FACTORAL BIPYRIDINIUM PHYTOTOXIC INTERACTIONS OF *CHLORELLA PYRENOIDOSA*, Chick. INDUCED WITH PARAQUAT

By

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CHAPTER I

INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'bipyridinium cation) is a non-specific contact herbicide inducing rapid chlorosis and desiccation of plants. This material has been utilized extensively for crop desiccation and rapid non-selective weed destruction because of its rapid action with no residual effects. In most cases, this herbicide apparently moves in an acropetal direction but basipetal movement may occur under some conditions in plant tissues. As a cation it is inactivated readily by strong soil adsorption and is apparently bound to some extent in cell wall materials. Light has been shown to enhance the herbicidal effectiveness of this dipyridylium cation indicating that the mode of action of this chemical is involved in photosynthesis. The unicellular green alga, Chlorella pyrenoidosa, has been a useful bioassay plant in quantitative determination of paraquat level. It was chosen for this study because of capabilities of being facultatively adaptive to growth in either light or dark conditions and because of its sensitivity to paraquat toxicity.

The objective of this study was to determine governing factors influencing phytotoxic effectiveness and the control mechanisms for paraquat activity with C. pyrenoidosa.

CHAPTER II

LITERATURE REVIEW

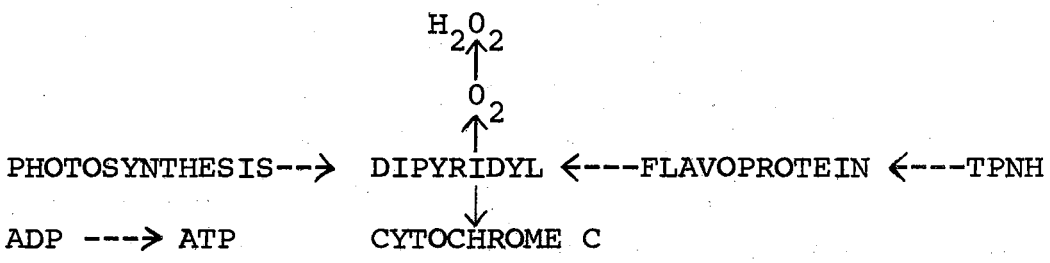
Paraquat, 1,1'-dimethyl-4,4'-bipyridinium salt, and diquat, 6,7- dihydrodipyrido (1,2-a: 2',1'-c) pyrazidinium salt were first described by Brian et al. (8) in 1955. Both diquat and paraquat act in a similar manner and are marketed and utilized as completely water soluble compounds. With powerful reducing agents such as sodium dithionite, paraquat was reduced in two step reactions. In the course of the reduction, the quaternary salts took up two electrons, one at each step. The first electron, formed the colored free radical, the second yielded the fully reduced compound (11). Investigators had reported that this herbicide was active on species of aquatic and terrestial weeds at exceedingly low concentrations (16). It also gave satisfactory postemergence weed control under young apple, peach and pecan trees (2) but failed to suppress the woody perennials (7). This chemical was absorbed rapidly and translocated acropetally and tended to concentrate in the vascular system within plant tissues. Apparently there was less movement when dipyridyls were applied to foliage. This apparently was due to the desiccating effect of par-

aquat on green plants and disruption of routes of transport.

Paraquat caused a stimulation of respiration and an inhibition in photosynthesis of duckweed at low concentration level. Inhibition of the Hill reaction by dipyridylium was directly related to redox potentials (12). The redox potentials for paraquat and diquat were -446 mV and -349 mV respectively. It seemed likely that phytotoxicity and reduction were related, and that herbicidal activity depended on the ability of active compounds to form toxic free radicals by the uptake of one electron within plants (12). Hormer (15) suggested that not only reduction with free radical formation but also reoxidation was probably required for herbicidal activity by processes connected with photosynthesis and respiration. Brian (9) found that light and chlorophyll were essential for free radical formation and that oxygen was required for toxic symptoms to occur in plants.

Among the possible components of the electron transport chain in photosynthesis are cytochrome b, cytochrome f, plastoquinone, plastocyanin, pigment 700, pteridine, ferredoxin and a ferredoxin-NADP reductase. Because of its high reducing potential (-432 mV) ferredoxin could readily reduce TPN to form TPNH (18). By using spinach chloroplasts, Black (4) was able to show that some dipyridyls could substitute for ferredoxin in the reduction of cytochrome c in the presence of TPN reductase and TPNH. Once being reduced, the dipyridyl

salt was quickly reoxidized in the presence of oxygen to produce hydrogen peroxide and acted as a catalytic electron carrier. It seemed unlikely that hydrogen peroxide was the toxic agent since catalase was common in plant tissues. A general scheme for the action mechanism of the quaternary dipyridylium is presented below:

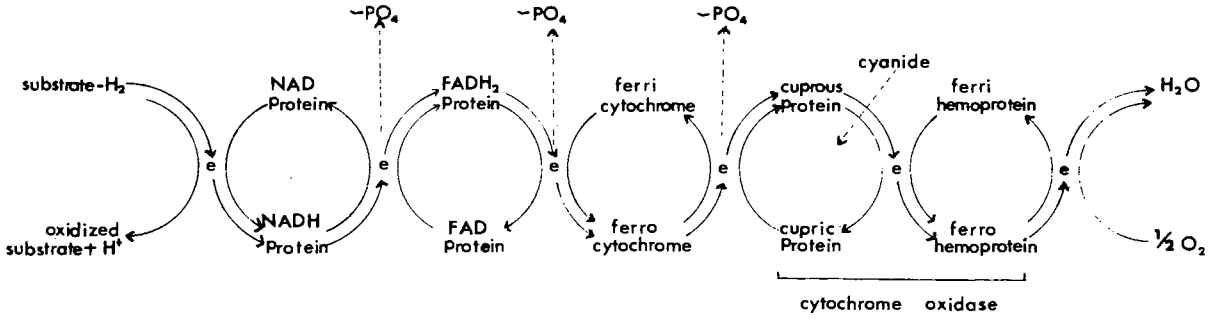


The reducing potential could arise from reduced pyridine nucleotides or an electron produced during photosynthesis. By this mechanism, plants lost a major energy supply, reduced pyridine nucleotides. The mechanism could account for toxicity both in light and in darkness. Previous hypotheses based on the light-dependent formation of free radicals failed to account for the dark toxicity of this herbicide (5). Black and Myers (5) also found that an outstanding feature of paraquat was the inhibition of ATP formation at concentrations in excess of the optimal concentration peak for photophosphorylation.

The toxicity of paraquat is governed by several factors. There was evidence by Mees (20) that oxygen was required if toxic symptoms were to follow free radical production in plants in light. Light was shown to increase the rate of

kill with the dipyridyl salts without being required for herbicidal action (20). The greater the light intensity the more rapid the chlorosis in duckweed (Lemna miner L,). The greatest activity of paraquat on giant duckweed was found under red light (600mμ) and the least activity under the green light (510-560 mμ) (6). Light was also essential for the changes in membrane permeability brought about by this chemical in mesquite, honeysuckle and broadleaf bean (21). Increased time, temperature and light intensity accelerated the rate and magnitude of Indian bean leaf disk chlorosis induced with active paraquat levels (3). It was found that monuron and diuron inhibited the action of diquat and paraquat in the light but not in the dark (25). However, potassium cyanide, which reduced carbon dioxide uptake and depressed respiration, did not reduce the speed of action in the light.

In previous work, Lee (17) indicated that inhibition of paraquat phytotoxicity to Chlorella by Cu^{++} addition was apparent at levels equivalent to paraquat levels. Increasing the Cu^{++} to equal the level of paraquat resulted in near complete reduction in paraquat activity. Indeed, because copper is an essential constituent of many enzymes and other biological catalysts, it can be described as one of the prime movers of the biochemical machine. So far, only three copper proteins, cytochrome oxidase, hemocyanin, and tyrosinase, have been investigated as to their roles in metabolism.



OXIDATIVE METABOLISM

Fig. 1. Sequence of oxidative reactions proposed for reduced substrate to H_2O and O_2.

Cytochrome oxidase is the principal "terminal" oxidase in all animals and in most plants and is involved in oxidative metabolism. Shown here is the final series of oxidative reactions following the order proposed by Gibson. Cyanide reacts with protein to block metabolism at the final stage (10).

It was speculated that cytochrome a_3, the terminal oxidase Cu^{++} containing member of the electron transfer sequence within the mitochondria, may be involved directly with paraquat phytotoxicity to C. pyrenoidosa (17).

The chemical nature of paraquat is unique because of its strong cationic nature. This compound is adsorbed in soils by the cation exchange mechanism and becomes inactivated (15). The paraquat adsorption capacity is approximately the cation exchange capacity of the clay minerals. Montmorillonite and kaolinite clays are effective in decreasing the toxicity of paraquat to plants. Both paraquat and diquat were found to be bound within interlayer spacing of the montmorillonite by coulombic and van der Walls forces, and to the surfaces of particles of kaolinite clay by coulombic ion exchange forces. Only the small amount of this chemical adsorbed on the exterior surfaces of montmorillonite and kaolinite clay is probably available to the plant roots (24). The amount of degradation by soil microorganism is very small in montmorillonite, but it can be degraded readily on kaolinite (11). Slade (23) showed that UV light formed by a mer-

cury vapor lamp, as well as sunlight, was capable of degrading paraquat to volatile compounds.

Determination of residues has been an important aspect of herbicidal research. Anion exchange chromatographic paper as well as electophoresis chromatography proved satisfactory for studying degradation of dipyridyl quaternary salts (12), whereas color development by the dithionite method was unsatisfactory. Funderburk and Lawrence (13) have devised a bioassay procedure for determining the amount of residue of paraquat applied for control of aquatic weeds. They found that duckweed was extremely sensitive to a very low concentration. Barnes (3) used the leaf disk of Phaseolus vulgaris, L. and Chlorella pyrenoidosa in bioassay studies of paraquat. The unicellular green alga, Chlorella pyrenoidosa has been introduced into plant physiology as a useful tool for research in photosynthesis, mineral nutrition and cellular growth and development. This alga is typified by the possession of photoautotrophic nutrition and chlorophyll endows it with the ability to use light as an energy source and carbon dioxide as a carbon source. Illuminated cells of C. pyrenoidosa have been found to form two different types of products, containing unpaired electrons, which can be detected by electron spin resonance techniques (22). While assessing the possibilities of using these algae as a rapid test of phytotoxicity of chemicals, it was noticed that paraquat

markedly inhibited algal growth. The magnitude of chlorosis can be quantitatively evaluated by spectral readings with a Spectronic 20 and a Perkin and Elmer 202 spectrophotometer at 434 and 670 mμ.

CHAPTER III

MATERIALS AND METHODS

Chemical quantitation with a dithionite reaction sequence is the standard assay method for paraquat. Salt concentrations of 1, 100, and 200 ppm were supplied as sulfates of potassium, calcium and magnesium, in ratio of $\frac{K^{+}}{\sqrt{\frac{Ca^{++} + Mg^{++}}{2}}} = 1$, then combined at each level with 0, 0.1, 0.5, 1.0, 2.0 and 5.0 ppm paraquat. Two ml sodium dithionite were added to 10 ml of paraquat-salt solution and the absorbance determined at 394 mμ using a Bausch and Lomb Spectronic 20 Spectrophotometer.

The test plant, Chlorella pyrenoidosa Chick, IAC 251, was obtained from the Culture Collection of Algae at Indiana University, Bloomington, Indiana. The paraquat used in these studies was a commercial formulation of the Chevron Chemical Corporation with 24% methyl sulfate salt active ingredient. The Cu^{++} medium was composed of sulfate salts.

The unicellular algae were cultured in 2.8 liter Erlenmeyer flasks with continuous day light (GE) and standard

Gro-lux (Sylvania) fluorescent lamps illumination at light intensity of 500 footcandles. Aluminum reflectors were used to improve light efficiency. Cultures were grown lithotrophically at 25-30°C in a completely inorganic distilled water medium and bubbled with compressed air (approximately 3% CO_2) in order to keep the cells agitated and in suspension. Components of the inorganic medium included three grams each of K_2SO_4, NH_4NO_3 and Phillips-Hart IV salt mixture per liter of distilled water. The composition of P. H. IV salt in per cent by weight was: NaCl, 16.7; $CaCO_3$, 30.0; $CaHPO_4$, 7.5; K_2HPO_4, 32.2; $MgSO_4$, 10.2; $MnSO_4$, 0.51; $CuSO_4$, 0.03; $ZnCl_2$, 0.025; KI, 0.08; ferric citrate, 2.75; and cobalt chloride, 0.005.

The stock Chlorella cultures were developed to near peak log phase of growth then transferred in 600 ml volumes into one liter Erlenmeyer flasks containing different levels of Cu^{++} salt solution. Cell suspensions were incubated under a light intensity of 500 foot-candles with aeration for three days. These cultures were then centrifuged to separate the cells from the nutrient solution. The harvested cells were washed three times with deionized water, then resuspended in deionized water. Cell concentration was determined turbidimetrically using a Baush & Lomb Spectronic 20 Spectrophotometer at 600 mμ. Equal 30 ml volumes of constant cell concentration from the suspended cultures were allotted to

50 ml flasks for differential paraquat treatment. Flask openings were covered with perforated parafilm to allow free gas exchange. Cultures were developed in a new Brunswick Controlled Environment Incubator Shaker at 30°C under continuous light and shaken at 180 RPM per minute for 45.5 hours. The effect of paraquat was estimated by the degree of chlorosis of Chlorella cells with the methanol extraction method. Following separation by centrifugation, cells were frozen at -10°C overnight, then chlorophyll was extracted with 10 ml of methanol using a tissue homogenizer. These extracts were read at 434 mμ and 670 mμ using a B. & L. Spectrophotometer or full spectra recordings from a Perkin-Elmer Model 202 Spectrophotometer. Chlorella dry cell weight was determined in g/30 ml following dehydration at 80°C overnight.

The procedure for studying the effect of time and paraquat levels on extractable chlorophyll absorbance of C. pyrenoidosa was similar to a previous study except that the incubation times were 16, 24, 32, 40, and 48 hours instead of 45.5 hours and the cell density was adjusted to 0.6 O. D.

The basic procedure was also modified for studying effects of Cu^{++}, CN^{-} and paraquat levels of C. pyrenoidosa. Copper treated algal cultures were subjected to 12 hours of 500 foot-candles of light at room temperature followed by 12 hours in the dark at 9°C for three complete cycles before cell washing, and suspended in 2% $NaHCO_3$ solution. Following

differential cyanide and paraquat treatments, the flask cultures were shaken at approximately 120 oscillations per minute in an Eberbach culture shaker for 40 hours or 45 hours.

The effect of light composition and paraquat levels on Chlorella was studied using the same procedure as for the Cu^{++}, CN^{-} and paraquat experiments, except that cultures were individually covered with red, blue, or black (combination of red and blue) filters during incubation in the shaker. The absorption region for the red filter was 380- 580 mμ, and for the blue filter it was 580- 700 mμ. For the predarkness treatment experiment, the algal cultures were placed in the dark at room temperature (25°C) for 24, 48, 72, and 96 hours in one study, and 12, 24, 36, and 48 hours in another study, prior to paraquat addition and light incubation.

CHAPTER IV

RESULTS AND DISCUSSION

The effects of various concentration levels of salt supplied as sulfates of potassium, calcium and magnesium in a ratio of $\frac{K^{+}}{\sqrt{\frac{Ca^{++} + Mg^{++}}{2}}} = 1$, are shown in Table 1 and Figure 2. Paraquat is easily reduced by 0.2% sodium dithionite solution (0.3 N NaOH) to an unstable free radical having an intense blue color and a strong absorption peak at 394 mμ. However, this dithionite reaction is not entirely specific for the paraquat cation and requires confirmation with bioassay methods. Other oxidation-reduction reactions within tissue or media containing paraquat may contribute to interferences and reduced precision.

Salt concentration at a 100 ppm level had little influence on dithionite reactions with paraquat at all levels of 0.1 to 5.0 ppm. Salt concentration at the 200 ppm level greatly influenced the dithionite-paraquat reactions at all paraquat levels. Absorption at 394 mμ was greatly increased, particularly at the four lower paraquat levels. Differences

as a function of treatments and interactions were all statistically highly significant with a very low coefficient of variation of 4.93%. Results of variable cation salt concentration on paraquat activity with Chlorella cultures as shown by Lee (17) may be explained by the effects shown in these dithionite quantitation data.

TABLE I

EFFECT OF SALT ON PARAQUAT QUANTITATION
USING A DITHIONITE METHOD

Paraquat ppm	ppm salt concentration			
	0	100	200	sum
0	0.0	0.0	0.0	0.0
.1	.034	.028	.49	.552
.5	.14	.165	.74	1.045
1.0	.37	.385	.90	1.655
2.0	.665	.59	1.06	2.315
5.0	1.36	1.40	1.84	4.60
SUM	2.569	2.568	5.03	10.167

Sum of duplicate determinations are expressed as O.D. at 394 mμ. Sulfate salt solution ratio:

$$\frac{K^{+}}{\sqrt{\frac{Ca^{++} + Mg^{++}}{2}}} = 1$$

Significant F values:

Treatment 803.1 Interaction 38.7

Paraquat levels 2307.2 C. V. = 4.93%

Salt levels 867.5

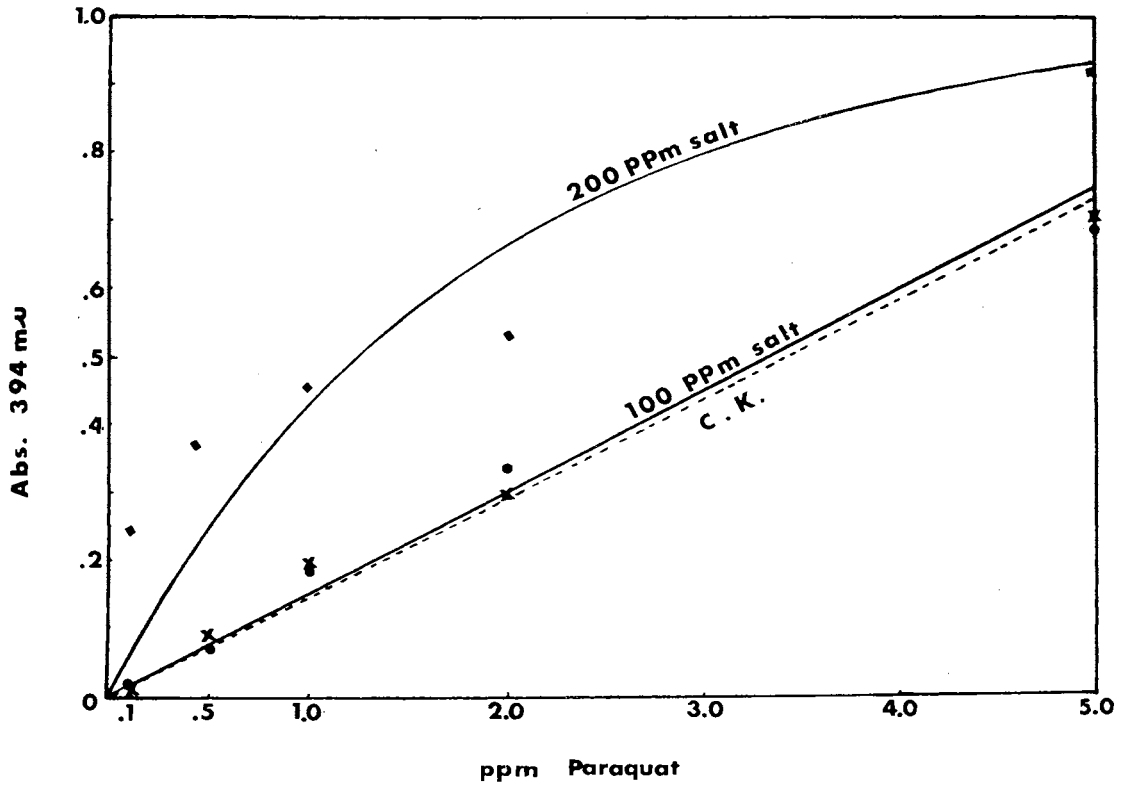


Fig. 2. Influence of salt concentrations on paraquat activity using a dithionite method.

Interaction of copper supplied as sulfate with paraquat on phytotoxicity to Chlorella is shown in Table II. Chlorella cultures were suspended in 2% $NaHCO_3$ solution for an enriched CO_2 source rather than in a water medium.

Little paraquat activity was apparent with the extractable chlorophyll absorption at 434 mμ with paraquat levels below 2.0 ppm. However, copper concentrations at 0.1, 0.5, 1.0 and 2.0 ppm significantly reduced paraquat toxicity at the 2.0 ppm level. Significant F values for treatment levels and interactions were apparent with a low coefficient of variation of 9.63%.

Differential changes in extractable chlorophyll absorption were obtained at 670 mμ as a function of both paraquat and copper concentration. Chlorosis generally increased with each increase in paraquat level. Copper levels apparently inhibited paraquat phytotoxicity but results were somewhat variable as to the trend and magnitude of these effects. The low copper level of 0.1 ppm apparently was more effective than the higher copper levels, although the response curves at each paraquat level were not quadratic. Treatment F values were significant with a coefficient of variation of 23.05%.

These data confirmed the results of Lee (17) showing an inhibitory effect from copper additions to phytotoxic activity of paraquat to this unicellular alga.

TABLE II

EFFECT OF COPPER AND PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Paraquat ppm	Absorption at 434 mμ ppm Cu^{++}					
	0	.1	.5	1.0	2.0	sum
0	3.0	3.0	3.0	3.0	3.0	15.0
.1	3.0	3.0	3.0	3.0	3.0	15.0
.5	3.0	3.0	3.0	2.99	3.0	14.99
1.0	3.0	3.0	2.99	2.99	3.0	14.98
2.0	.73	.93	1.96	1.37	2.1	7.09
sum	12.73	12.93	13.95	13.35	14.10	67.06

Significant F values:

Treatment 13.9
Paraquat level 72.6
Paraquat x Cu^{++} interaction 2.1
C. V. = 9.63%

Paraquat ppm	Absorption at 670 mμ ppm Cu^{++}					
	0	.1	.5	1.0	2.0	sum
0	2.9	2.86	2.79	2.43	2.7	13.68
.1	2.9	2.75	2.66	2.48	2.7	13.49
.5	1.47	2.50	2.10	1.28	2.06	9.41
1.0	1.26	2.09	1.80	1.77	1.53	8.45
2.0	.23	.31	.68	.54	1.07	2.83
sum	8.76	10.51	10.03	8.50	10.06	47.86

Significant F values:

Treatment 7.6
Paraquat level 40.5
C. V. = 23.05%

Figures are sums of duplicate synchronized cultures with absorption O. D. determined from full spectra recordings.

Results from two successive studies using differential paraquat and copper treatments with Chlorella are presented in Table III. Paraquat levels were 0, 0.5, 1.0, and 2.0 ppm and Cu^{++} as sulfate was supplied at these same levels.

Highly significant F values were apparent for treatments, paraquat levels, copper levels, and treatment interactions at both wave-lengths. Coefficients of variation were 14.12% for 434 mμ and 20.76% for 670 mμ. In general, the phytotoxicity of paraquat increased with increased concentration. Although copper significantly inhibited the reaction of paraquat, the highest inhibitory concentration was not obtained at 2 ppm but at the 0.5 ppm level. Copper at 2 ppm was toxic to Chlorella, however; $CuSO_4$ is known as one of the most effective algaesides. The 1.0 ppm level was intermediate between responses at the 0.5 and 2.0 ppm concentrations.

Response surfaces of relative chlorosis with absorbance at 434 mμ were indicative of the consistent positive effect attained with different copper levels when combined with equivalent paraquat levels (Figure 3). These differences were of greater magnitude than the surface response obtained with 670 mμ. Although less apparent at the 670 mμ wavelength, reduction in chlorosis as a function of Cu^{++} concentration was significant.

Chlorella dry cell weight after chlorophyll extraction was also affected by copper and paraquat treatments as shown

TABLE III

EFFECT OF Cu^{++} AND PARAQUAT LEVELS ON CHLORELLA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ					Absorption at 670 mμ				
ppm Cu^{++}	ppm paraquat	Experiment I	Experiment II	sum	ppm Cu^{++}	ppm paraquat	Experiment I	Experiment II	sum
0	0	5.4	5.34	10.74	0	0	2.34	2.14	4.48
0	.5	.967	1.184	2.151	0	.5	.355	.488	.843
0	1.0	.615	.512	1.127	0	1.0	.226	.199	.425
0	2.0	.496	.505	1.201	0	2.0	.172	.187	.359
.5	0	5.6	5.57	11.17	.5	0	2.68	2.82	5.5
.5	.5	2.4	1.778	4.178	.5	.5	1.03	.753	1.783
.5	1.0	2.25	1.60	3.85	.5	1.0	.948	.660	1.608
.5	2.0	1.88	1.286	3.166	.5	2.0	.775	.521	1.296
1.0	0	5.52	4.24	9.76	1.0	0	2.41	1.415	3.825
1.0	.5	1.755	1.128	2.883	1.0	.5	.736	.420	1.156
1.0	1.0	1.840	1.205	3.045	1.0	1.0	.745	.440	1.185
1.0	2.0	1.480	1.133	2.613	1.0	2.0	.572	.405	.977
2.0	0	2.50	1.025	3.525	2.0	0	1.105	.417	1.522
2.0	.5	2.08	.933	3.013	2.0	.5	.920	.353	1.273
2.0	1.0	2.16	.790	2.950	2.0	1.0	.970	.299	1.269
2.0	2.0	2.03	.935	2.965	2.0	2.0	.875	.359	1.254
sum		38.973	29.164	68.137	sum		16.679	11.876	28.755

Significant F values:
- Treatment 174.5
- Paraquat 611.9
- Cu^{++} 76.1
- Interaction 61.4
- C. V. 14.12%

Significant F values:
- Treatment 93.4
- Paraquat 319.2
- Cu^{++} 49.0
- Interaction 32.9
- C. V. 20.76%

Figures are sums of three replicate cultures in each experiment I and II with absorption O. D. determined with a Spectronic 20 at the specified wavelengths.

in Table IV. The higher the paraquat and copper levels added, the less dry cell weight was recovered. Treatment F values were significant, paraquat levels 8.9, Cu^{++} levels 34.1 and interaction levels 2.9, with C. V. 11.94%.

TABLE IV

EFFECT OF Cu^{++} AND PARAQUAT LEVELS ON CHLORELLA DRY CELL WEIGHT AFTER CHLOROPHYLL EXTRACTION

	Dry Cell Weight				
ppm Cu^{++}	ppm paraquat				
	0	.5	1.0	2.0	sum
0	.0531	.0510	.0578	.0429	.2048
.5	.0463	.0420	.0391	.0367	.1641
1.0	.0437	.0389	.0355	.0385	.1566
2.0	.0422	.0372	.0358	.0370	.1522
sum	.1853	.1691	.1682	.1551	.6777

Significant F values: Treatment 10.3, paraquat levels 8.9, Cu^{++} levels 34.1, interaction 2.9, C. V. 11.94%

Figures are sums of all replicate cultures from both experiments with dry cell weight in g/ 30 ml culture.

The influence of copper levels on paraquat effectiveness on Chlorella cultures was similar to that described in previous studies by Lee (17). As shown in Table V, not only paraquat and copper levels affected Chlorella reactions but

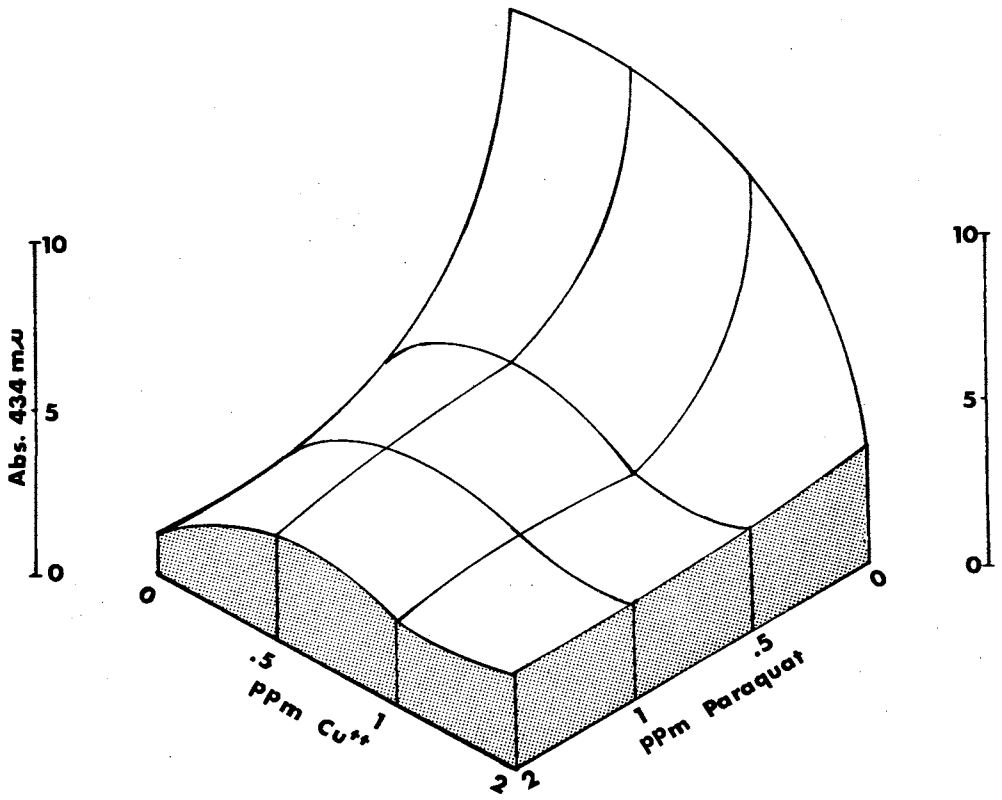


Fig. 3. Interaction of Cu^{++} and paraquat levels on C. pyrenoidosa extractable chlorophyll absorbance at 434 mμ.

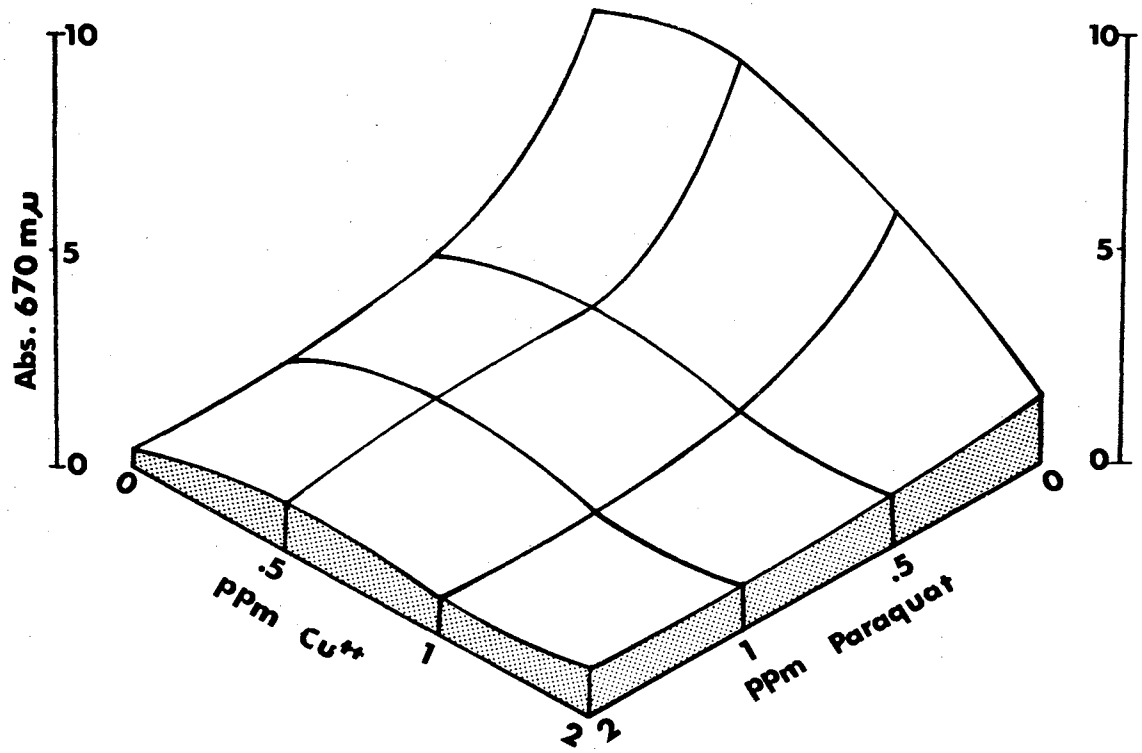


Fig. 4. Interaction of Cu^{++} and paraquat levels on C. pyrenoidosa extractable chlorophyll absorbance at 670 mμ.

also cyanide levels interacted with these other two factors. Highly significant F values resulted from treatments of paraquat level, copper level, cyanide level and with interactions, at both wavelengths, with a low C. V. of 8.65% at 434 mμ and 13.32% at 670 mμ wavelength.

TABLE V

EFFECT OF Cu^{++}, CN^{-} AND PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ

ppm paraquat	ppm CN^{-}	ppm Cu^{++} 0	2	sum
0	0	2.97	3.44	6.41
0	4	2.51	3.66	6.17
0	8	3.06	3.50	6.56
.5	0	2.85	3.13	5.98
.5	4	2.40	2.45	4.85
.5	8	2.58	1.82	4.40
2.0	0	2.40	1.25	3.65
2.0	4	2.06	.30	2.36
2.0	8	1.57	.36	1.93
sum		22.40	19.91	42.31

Significant F values:

Treatment 69.9
paraquat levels 390.2
Cu^{++} levels 24.9
CN^{-} levels 34.7
Interaction 26.2
C. V. 8.65%

Absorption at 670 mμ

ppm paraquat	ppm CN^{-}	ppm Cu^{++} 0	2	sum
0	0	.96	1.31	2.27
0	4	.90	1.32	2.22
0	8	1.01	1.24	2.25
.5	0	.87	1.05	1.92
.5	4	.80	.76	1.56
.5	8	.88	.66	1.54
2.0	0	.82	.44	1.26
2.0	4	.75	.06	.81
2.0	8	.64	.15	.79
sum		7.63	6.99	14.62

Significant F values:

Treatment 30.5
paraquat levels 161.5
Cu^{++} levels 5.8
CN^{-} levels 10.7
Interaction 14.1
C. V. 13.32%

Figures are sums of three replicate synchronized cultures with absorption O. D. determined from full spectra recordings.

The addition of copper at 2 ppm apparently did not reduce paraquat activity but was toxic to Chlorella cells at that level. However, cyanide concentrations of 4 and 8 ppm significantly reduced the inhibitory effect of copper as paraquat levels increased. It is believed that cyanide may block the electron transfer system for cupric to cuprous protein in oxidative metabolism, thus it may also block the inhibitory effect of copper in paraquat activity. The interaction between copper and cyanide was antagonistic, while the interaction between paraquat and cyanide was additive.

The treatments for the effect of CN^-, Cu^{++} and paraquat on absorbance recordings of Chlorella reported in Table VI were similar to those reported in Table V, except that CN^- levels were changed to 0, 16, and 32 ppm. Cyanide markedly reduced the activity of copper as an inhibition of paraquat. A comparison of the difference in the F values for these two experiments indicated higher significant F values for copper levels and cyanide treatment at both wavelengths than shown in Table V. An enhancement of cyanide activity with copper was found with the increased concentrations of cyanide. Rate of change at two cyanide levels was about the same for copper treated cultures.

TABLE VI

EFFECT OF CN^-, Cu^{++} AND PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ					Absorption at 670 mμ				
ppm paraquat	ppm CN^-	ppm Cu^{++} 0	2	sum	ppm paraquat	ppm CN^-	ppm Cu^{++} 0	2	sum
0	0	4.31	4.06	8.37	0	0	1.44	1.24	2.68
0	16	3.92	3.10	7.02	0	16	1.23	.98	2.21
0	32	4.19	3.07	7.26	0	32	1.41	1.20	2.61
.5	0	4.33	3.52	7.85	.5	0	1.38	1.20	2.58
.5	16	3.16	1.92	5.08	.5	16	.95	.54	1.49
.5	32	3.34	1.84	5.18	.5	32	.97	.43	1.40
2.0	0	3.91	1.04	4.95	2.0	0	1.28	.20	1.48
2.0	16	1.69	.00	1.69	2.0	16	.46	.00	.46
2.0	32	.55	.00	.55	2.0	32	.04	.00	.04
sum		29.40	18.55	47.95	sum		9.16	5.79	14.95

Significant F values:

Treatment 43.0
paraquat levels 201.6
Cu^{++} levels 125.3
CN^- levels 64.9
Interaction 6.1
C. V. 11.4%

Significant F values:

Treatment 19.8
paraquat levels 94.2
Cu^{++} levels 45.7
CN^- levels 27.9
Interaction 3.7
C. V. 24.49%

Figures are sums of three synchronized cultures with absorption O. D. determined from full spectra recordings.

The influence of time of incubation (16 to 48 hours) on 1 ppm paraquat reactions with Chlorella, using cell concentrations adjusted to O. D. readings of 0.2, 0.6, 0.9, levels, and extractable chlorophyll at 434 mμ and 670 mμ, is shown in Table VII. Differences in O. D. of non-treated and 1 ppm paraquat-treated cultures were increased with time period for all cell concentrations. Treatment F values at 434 mμ for an 0.2 O. D. cell concentration was 5.8 with C. V. of 5.46%, for the 0.6 O. D. level F was 41.4, with C. V. of 13.65% and for the 0.9 O. D. level F was 19.3, with C. V. of 45.76%. At 670 mμ the F value for treatment response at the 0.2 O. D. level was 1.9 which was not statistically significant. However, for 0.6 O.D. the F value was 24.1 and for 0.9 O. D., F was 35.9. The coefficients of variation were: 8.19% for 0.2 O. D., 12.05% for 0.6 O. D. and 8.21% for 0.9 O. D. These C. V. values were less than C. V. values at 434 mμ. Calculated equivalent dry cell concentrations as mg/ml at the respective readings were: 0.2 O. D. = 0.108 mg, 0.6 O. D. = 0.324 mg, and 0.9 O. D. = 0.486 mg. The concentration of cells at an O. D. of 1.0 was given by Lee (17) as 50×10^6 cells/ml.

TABLE VII

EFFECT OF TIME AND PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ — O. D. Cell concentration 0.2

Time	Exp. I	II	sum
16	1.295	1.46	2.755
24	1.525	1.671	3.196
32	1.537	1.566	3.103
40	1.395	1.705	3.10
48	1.405	1.86	3.265
sum	7.157	8.262	15.419

Significant F value:
Treatment 5.8
C. V. 5.46%

Absorption at 670 mμ — O. D. Cell concentration 0.2

Time	Exp. I	II	sum
16	.540	.560	1.10
24	.596	.677	1.273
32	.583	.615	1.198
40	.555	.649	1.204
48	.506	.749	1.255
sum	2.78	3.25	6.03

Significant F value:
Treatment 1.9
C. V. 8.19%

O. D. Cell concentration 0.6

Time	Exp. I	II	sum
16	1.07	.38	1.45
24	2.53	2.035	4.565
32	2.45	2.62	5.07
40	2.53	3.344	5.874
48	3.585	3.527	7.112
sum	12.165	11.906	24.071

Significant F value:
Treatment 41.4
C. V. 13.65%

Time	Exp. I	II	sum
16	.88	.74	1.62
24	1.484	1.662	3.146
32	1.595	1.793	3.388
40	1.210	1.995	3.205
48	1.895	2.233	4.128
sum	7.064	8.423	15.487

Significant F value:
Treatment 24.1
C. V. 12.05%

O. D. Cell concentration 0.9

Time	Exp. I	II	sum
16	.18	.05	.23
24	.47	.25	.72
32	.10	1.05	1.15
40	.90	2.52	3.42
48	3.03	2.49	5.52
sum	4.68	6.36	11.04

Significant F value:
Treatment 19.3, C.V. 45.76%

Time	Exp. I	II	sum
16	1.30	1.444	2.744
24	1.78	1.65	3.43
32	1.44	2.095	3.535
40	2.20	2.681	4.881
48	2.269	2.645	4.914
sum	8.989	10.515	19.504

Significant F value:
Treatment 35.9, C.V. 8.21%

Figures are sums from three replicates for two experiments, expressed as difference of O.D. of non-treated and 1 ppm paraquat-treated cultures.

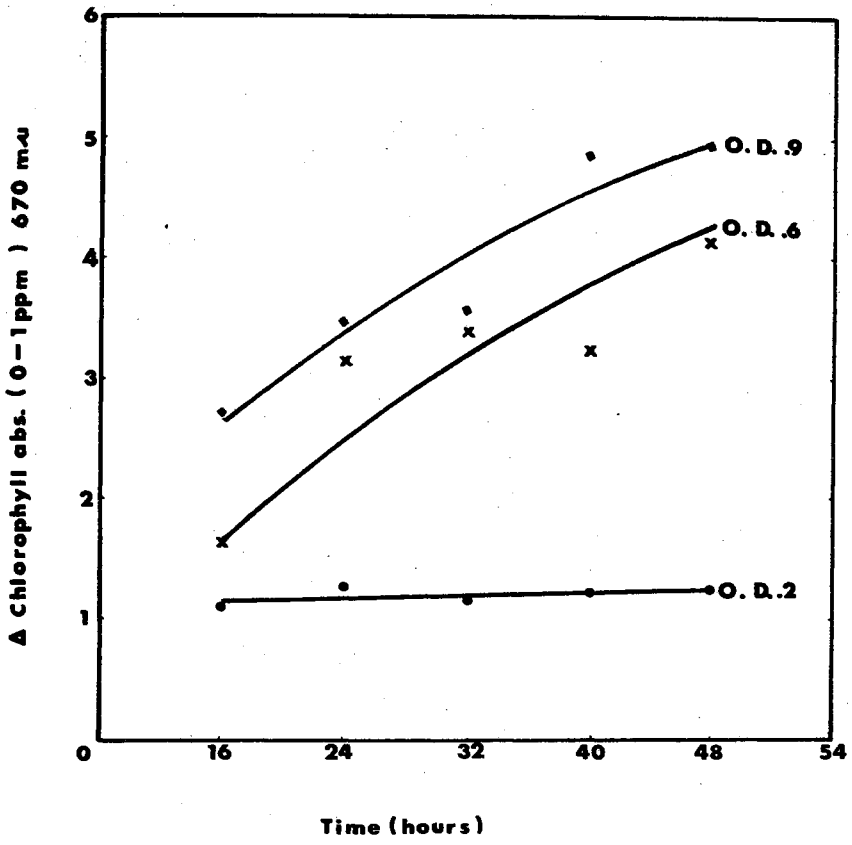


Fig. 5. Deviations in chlorophyll absorption with various Chlorella cell concentration and differential incubation periods at 434 mμ.

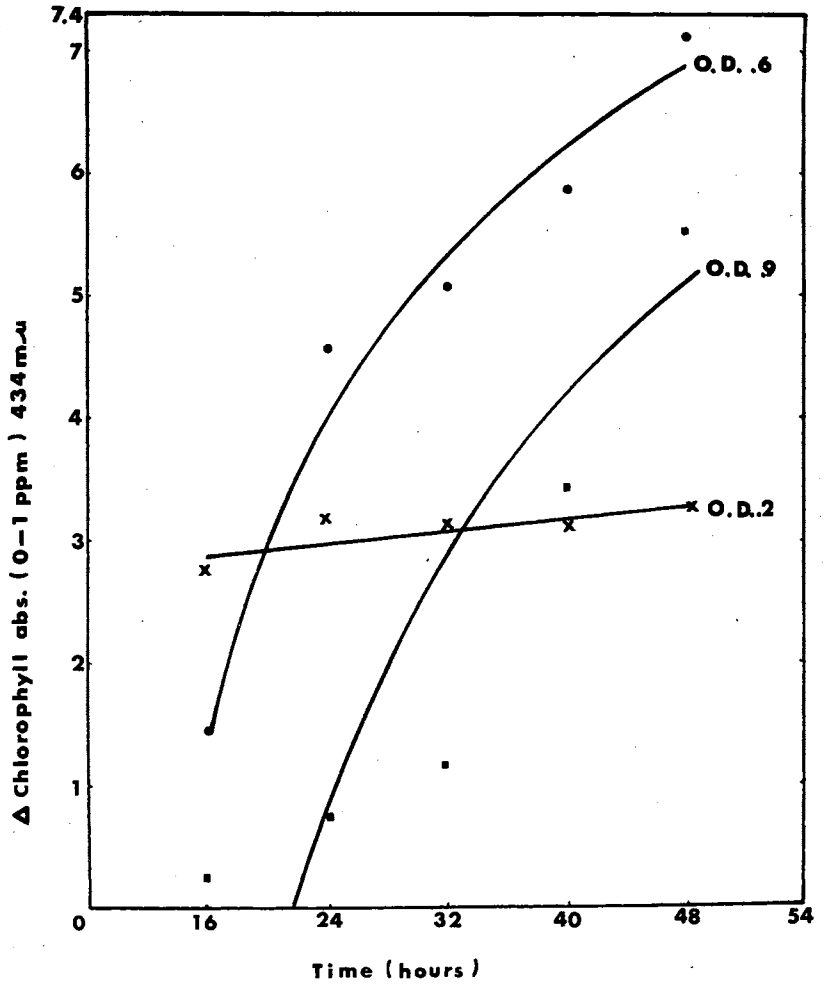


Fig. 6. Deviations in chlorophyll absorption with various Chlorella cell concentration and differential incubation periods at 670 mμ.

Adjustment of constant population per herbicide concentration resulted in reliable reaction rate characteristics for a uniform substrate per unit concentration of paraquat. Figures 5 and 6 illustrate this response. These results indicated a real influence of incubation time on rates of paraquat toxicity with all Chlorella cell concentrations. Also, these data indicated more effective detection of paraquat inhibition at 434 mμ than at 670 mμ. Differential effects of time on paraquat activity were apparently also of greater magnitude at 434 mμ wavelengths.

Effects of paraquat on extractable chlorophyll of Chlorella as a function of pre-darkness period before treatments are shown in Table VIII and Figure 7. Although some variations were apparent, a general trend for increase in paraquat phytotoxicity resulted with increased periods of pre-darkness treatment. Highly significant F values were obtained at 434 mμ for treatment 36.6, paraquat levels 241.3, dark periods 17.9, and for interactions 5.0, with a C. V. of 25.2% at 434 mμ.

A somewhat uniform trend, showing progressive paraquat activity with increased pre-darkness periods, was apparent at 670 mμ as shown in Table VIII and Figure 7. Highly significant F values were apparent for treatments 26.9, paraquat levels 164.1, dark periods 20.6 and for interactions 2.6 with C. V. of 34.93%. Near complete chlorosis was obtained at the

2.0 ppm paraquat level with all the pre-darkness treatments except at time 0.

Results indicate the influence of cell age, as determined by length of pre-darkness treatments, related to paraquat phytotoxicity.

TABLE VIII

EFFECTS OF DARK PERIODS BEFORE 40 HOURS LIGHT TREATMENT WITH PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ

ppm paraquat	Hours of dark period 0	24	48	72	96	120	sum
0	4.48	4.44	4.10	4.44	3.97	3.49	24.92
1	4.12	3.06	.93	2.07	.19	.69	11.06
2	1.21	.15	.00	.43	.00	.00	1.79
sum	9.81	7.65	5.03	6.94	4.16	4.18	37.77

Significant F values:
Treatment 36.6 paraquat levels 241.3
dark periods 17.9 interaction 5.0
C. V. 25.2%

Absorption at 670 mμ

ppm paraquat	Hours of dark period 0	24	48	72	96	120	sum
0	1.92	1.49	1.44	1.32	1.26	.93	8.36
1	1.31	.76	.02	.35	.00	.00	2.44
2	.49	.14	.00	.01	.00	.02	.66
sum	3.72	2.39	1.46	1.68	1.26	.95	11.46

Significant F values:
Treatment 26.9 paraquat levels 164.1
dark periods 20.6 interaction 2.6
C. V. 34.93%

Figures are sums of three replicate synchronized cultures with absorption O. D. determined from full spectra recordings.

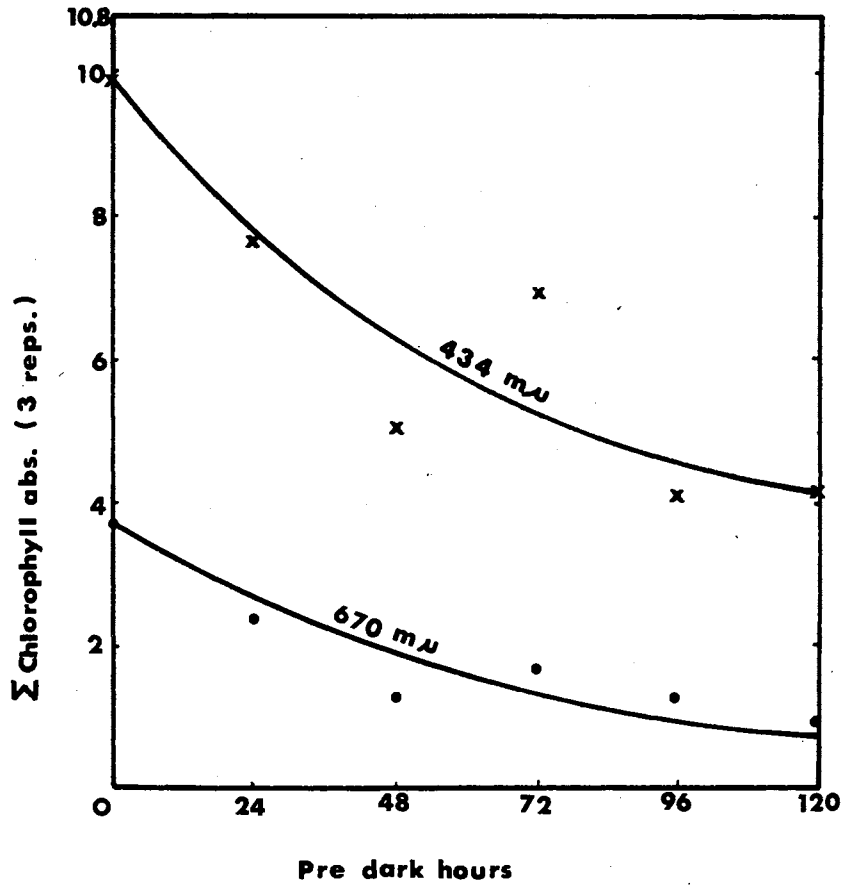


Fig. 7. Pre-darkness treatment effects on paraquat toxicity with C. pyrenoidosa.

Results in Table IX show the effect of pre-darkness treatment and light incubation before 1 ppm paraquat addition on Chlorella. Highly significant F values were obtained for treatments, 133.8, with C. V. 18.31% at 434 mμ and 73.2 for treatment F with C. V. 31.22% at 670 mμ.

In general, each additional increment of pre-darkness period resulted in increased paraquat activity, particularly at the 36 hour level at 434 mμ. However, the effect of light incubation before 1 ppm paraquat addition was less obvious than dark treatments. Non-paraquat treated Chlorella cultures were not affected by the pre-darkness treatments. Although less apparent at the 670 mμ wavelength, similar trends were also noted. Enhancement in chlorosis as a function of pre-dark treatment was apparent.

Differential pre-darkness periods prior to light incubation and paraquat treatments affected chlorophyll extraction of Chlorella as shown in Table X. Pre-darkness treatment significantly reduced Chlorella growth in paraquat treated cultures at 434 mμ. A similar response was obtained at 670 mμ wavelength, but it was not as well defined. Light incubation before addition of paraquat apparently reduced O.D. readings from extractable chlorophyll of Chlorella. Light incubation up to the 48 hour level resulted in near complete chlorosis for algal cells. This somewhat confirmed that light influence was responsible for the accelerated phytotoxicity

of paraquat in plant tissues.

TABLE IX

EFFECT OF DARK PERIOD TREATMENT BEFORE 1 PPM PARAQUAT AND LIGHT INCUBATION ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ

Pre-darkness hours	Paraquat addition at 1 ppm Hours incubation				
	none	0	12	24	36
12	4.5	.74			
24	4.5	.58	1.08		
36	4.5	.40	.47	.44	
48	3.65	.09	.07	.09	.07

Significant F value: Treatment 133.8 C. V. 18.31%

Absorption at 670 mμ

Pre-darkness hours	Paraquat addition at 1 ppm Hours incubation				
	none	0	12	24	36
12	2.77	.14			
24	2.06	.08	.25		
36	2.28	.02	.06	.03	
48	1.20	.04	.03	.03	.03

Significant F value: Treatment 73.2 C. V. 31.22%

Absorption as O. D. of three replicate cultures determined from full spectra recordings.

TABLE X

EFFECT OF DARK PERIOD TREATMENT BEFORE 1 PPM PARAQUAT ADDITION AND LIGHT INCUBATION ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ

Pre-darkness	Paraquat addition at 1 ppm Hours incubation				
hours	none	0	24	48	72
24	4.5	1.87			
48	4.5	1.35	.66		
72	3.99	.74	.20	.00	
96	.83	.08	.04	.01	.01

Significant F values: Treatment 84.6 C. V. 23.96%

Absorption at 670 mμ

Pre-darkness	Paraquat addition at 1 ppm Hours incubation				
hours	none	0	24	48	72
24	2.15	.64			
48	1.85	.34	.16		
72	1.07	.13	.01	.00	
96	.160	.01	.00	.00	.00

Significant F values: Treatment 54.5 C. V. 36.29%

Absorption as O. D. of three replicate cultures determined from full spectra recordings.

Table XI presents data from studies with light composition and paraquat phytotoxicity to Chlorella. As paraquat concentration increased, the absorption readings of extractable chlorophyll from algae gradually decreased.

Influence of light composition on paraquat activity was somewhat erratic at 434 mμ, with a high C. V. of 41.6%. Significant F values were obtained: for treatment levels 12.1, paraquat levels 25.8, light composition 19.5, and for interaction 2.8. Extractable chlorophyll readings from the cultures that were covered with the blue filter, showed the most effective recovery from paraquat toxicity, but there was little difference between the red filter and nonfiltered light treatments. The absorption region of Chlorella was in the red region as shown by covering with red filter (long wavelengths, 580 to 700 mμ), while with the blue filter it was in the blue region (short wavelengths, 380 to 580 mμ). The decline in photosynthetic efficiency at long wavelengths within the red absorption band of chlorophyll, discovered by Emerson & Lewis (19), might be attributed to coexisting active and inactive forms of chlorophyll "a" with the inactive form absorbing at longer wavelengths. Wavelengths longer than 680 mμ were beyond the range of maximum efficiency of chlorophyll for photosynthesis in Chlorella. The sum of absorbance readings for the blue filter treatment was about double those from the control and red filter treatments.

This suggested an enhancement in photosynthesis by short wavelengths for Chlorella.

Similar results were obtained at the 670 mμ level, except that the F value for interaction between paraquat and light was not significant. The algal cells appeared to react to paraquat and light composition treatments separately rather than showing interactions to the combination.

TABLE XI

EFFECT OF LIGHT COMPOSITION AND PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ

Light filter	ppm paraquat 0	1	2	10	sum
None	4.5	1.0	.065	.060	5.625
Blue	4.5	3.85	3.78	2.29	14.42
Red	4.5	.75	1.516	.10	6.866
sum	13.5	5.60	5.361	2.45	26.911

Significant F values:
Treatment 12.1 paraquat levels 25.8
light compositions 19.5, interaction 2.8
C. V. 41.6%

Absorption at 670 mμ

Light filter	ppm paraquat 0	1	2	10	sum
None	2.03	.365	.01	.05	2.455
Blue	2.84	1.48	1.46	.94	6.72
Red	1.72	.22	.481	.05	2.471
sum	6.59	2.065	1.951	1.04	11.646

Significant F values:
Treatments 20.3 paraquat levels 49.0
light compositions 35.7 C. V. 36.37%

Figures are sums of three replicate synchronized cultures with absorption O. D. determination from full spectra recordings.

Light composition resulted in varied effects on paraquat toxicity with Chlorella cells as indicated in Table XII. Significant F values were obtained for treatments, paraquat levels, light composition and interactions between paraquat and light at both wavelengths. Symptoms of paraquat toxicity accelerated with increased concentration up to 2.0 ppm in Chlorella cells. All the paraquat levels of 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ppm, with both blue and black light filter treatments, resulted in an inhibitory effect of the herbicide on algal growth at 434 mμ.

Marked reduction in paraquat activity was evident for blue and black filter treatments as indicated by extractable chlorophyll absorbance at 670 mμ. F values for paraquat and light were higher at this wavelength indicating greater response at 670 mμ in this experiment.

Light composition and copper levels modified paraquat phytotoxicity to Chlorella as shown in Table XIII. These data confirmed an apparent ability of the blue filter for algae cells to offset paraquat toxicity as indicated in Table XI. Statistical analyses of these results showed highly significant values from treatments, paraquat, copper effects and light compositions with C. V. of 13.39% at 434 mμ and 24.23% at 670 mμ respectively. A significant F value for treatment interaction was 6.0 at 434 mμ and 3.4 at 670 mμ. It was apparent that more effective paraquat inhibition by

the blue filter and copper addition treatment was detected at 434 mμ than at 670 mμ. However, in comparison with the paraquat and light effects, copper levels at both wavelengths resulted in the least significant F values.

TABLE XII

EFFECT OF LIGHT COMPOSITION AND PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ					Absorption at 670 mμ				
ppm paraquat	Light filter None	Black	Blue	Sum	ppm paraquat	Light filter None	Black	Blue	Sum
0	3.0	3.0	3.0	9.0	0	2.46	2.83	2.85	8.14
.5	2.99	3.0	3.0	8.99	.5	2.12	2.63	2.70	7.45
1.0	2.96	3.0	3.0	8.96	1.0	1.36	2.78	2.82	6.96
1.5	2.99	2.99	2.99	8.97	1.5	1.91	2.93	2.52	7.36
2.0	1.85	3.0	3.0	7.85	2.0	.70	2.63	2.74	6.07
2.5	1.72	2.98	2.98	7.68	2.5	.76	2.6	2.73	6.09
3.0	.36	2.98	2.99	6.33	3.0	.04	1.89	2.5	4.43
sum	15.87	20.95	20.96	57.78	sum	9.35	18.29	18.86	46.5

Significant F values:
Treatment 5.40
paraquat levels 4.5
light 13.1
interaction 4.3
C. V. 15.73%

Significant F values:
Treatment 12.8
paraquat levels 9.7
light 65.1
interaction 3.9
C. V. 15.95%

Figures are sums of duplicate synchronized cultures with absorption O. D. determined from full spectra recordings.

TABLE XIII

EFFECT OF LIGHT COMPOSITION, COPPER AND PARAQUAT LEVELS ON C. PYRENOIDOSA EXTRACTABLE CHLOROPHYLL ABSORBANCE

Absorption at 434 mμ

ppm Cu^{++}	ppm paraquat	Red	None	Blue	Sum
0	0	3.10	3.10	3.25	9.45
.5	0	1.95	1.73	3.00	6.68
2.0	0	1.35	2.80	3.25	7.40
0	.5	1.555	1.37	3.15	6.075
.5	.5	.94	1.53	2.895	5.365
2.0	.5	1.175	1.44	3.20	5.815
0	2.0	.39	.57	2.90	3.86
.5	2.0	.28	.151	2.00	2.431
2.0	2.0	.46	.340	2.35	3.15
Sum		11.20	13.031	25.995	50.226

Significant F values:
Treatment 37.2 paraquat levels 174.1 interaction 6.0
Cu^{++} levels 21.4 light composition 227.3, C. V. 13.93%

Absorption at 670 mμ

ppm Cu^{++}	ppm paraquat	Red	None	Blue	Sum
0	0	.93	.97	.895	2.795
.5	0	.515	.415	.66	1.59
2.0	0	.383	.665	.85	1.898
0	.5	.45	.39	.97	1.81
.5	.5	.251	.455	.79	1.496
2.0	.5	.37	.365	.88	1.615
0	2.0	.136	.183	.87	1.189
.5	2.0	.083	.051	.482	.616
2.0	2.0	.144	.115	.65	.909
Sum		3.262	3.609	7.047	13.918

Significant F values:
Treatment 11.6 paraquat levels 46.2 interaction 3.4
Cu^{++} levels 16.1 light composition 62.4 C. V. 24.23%

Figures are sums of duplicate synchronized cultures with absorption O. D. determination using Spectronic 20 Spectrophometer at the specified wavelengths.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objective of this study was to determine factors that affect the herbicidal activity of paraquat and that control its mechanisms in plants. The present investigation described the results of culture studies using unicellular green alga, C. pyrenoidosa, in experiments designed to provide an insight into the mode of action of paraquat.

Sulfate salts at 200 ppm level, in ratio of $\frac{K^{+}}{\sqrt{\frac{Ca^{++}+Mg^{++}}{2}}} = 1$, greatly influenced the dithionite-paraquat reaction with absorption at 394 mμ. Salt concentration at the 100 ppm level had little effect.

Copper apparently inhibited paraquat phytotoxicity to Chlorella cultures. However, the highest inhibiting concentration was 0.5 ppm Cu^{++} with apparent toxic effects at the 2 ppm level. Concentrations of CN^{-} markedly reduced the inhibitory effect of copper as paraquat levels increased. It was believed that cyanide might react with cuprous protein to block oxidative metabolism, thus it might interfere with the copper-paraquat interaction.

The effect of incubation time and pre-darkness treatments on paraquat activity indicated a general trend for increase in paraquat phytotoxicity with increased periods of pre-darkness before culture incubation in light. Significant reduction in paraquat activity was evident for blue and black light filter treatments as indicated by extractable chlorophyll absorbance at both 434 and 670 mμ.

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