Mode of Action of Dipyridyls and Certain Quinone Herbicides

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Experimental results clearly demonstrate that a number of quinones exhibit algicidal properties. Free radical formation seems feasible, based on the observation of photoreduction of quinones to the corresponding hydroquinones, and was demonstrated indirectly in the case of chloranil. The mode of action of quinone algicides may be similar to the postulated free radical theory of the dipyridyl herbicides. It may also be related to an interference with the electron transport system in photosynthesis based on the oxidation of reduced pyridine nucleotides.

Since the discovery of the dipyridylium salts as herbicides, it has become an accepted theory that a photochemically produced free radical by a one-electron transfer was the causative agent for their phytotoxicity (Mees, 1960). Several years later it was shown that diquat and paraquat, two dipyridyl herbicides, inhibited $NADPH_2$ formation by illuminated isolated chloroplasts (Zweig *et al.*, 1964). Cyclic ATP formation was competitively inhibited. The same studies demonstrated that the anaerobically stable free radical of diquat is produced by illuminated isolated chloroplasts from Swiss chard, but that this reaction was stopped by 1-(3,4-dichlorophenyl)-3,3-dimethylurea (DCMU), a powerful Hill reaction inhibitor.

Based on these observations, it might be postulated that dipyridyls acted by blocking $NADPH_2$ and ATP formation. This explanation may be an alternative to the postulated free radical theory, in which catalytic amounts of dipyridyls rapidly generate toxic free radicals.

Several substituted quinones such as chloranil (tetrachlorobenzoquinone) and dichlone (2,3-dichloro-1,4-naphthoquinone) have been known as effective fungicides due to their inhibitory action on sulfhydryl enzymes and redox enzyme systems (Owens and Blaak, 1960). 2-Chloro-3-amino-1,4-naphthoquinone, 06K-quinone (Uniroyal Co.), has shown herbicidal activity.

Warburg (1944) observed that unsubstituted 1,4-benzoquinone was reduced to hydroquinone by isolated chloroplasts evolving stoichiometric amounts of oxygen. Other quinones were studied in detail by Wessels (1954) as suitable Hill oxidants, presumably being reduced to the corresponding hydroquinones concomitant with oxygen evolution. Michaelis (1951) had postulated that the reduction of bivalent organic compounds proceeded in two successive univalent steps. Thus, during the photosynthetic reduction of quinones to hydroquinones by green plants, one of the intermediates would be the semiquinone free radical. Due to the similarity of dipyridyl and quinone herbicides with respect to their property of photoreduction, it seemed appropriate to study in detail the physiological and chemical behavior of quinones by in vitro reactions with isolated chloroplasts and with the unicellular alga. *Chlorella pyrenoidosa.*

PHOTOREDUCTION WITH ISOLATED CHLOROPLASTS

By spectrophotometric methods, Cho *et al.* (1966) demonstrated that illuminated, isolated chloroplasts were capable of quantitatively reducing several substituted quinones to their corresponding hydroquinones. However, semiquinone formation could not be demonstrated by this technique. The compounds which were studied included 1,4-naphthoquinone, menadione (3-methyl-1,4-naphthoquinone), dichlone, and 06K-quinone. These compounds were reduced under anaerobic conditions, but two other quinones, 1,4-benzoquinone and chloranil, were fully reduced even under aerobic conditions. These results clearly showed that electron transport by illuminated chloroplasts was proceeding at high efficiency, and also that free radical formation (semiquinone) might be feasible, according to Michaelis' theory (Michaelis, 1951).

A detailed examination of the time-course photoreduction of chloranil showed that quinone disappeared, while semiquinone and hydroquinone were formed, as indicated by changes in their respective characteristic absorption maxima (Figure 1). The reaction mixture contained, in μmoles: phosphate 150, sucrose 1200, NaCl 30, chloranil 0.08; chloroplast suspension containing about 50 μg. of chlorophyll, and water to a final volume of 3.0 ml., pH 6.80. The reaction mixture was illuminated by 450-lux light from a 100-watt red flood light in an anaerobic Thunberg tube at 25° C. Extinction coefficients were determined as: chloranil 15,600 $mole^{-1}$ at 292 mμ, hydroquinone 6900 $mole^{-1}$ at 327.5 mμ, and semiquinone 7500 $mole^{-1}$ at 455 mμ. Absorption maxima at 325, 406, 426, and 455 mμ were assigned to semiquinone, as verified from the spectrum of authentic potassium semiquinone salt prepared by the method of Torrey and Hunter (1912) and described by Foster and Thomson (1962). Since the absorption peak at 325 mμ was due to semiquinone and hydroquinone, appropriate corrections were made for the actual concentration of hydroquinone. The actual change in concentration of each species was calculated from absorbance data and plotted as shown in Figure 2. If these changes were represented by a one-step, two-electron transfer, quinone and hydroquinone would be in equilibrium with semiquinone, as

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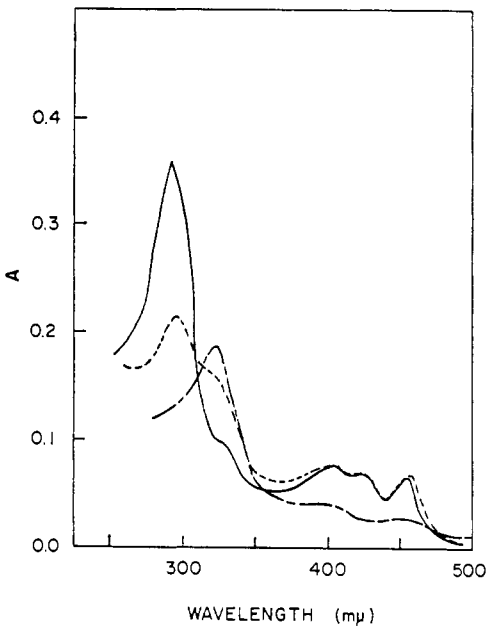

Figure 1. Photoreduction of chloranil by isolated spinach chloroplasts

——— before illumination
– – – – – after 30 seconds' illumination
— – — – – after 2.5 minutes' illumination

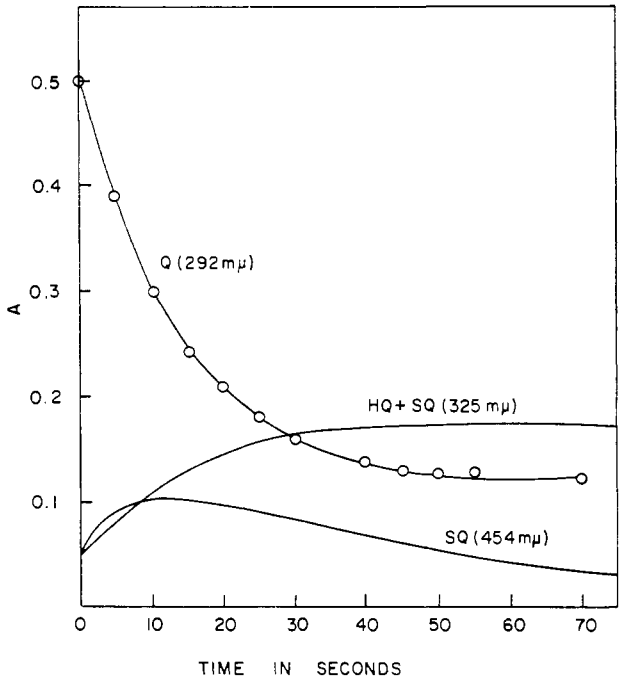

Figure 2. Time course study of photoreduction of chloranil by isolated Swiss chard chloroplasts

shown in Figure 3. Figure 3 is a plot of equilibrium concentrations of all three species calculated from spectrophotometric data by mixing varying amounts of chloranil and hydroquinone in 0.05*M* phosphate buffer, pH 6.80, so that the sum of their concentrations was always 0.1 μmole. This experiment simulates the photo-

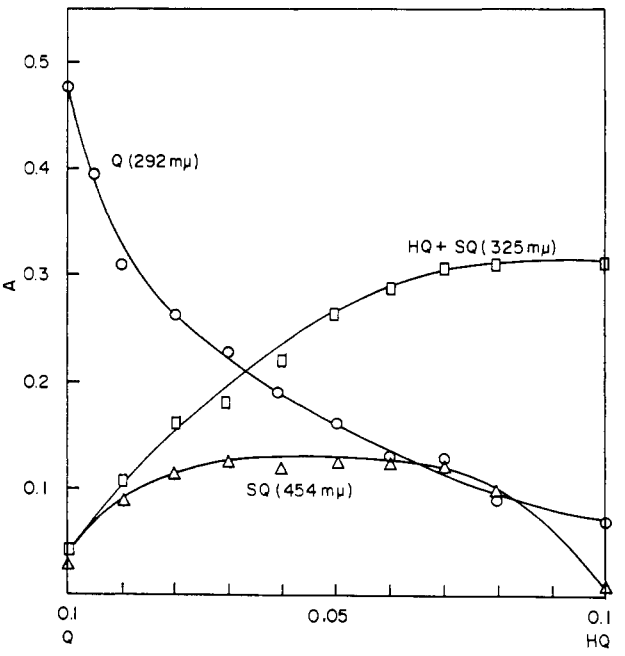

Figure 3. Equilibrium mixtures of quinone (chloranil), semiquinone, and hydroquinone produced by mixing quinone and hydroquinone, the sum of which is always 0.1 μmole

reduction of chloranil to hydroquinone followed by the reaction between quinone and hydroquinone, as already described by Foster (1964):

$$Q \xrightarrow{k_1} HQ \qquad (1)$$

$$Q + HQ \xrightarrow{k_2} 2SQ \qquad (2)$$

The second alternate scheme to explain the photoreduction of chloranil is as follows:

$$Q \xrightarrow{k_3} SQ \qquad (3)$$

$$SQ \xrightarrow{k_4} HQ \qquad (4)$$

$$Q + HQ \xrightarrow{k_2} 2SQ \qquad (5)$$

To decide between the two alternate reduction schemes, an analog computer program was written to simulate reaction sequences 3, 4, and 5. When rate constants were selected, $k_3 = 0.0774$ sec.$^{-1}$, $k_4 = 0.0412$ sec.$^{-1}$, and $k_3 = 13.64$ moles^{-1} sec., the computer drew a set of curves shown in Figure 4, giving the best fit to the actual situation as depicted in Figure 2.

Another analog model was constructed to simulate reaction sequences 1, 2, but no set of constants could be found that would draw curves resembling the experimental plot shown in Figure 2 or 3. On the basis of these studies, it was decided that photoreduction of chloranil by means of isolated chloroplasts proceeded by a two-step, one-electron pathway already predicted by Michaelis (1951), indicating that free radical (semiquinone) formation was feasible.

Other experiments attempting to demonstrate electron

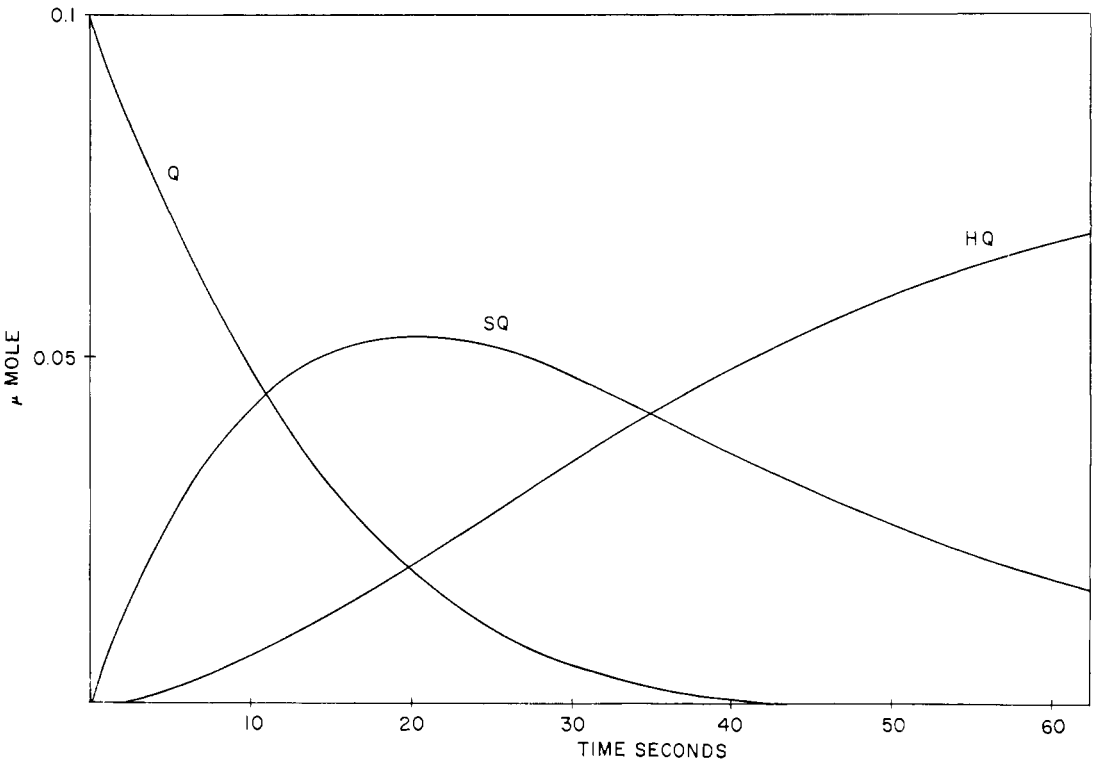

Figure 4. Time course of process of photoreduction of chloranil simulated by analog computer based on the mechanism

$$Q \xrightarrow{k_3} SQ \qquad SQ \xrightarrow{k_4} HQ \qquad Q + HQ \xrightarrow{k_2} 2\,SQ$$

transport and coupled photophosphorylation were reported by Zweig *et al.* (1965) and Black and Myers (1966). These results are summarized in Table I and represent optimum concentration of quinones. The quinones which catalyzed cyclic photophosphorylation were not as effective as phenazinemethosulfate (PMS), for example, but dichlone and naphthoquinone failed completely to stimulate photo-ATP formation. Diquat had been shown previously to catalyze cyclic photophosphorylation (Zweig *et al.*, 1964), but DCMU, a Hill reaction inhibitor, was not a catalyst.

INHIBITION STUDIES

To study the possible inhibition by quinones of the two light reactions in photosynthesis, ferricyanide reduction (System II) and phenazinemethosulfate-mediated photophosphorylation (System I) were chosen as model systems. These results are summarized in Table II. Although it seems from these data that dichlone mimics the action of DCMU, it did not block electron transport from water, as is shown by its photoreduction to hydroquinone (Cho *et al.*, 1966). On the other hand, the overwhelming evidence suggests that DCMU does block the Hill reaction—i.e., electron transport from water (Bishop, 1958; Good, 1961; Moreland *et al.*, 1959). Furthermore, some of the other quinones shown in Table I were catalysts of photophosphorylation and

Table I. Photophosphorylation Catalyzed by Quinones[a]

Compound[b]	Spec. Activity, μMoles ATP/Mg. Chlorophyll/Hr.
06K-Quinone	43
Chloranil	45
Benzoquinone	28
Dichlone	6
Naphthoquinone	6
Diquat	105
DCMU	0

[a] Zweig *et al.*, 1965.
[b] $6.6 \times 10^{-6} M$.

Table II. Effect of Quinones on Cyclic Photophosphorylation and Ferricyanide Reduction[a]

Compound[b]	% of Control	
	FeCN	PHP
06K-Quinone	85	26
Naphthoquinone	40	50
Dichlone	21	61
Chloranil	69	97
Benzoquinone	100	100
DCMU	28	90

[a] Zweig *et al.*, 1965.
[b] $6.6 \times 10^{-6} M$.

did not inhibit the two light reactions very strongly. Thus, the quinones seem to act differently from DCMU and other known Hill reaction inhibitors (triazines, anilides, and other substituted ureas).

An additional experiment with 06K-quinone is shown in Figure 5. A Lineweaver-Burke plot of the inhibition of cyclic photophosphorylation by 06K-quinone indicates that this quinone acts like diquat by competitive inhibition of PMS (Zweig *et al.*, 1965).

REDOX POTENTIAL STUDIES

Based on the evidence presented so far, the phytotoxic quinones do not seem to block the primary electron transport from water, but might impede electron flow at other sites of the light reactions during photosynthesis. To this end, standard potentials of the quinones were determined by polarographic titrations (Table III). The standard potentials of the quinones tested are well within optimum range of the maximum reduction potential of chloroplasts calculated by Zweig and Avron (1965) and make it possible for the photoreduction of these compounds (Cho *et al.*, 1966).

EFFECT OF QUINONES ON *Chlorella pyrenoidosa*

Chlorella p. cultures were treated with various quinones at a concentration of $3 \times 10^{-5}M$. Cell population, chlorophyll content, oxygen evolution, and cell viability were measured during several growth stages (Zweig *et al.*, 1968a). Of the compounds studied, dichlone, 06K-quinone, naphthoquinone, and DCMU had an immediate effect on oxygen evolution by illuminated *Chlorella*. When treated with quinones, washed cells recovered oxygen evolution only partially, while DCMU treatment could be completely reversed, and the oxygen evolution restored to its original rate.

Long-term studies (up to 120 hours' treatment) resulted in the following: Dichlone, chloranil, 06K-quinone, and naphthoquinone caused total bleaching of the cells within 16 to 48 hours, as summarized in Table IV, and benzoquinone had no effect. Dichlone also

Table III. Standard Potentials of Quinones[a]

Compound	Electrolyte	E°, pH 0,[b] Mv.
1,4-Benzoquinone	0.1*M* phosphate, pH 6.86	+699
1,4-Naphthoquinone	0.1*M* phosphate, pH 6.86 + minimum ethanol	+447
Chloranil	0.1*M* phosphate, pH 4.5 + 50% dioxane	+482
Dichlone	0.1*M* tartrate, pH 5.55 + 50% dioxane	+463
06K-Quinone	0.1*M* phosphate, pH 6.86 + minimum ethanol	+268
Diquat	0.1*M* phosphate, pH 6.86	−354 −714

[a] Below and Zweig, 1966.

[b] Experimental procedure: A Sargent Model XV polarograph was used with standard calomel electrode. Standard base electrolyte indicated in table; ethanol or dioxane was used to effect solution of quinones.

Table IV. Time Required to Bleach *Chlorella* by Quinones, Diquat, and Diuron[a]

Compound[b]	Time of Exposure, Hr.[c] 16	48	120
Dichlone	++	+++	+++
Chloranil	++	+++	+++
06K-Quinone	+	+++	+++
Naphthoquinone	++	+++	−++
Benzoquinone	−	−	−
Diuron	−	+	++
Diquat	−	−	−

[a] Zweig *et al.*, 1968a.

[b] $3 \times 10^{-5}M$.

[c] − no effect, + slight bleaching, +− partial bleaching, +++ complete bleaching.

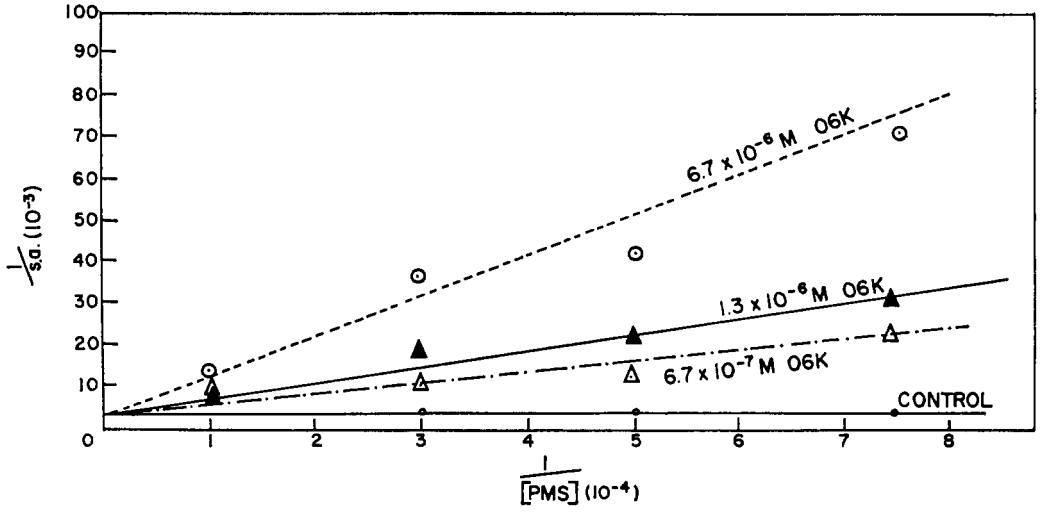

Figure 5. Lineweaver-Burke plot of phenazine-methosulfate photophosphorylation, and inhibited by three different concentrations of 06K-quinone. Experimental conditions of chloroplast reaction are identical to those described by Zweig *et al.* (1964)

caused the death of cells after 65 hours' treatment, as shown by viability studies (Table V). As a typical example, dichlone caused a depression of oxygen evolution before the destruction of chlorophyll and cell integrity became apparent. At the end of the experiment, oxygen evolution and chlorophyll concentration had reached zero, while some cellular structure seemed to remain intact. Similar observations were made with 06K-quinone, chloranil, and naphthoquinone; the damage was not as dramatic, but severe enough to classify these quinones as algicides.

DCMU, diquat, and benzoquinone had little deleterious effect on chlorophyll synthesis, cell number, and oxygen evolution, and in some cases were even stimulatory, as seen from the results of Zweig *et al.* (1968a). At best. these compounds may be classed as algistats. Menadione had no effect at all on *Chlorella p.* Although diquat is an effective herbicide for higher plants, it seems to have little effect on *Chlorella pyrenoidosa.*

$^{14}CO_2$-FIXATION STUDIES

Five milliliters of *Chlorella pyrenoidosa* suspension (50 μg. of chlorophyll per ml.) in water, 40 μc. of $NaH^{14}CO_3$ was illuminated with 300 foot-candles for 10 minutes (Zweig *et al.,* 1968b). At the conclusion of the experiment, the cells were extracted with hot 80% ethanol and aliquots counted with a liquid scintillation spectrometer. Quinones were added as 0.1 ml. of stock solution in 30% methanol, 10 minutes before illumination.

Experiments were conducted to study the effect of quinones on $^{14}CO_2$-fixation by illuminated *Chlorella* during 10 minutes of photosynthesis. A summary of these results is shown in Table VI and indicates that the effectiveness of the quinones as inhibitors of $^{14}CO_2$-fixation is in the order of: dichlone (most effective), 06K-quinone, naphthoquinone, benzoquinone.

Table V. Viability Studies of *Chlorella* Treated with Diuron and Dichlone[a]

Treatment	Time of Treatment, Hr. 0	65	90
	% Viability—100 Cells		
Control	90	90	78
Diuron[b]	90	90	39
Dichlone	90	0	0

[a] Zweig *et al.,* 1968a. [b] $3 \times 10^{-5}M$.

Table VI. Effect of Quinones, Diquat, and Diuron on $^{14}CO_2$-Fixation by *Chlorella*

Compound[a]	% of Control
Dichlone	5.2
06K-Quinone	60.5
Chloranil	62.6
Naphthoquinone	84.5
Benzoquinone	85.7
Diquat	72.8
Diuron	10.3

[a] $3 \times 10^{-5}M$.

EFFECT OF QUINONES ON $NADPH_2$

The reaction mixture contained 0.3 μmole of $NADPH_2$, 0.1 ml. of $0.9 \times 10^{-3}M$ quinone, 0.04 ml. of $NADPH_2$-diaphorase containing 0.5 unit of enzyme activity per 0.68 mg. of protein; 0.3 ml. of $0.5M$ Tris buffer of pH = 7.5, and water to make a total volume of 3 ml. in a silica cuvet, 1 cm. in diameter. The absorbance at 340 mμ for $NADPH_2$ was measured before and after the addition of quinones. The reaction mixture was gently shaken three times so that air would dissolve in the solution.

As had already been postulated by Black and Myers (1966), the phytotoxic effect of quinones might be related to their ability to catalyze the enzymic oxidation of $NADPH_2$. This postulate has now been verified by studying the rate of enzymatic oxidation of $NADPH_2$ in the presence of catalytic amounts of quinones, and is shown in Table VII. These data indicate that the catalytic effect of quinones is in the same order as their inhibitory effect on $^{14}CO_2$-fixation—i.e., dichlone, 06K-quinone, menadione, naphthoquinone, benzoquinone.

DISCUSSION

The case for the phytotoxicity of dipyridyl compounds due to free radical formation (Mees, 1960; Zweig *et al.,* 1964) seems to be based on good evidence. For a compound to serve as a causative agent for the free radical formation within a biological system, the compound in turn must be readily autooxidizable, and the dipyridyls fulfill this requirement. However, the studies by Zweig *et al.* (1964) which showed that diquat inhibited $NADPH_2$ production and competitively inhibited PMS-mediated cyclic photophosphorylation raised the possibility that diquat could effectively exert its phytotoxic action by blocking these reactions. This would also be manifested by the inhibition of CO_2-fixation by green plants. However, the results in Table IV show that $^{14}CO_2$-fixation by *Chlorella p.* is only moderately inhibited by diquat, in contrast to the Hill reaction inhibitor, DCMU, which severely affected $^{14}CO_2$-fixation. The observations by Mees (1960) and Zweig *et al.* (1964) that DCMU inhibited free radical formation, which was

Table VII. Rate of Enzymic Oxidation of $NADPH_2$ Catalyzed by Quinones

Compound	Rate of $NADPH_2$ Disappearance, μMoles/Min.
Dichlone	>2.67
06K-Quinone	2.33
Menadione	0.29
Naphthoquinone	0.28
Benzoquinone	0.25

manifested by the delay of diquat-symptoms, gave additional evidence to the free radical theory.

The similarity between quinones and dipyridyls is their reduction by illuminated chloroplasts (Cho *et al.*, 1966). Furthermore, it has now been shown, using chloranil as a model compound, that the photoreduction of quinone to the corresponding hydroquinone probably proceeded via semiquinone. However, not all of the studied quinones exhibited algicidal properties against *Chlorella p.*—e.g., menadione and 1,4-benzoquinone. Benzoquinone and chloranil, once reduced, were not autooxidizable, thus precluding the important requirement for a causative agent of free radical formation. Also, dichlone, in contrast to the other quinones studied, was a potent inhibitor of $^{14}CO_2$-fixation by *Chlorella p.* 06K-quinone resembled diquat in its behavior toward reactions catalyzed by isolated chloroplasts (Zweig *et al.*, 1964; Table II). The conclusion one must reach, therefore, is that the quinones may be acting at different sites in the electron-transport chain and depleting the supply of electrons.

The similarity between dichlone and DCMU—i.e., inhibition of $^{14}CO_2$-fixation by *Chlorella p.*—might suggest that dichlone inhibits the Hill reaction. This, however, is not the case, since dichlone is photoreduced by illuminated isolated chloroplasts, indicating that electron transport mediated by System II is proceeding unimpeded. The fact that oxygen evolution due to dichlone was not totally inhibited in *Chlorella p.* makes this explanation feasible. It is suggested, therefore, that dichlone acts at a site in the electron transport system near the first oxidant and not coupled to any phosphorylation.

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