgaris* were pre-illuminated for 1 hr in Warburg buffer No. 9 and diquat was added to some samples immediately after turning off the light, and to others after increasing periods in the dark. The greatest stimulation of oxygen uptake occurred when diquat was added immediately the lights were turned off [Fig. 4(*b*)]. Increasing periods in the dark after pre-illumination reduced the magnitude of the diquat stimulated oxygen uptake. However, the diquat-stimulated oxygen uptake was of the type characteristic of pre-illuminated *C. vulgaris* even after 120 min in the dark [Fig. 4(*b*)]. Hence, the substance or substances produced in the light, which cause the marked stimulation of oxygen uptake with diquat in the dark, survive in part for at least 120 min after the light is turned off.

(*e*) *The Path of Carbon*

(i) *Experiment A*

C. vulgaris was labelled in the light during a pre-illumination period (see Section II). A parallel manometric experiment (Fig. 5) showed that a high rate of photosynthesis was maintained in the weak buffer throughout the labelling period, and that diquat added in the dark caused a marked temporary stimulation of oxygen uptake characteristic of effects with pre-illuminated cells.

The results from the autoradiographs are presented in Figure 6. In the alcohol-soluble fraction most of the counts were in sucrose, alanine, serine, glutamate, aspartate, malate, and the phosphate esters. Diquat caused an acceleration of the disappearance of radioactivity from most compounds, in particular from sucrose and glutamate. The exception to this is that diquat brought about a marked accumulation of labelled citric acid.

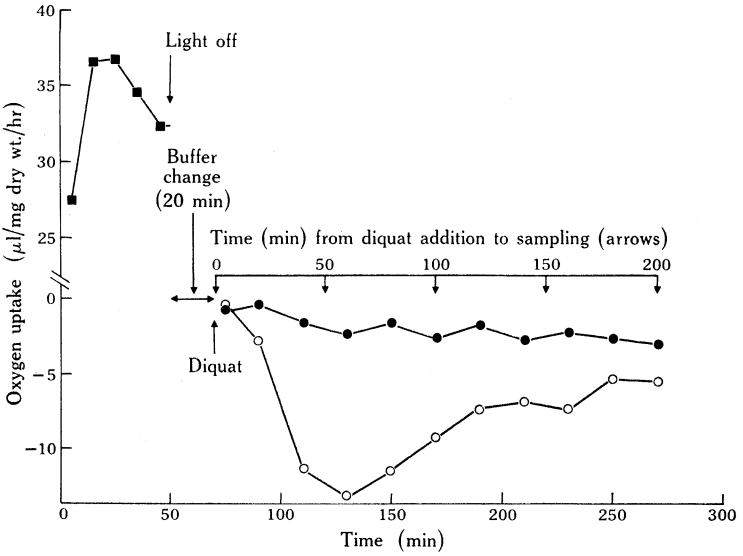

Fig. 5.—Oxygen-exchange curves for *C. vulgaris* for experiment A at 25°C. Pre-illumination at 600 f.c. in dilute carbonate–bicarbonate buffer (see text): transfer to phosphate buffer for oxygen-uptake measurements after centrifugation. ■ Control: apparent photosynthesis. ● Control: oxygen uptake after pre-illumination. ○ Oxygen uptake after pre-illumination with 3×10^{-4}M diquat added in darkness.

Fig. 6

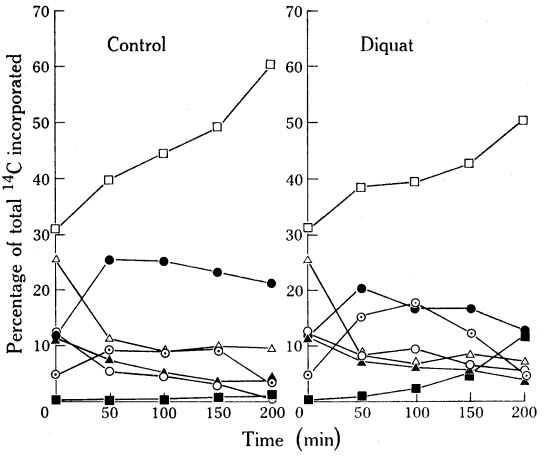

Fig. 7

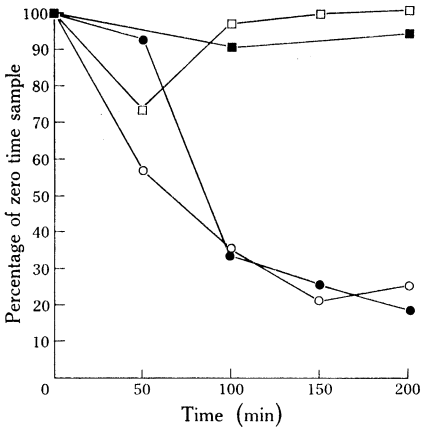

Fig. 6.—Experiment A: radioactivity in alcohol-soluble fractions of the control and diquat treatments of *C. vulgaris* expressed as a percentage of the total radioactivity on the chromatogram at any one time. □ Sucrose. ● Glutamate. △ Phosphate esters. ■ Citrate. ○ Alanine. ▲ Serine. ⊙ Aspartate.

Fig. 7.—Experiment A: effect of 3×10^{-4}M diquat (○, ●) on acid-hydrolysable polysaccharide content of *C. vulgaris*. Counts per minute expressed as a percentage of counts per minute of zero time sample. □, ■ Controls.

Two experiments to determine the effect of diquat on the total acid-hydrolysable polysaccharide are shown in Figure 7. The disappearance of radioactivity from this

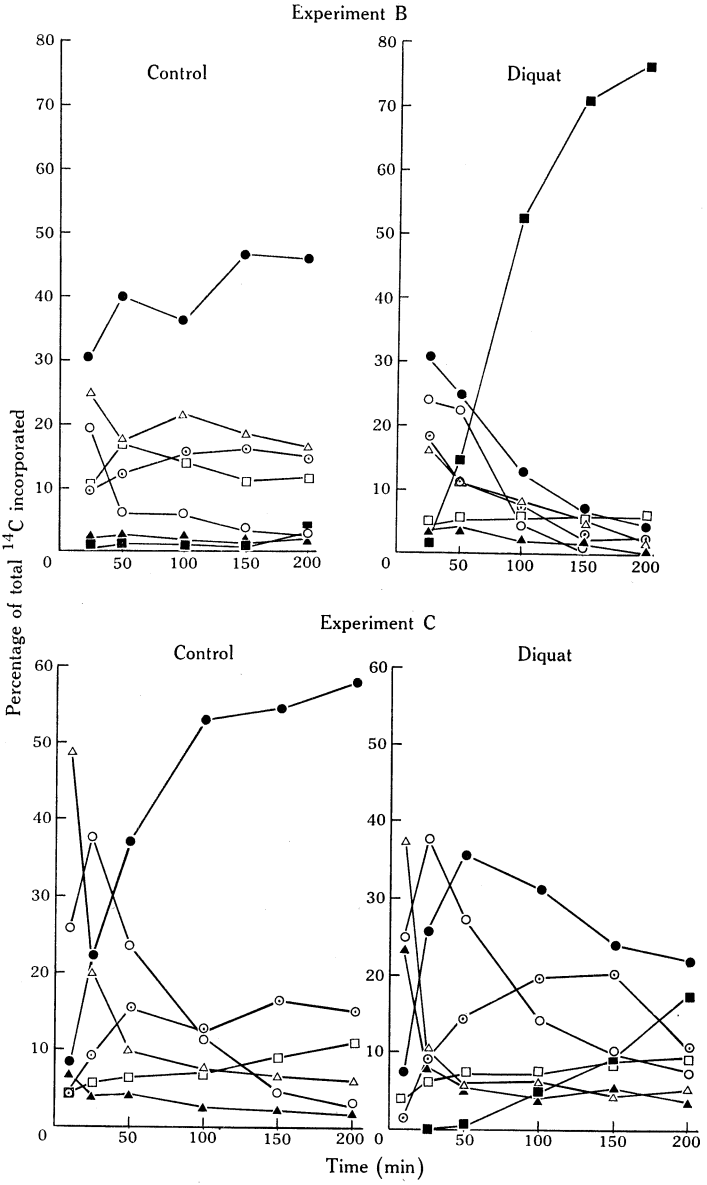

Fig. 8.—Experiments B and C: radioactivity in alcohol-soluble fractions of the control and diquat treatments of labelled *C. vulgaris*, expressed as a percentage of the total radioactivity on the chromatogram at any one time. □ Sucrose. ● Glutamate. ■ Citrate. △ Phosphate esters. ⊙ Aspartate. ▲ Serine. ○ Alanine.

fraction was markedly accelerated by diquat. Analysis of the acid hydrolysate by chromatography showed that it was composed entirely of glucose.

(ii) *Experiment B*

Here the *C. vulgaris* was pre-illuminated in the absence of ^{14}C, darkened, and then labelled with [^{14}C]glucose. The path of carbon was followed in the presence and absence of diquat. The results are shown in Figure 8. Diquat accelerated the disappearance of total radioactivity from the soluble fraction, in particular from glutamate and aspartate. In the control samples the high level of counts in glutamate and aspartate persisted throughout the experiment. However, in the diquat-treated samples radioactivity in citric acid markedly increased.

(iii) *Experiment C*

Here the *C. vulgaris* was not pre-illuminated but equilibrated in the dark at 20°C in $6{\cdot}7 \times 10^{-5}$M phosphate buffer, pH 6·7. Diquat was then added to one batch in the dark. The path of carbon was followed by the analysis of samples taken at various intervals after the addition of [^{14}C]glucose 10 min after diquat treatment. The results are given in Figure 8. As in previous experiments, diquat accelerated the disappearance of total radioactivity from the soluble fraction. High levels of radioactivity persisted throughout the experiment in the control treatment. In the diquat treatment, as the radioactivity in glutamate decreased, the radioactivity in citric acid increased.

IV. Discussion

Diquat, when added to pre-darkened *C. vulgaris* in the dark, causes a stimulation of oxygen uptake and carbon dioxide output with an R.Q. similar to or slightly lower than that for the control endogenous respiration.

If the *C. vulgaris* is pre-illuminated in the presence of carbon dioxide, if it is grown with glucose, or if glucose is added to mineral-grown *C. vulgaris*, polysaccharide is stored and the rate of respiration is increased. The polysaccharide is probably mainly starch (see Stokes, Turner, and Markus 1970). Addition of diquat to such cells (even after they have been darkened for up to 180 min) initially increases the respiration still further and the amount of acid-hydrolysable polysaccharide rapidly falls. It appears then that the effects of diquat on the respiration of pre-darkened cells are not fully expressed because of substrate limitation.

According to Hiller and Whittingham (1964), when radioglucose is fed to *Chlorella* in the dark the major early products are alanine, glutamate, and aspartate. The results presented above are consistent with this work. In experiments B and C these three compounds were the major products labelled, while serine and sucrose were slightly less radioactive. In experiment A, sucrose and glutamate were the major products, serine, alanine, and aspartate being labelled to a lesser extent. In all three experiments with diquat, as the radioactivity in glutamate fell the radioactivity in citric acid increased. Diquat accelerated the disappearance of radioactivity from other metabolic intermediates.

According to Davenport (1963), Zweig, Shavit, and Avron (1965), Black (1966), and Black and Myers (1966), dipyridyls such as diquat can be reduced by NADPH in the presence of NADPH reductase and an NADPH generating system such as isocitrate dehydrogenase. Gage (1968) has shown with rat liver mitochondrial fragments that NADH dehydrogenase appears to be involved in diquat reduction. Diquat and

paraquat stimulated NADPH oxidase activity in microsomes. Diquat had little effect on whole rat liver mitochondria, and it was concluded that diquat could not penetrate the mitochondrial membrane. Preliminary studies with isolated potato mitochondria (Stokes, unpublished data) support this view; diquat had no effect on the phosphorus : oxygen or respiratory control ratios for the oxidation of succinate, malate, and citrate. This suggests that diquat does not interfere directly with mitochondrial function or that its penetration is slow. It is possible that an enzyme essential for diquat reduction is lost in the preparation of mitochondria; alternatively, that diquat acts on cytoplasmic NADH and NADPH and thus reduces the level of reduced pyridines within the whole cell, with secondary effects on the mitochondria.

We propose, therefore, that diquat initially oxidizes pyridine nucleotides, either those associated with mitochondria or those located in the cytoplasm, and as a consequence the diquat is itself reduced. According to Mees (1960) and Zweig, Shavit, and Avron (1965), reduced diquat is rapidly reoxidized by molecular oxygen, with the formation of hydrogen peroxide. Thus the stimulation of oxygen uptake and carbon dioxide output in diquat-treated dark cells could be due to the uncoupling of phosphorylation beyond NAD or NADP, with diquat acting as the electron acceptor just prior to oxygen, as shown in the following two equations:

$$\underset{\text{(reduced)}}{\text{Pyridine nucleotide}} + 2\ \text{diquat} \rightarrow \underset{\text{(reduced)}}{2\ \text{diquat}} + 2H^+ + \underset{\text{(oxidized)}}{\text{pyridine nucleotide}} \qquad (1)$$

$$\underset{\text{(reduced)}}{2\ \text{diquat}} + 2H^+ + O_2 \rightarrow 2\ \text{diquat} + H_2O_2 \qquad (2)$$

In the presence of catalase the R.Q. of respiration should be unchanged on the addition of diquat, as we have found. With glucose feeding or pre-illumination in the presence of carbon dioxide, the increased level of carbohydrate allows a still greater stimulation of respiration with a consequent decrease in stored polysaccharide in the cell.

If the respiratory process is uncoupled from phosphorylation beyond the pyridine nucleotides, the plants would lose an important source of ATP. Sund and Nomura (1963) showed that diquat prevented germination in radish and Sudan grass seeds. We have also shown (unpublished data) that diquat applied in complete darkness inhibited germination of wheat seeds (cv. Olympia). At 10^{-4}M it also inhibited heterotrophic growth in darkness of *C. vulgaris* over a period of 4 days. It has been suggested by Black and Myers (1966) that the dipyridyl compounds may damage plants in the dark by depriving them of a source of reduced pyridine nucleotides and of ATP. We have, as yet, no data to show that diquat actually kills cells when applied in complete darkness at the concentrations we used in our experiments. Certainly there is no rapid toxic effect as there is in the light. Sund and Nomura (1963) showed that diquat slowly kills etiolated seedlings, but it is not clear whether the experiments were carried out in complete absence of light. Further evidence on this point is required.

We still have to explain the effects of diquat in causing citric acid accumulation in all three experiments (A, B, and C) and the decline in respiration (after the initial acceleration by diquat) in those experiments in which *C. vulgaris* had been previously

illuminated or directly supplied with substrate. The accumulation of citrate, together with a decrease in radioactivity in glutamate and aspartate, is consistent with a block in the tricarboxylic acid cycle, after citrate, but before α-ketoglutarate. If the increased radioactivity in citrate were due to an increase in its synthesis it would be expected that the radioactivity in glutamate would increase rather than decrease. The block in the tricarboxylic acid cycle could be related directly to the oxidation of the pyridine nucleotides by diquat. However, we think it more likely that it is due to the inhibition by hydrogen peroxide (see equation 2) of an enzyme or enzymes associated with citric acid oxidation. The actual concentration of hydrogen peroxide, and hence the degree of toxicity, will depend on (1) the rate of diquat reduction which, of course, would be slower in darkness than in light, and greater in the presence of added substrate; (2) the concentration of oxygen which, again, would be lower in darkness than in light in green tissue; and (3) the rate of destruction of hydrogen peroxide by catalase. We suggest that the differences in the respiratory effects between pre-darkened and pre-illuminated *C. vulgaris* are due to the differences in the net rate of hydrogen peroxide production and its subsequent effects upon citric acid oxidation. In the case of pre-darkened *C. vulgaris*, production of hydrogen peroxide would be very slow and most of it could be removed by catalase, hence a small accumulation of citrate and only a very slow fall in the rate of the enhanced (uncoupled) respiration. At higher substrate levels the respiratory rate is raised by diquat to a greater extent and more hydrogen peroxide is produced (equations 1 and 2). The increased concentration of hydrogen peroxide causes more extensive damage to enzymes associated with citric acid oxidation, more citrate accumulates, and the rate of respiration rapidly falls.

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VI. References

Bacon, J. S. R., and Edelman, J. (1951).—The carbohydrates of the Jerusalem artichoke and other Compositae. *Biochem. J.* **48**, 114–26.

Bassham, J. A., and Calvin, M. (1960).—The path of carbon in photosynthesis. In "Encyclopaedia of Plant Physiology". (Ed. W. Ruhland.) Vol. 1. p. 884. (Springer-Verlag: Berlin.)

Benson, A. A., Bassham, J. A., Calvin, M., Goodall, T. C., Hass, V. A., and Stepka, W. (1950).—The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. *J. Am. chem. Soc.* **72**, 1710–18.

Black, C. C. (1966).—Chloroplast reactions with dipyridyl salts. *Biochim. Biophys. Acta* **120**, 332–40.

Black, C. C., and Myers, L. (1966).—Some biochemical aspects of the mechanisms of herbicidal activity. *Weeds* **14**, 331–8.

Brian, R. C. (1967).—The uptake and adsorption of diquat and paraquat by tomato, sugar beet and cocksfoot. *Ann. appl. Biol.* **59**, 91–9.

Buch, M. L., Montgomery, R., and Porter, W. L. (1952).—Identification of organic acids on paper chromatograms. *Analyt. Chem.* **24**, 489–91.

DAVENPORT, H. E. (1963).—The mechanism of cyclic phosphorylation by illuminated chloroplasts. *Proc. R. Soc.* B **157**, 332–45.

DAVIES, J. W., and COCKING, E. C. (1966).—Liquid scintillation counting of ^{14}C and ^{3}H samples using glass fibre or filter paper discs. *Biochim. Biophys. Acta* **115**, 511–13.

EMERSON, R. (1926).—The effect of certain respiratory inhibitors on the respiration of *Chlorella*. *J. gen. Physiol.* **10**, 469–77.

FUNDERBURK, H. H., and LAWRENCE, J. M. (1964).—Mode of action and metabolism of diquat and paraquat. *Weeds* **12**, 259–64.

GAGE, J. C. (1968).—The action of paraquat and diquat on the respiration of liver cell fractions. *Biochem. J.* **109**, 757–61.

GRAHAM, D., and WALKER, D. A. (1962).—Some effect of light on the interconversion of metabolites in green leaves. *Biochem. J.* **82**, 554–60.

HILLER, R. G., and WHITTINGHAM, C. P. (1964).—The synthesis of amino-acids in *Chlorella* in light and dark. 1. Photosynthetic products in *Chlorella*. *J. exp. Bot.* **15**, 21–8.

KANDLER, O., and SIRONVAL, C. (1959).—Photoxidation processes in normal green *Chlorella* cells. II. Effects on metabolism. *Biochim. Biophys. Acta* **33**, 207–15.

LYNCH, V. H., and GILLMOUR, R. G. (1966).—Utilization of glutamine and glutamic acid by *Chlorella pyrenoidosa*. *Biochim. Biophys. Acta* **115**, 253–9.

MEES, G. C. (1960).—Experiments on the herbicidal action of 1,1′-ethylene-2,2′ dipyridylium dibromide. *Ann. appl. Biol.* **48**, 604–12.

MERRETT, M. J., and GOULDING, K. H. (1967).—Short term products of ^{14}C-acetate assimilation by *Chlorella pyrenoidosa* in the light. *J. exp. Bot.* **18**, 128–39.

MYERS, J. (1946).—Oxidative assimilation in relation to photosynthesis in *Chlorella*. *J. gen. Physiol.* **30**, 217–27.

NEGELEIN, E. (1925).—Über die Wirkung des Schwefwasserstoffs auf chemische Vorgange in Zellen. *Biochem. Z.* **165**, 203–13.

SOROKIN, C., and MYERS, J. (1956).—The course of respiration during the life cycle of *Chlorella* cells. *J. gen. Physiol.* **40**, 579–92.

STOKES, D. M., TURNER, J. S., and MARKUS, K. (1970).—The effects of the dipyridyl diquat on the metabolism of *Chlorella vulgaris*. II. Effects of diquat in the light on chlorophyll bleaching and plastid structure. *Aust. J. biol. Sci.* **23**, 265–74.

SUND, K. A., and NOMURA, N. (1963).—Laboratory evaluation of several herbicides. *Weed Res.* **3**, 34–43.

SYRETT, P. J. (1951).—The effect of cyanide on the respiration and the oxidative assimilation of glucose by *Chlorella vulgaris*. *Ann. Bot.* **15**, 473–92.

TURNER, J. S., STOKES, D. M., and GILMORE, L. B. (1970).—The effects of the dipyridyl diquat on the metabolism of *Chlorella vulgaris*. I. Gas exchange in the light. *Aust. J. biol. Sci.* **23**, 43–61.

WARBURG, O. (1919).—Über die Geschwindigkeit der photochemischen Kohlensaurezersetzung in lebenden Zellen. *Biochem. Z.* **100**, 230–70.

ZWEIG, G., SHAVIT, N., and AVRON, M. (1965).—Diquat (1,1′-ethylene-2,2′ dipyridylium dibromide) in photoreactions of isolated chloroplasts. *Biochim. Biophys. Acta* **109**, 332–46.