EFFECTS OF THE BIPYRIDYLIUM HERBICIDES PARAQUAT AND DIQUAT ON THE ULTRASTRUCTURE AND PHYSIOLOGY OF A DUCKWEED AND A BLUE-GREEN ALGA

by

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being a thesis presented in canditature for the degree of

DOCTOR OF PHILOSOPHY

of

THE UNIVERSITY OF STRATHCLYDE

GLASGOW

1976

DEPARTMENT OF BIOLOGY

CONTENTS

SUMMARY

Electron microscopical techniques were used to investigate the effects of treatment with paraquat and diquat on cell structure in the duckweed Spirodela oligorrhiza and the blue-green alga Anabaena cylindrica.

Treatment of fronds of S. oligorrhiza with both herbicides in the light resulted in ultrastructural damage which clearly preceded any outwardly visible changes in their appearance. Ultrastructural changes developed rapidly, occurring first in the presence of diquat. Paraquat and diquat also caused ultrastructural damage when applied in complete darkness but did so at a much slower rate.

The absence of appreciable amounts of catalase or peroxidase activity within the chloroplasts and mitochondria of frond cells was demonstrated by a histochemical technique.

The growth of S. oligorrhiza in the light was inhibited by low concentrations of paraquat and diquat. Diquat had the greater inhibitory influence in terms of the reduction of frond number and fresh and dry weights compared with controls. Reduction of the growth rate was detected with both herbicides before the appearance of outwardly visible symptoms of toxicity.

Paraquat and diquat caused a rapid destruction of chlorophyll in light-treated fronds, the loss being greater in the presence of diquat. The continued loss of chlorophyll from diquat-treated fronds was shown to be light-dependent. The ability of diquat to cause the loss of chlorophyll from treated fronds could be lessened by the simultaneous application of a tetrazolium salt.

Both paraquat and diquat significantly increased the rate of respiration in the dark and caused a rapid inhibition of apparent photosynthesis in the light. The stimulation of respiration and the inhibition of photosynthesis was greater with diquat.

Low concentrations of paraquat and diquat caused rapid ultrastructural damage in vegetative cells of A. cylindrica in the light. Under the conditions employed, both herbicides appeared to produce identical effects at about the same rate. The first observable symptom was the gradual disappearance of the polyglucoside granules. This occurred prior to any visible membrane damage. Subsequently, the plasma membrane disintegrated and cell structure deteriorated rapidly. Damage to the heterocysts also occurred in the light but at a slower rate than in vegetative cells. In the akinetes no effects were noted after periods sufficiently long to allow the total destruction of the other cell types.

In darkness, diquat caused no visible effects in vegetative cells after durations causing their complete destruction in the light.

Electron microscopical evidence was obtained pointing to the inhibition of carbon fixation in diquat-treated vegetative cells in the light prior to ultrastructural damage. Manometric investigations with A. cylindrica also revealed the rapid complete inhibition of apparent photosynthesis in the presence of paraquat or diquat. There was no significant difference between the effects of paraquat and diquat on the rate of apparent photosynthesis. The time required to completely inhibit apparent photosynthesis was far shorter than the period required to cause visible ultrastructural damage to the cells.

The results of the investigations carried out with S. oligorrhiza

and A. cylindrica are discussed in relation to existing information concerning the effects and mode of action of the bipyridylium herbicides.

INTRODUCTION

Paraquat (1,1' - dimethyl - 4,4' - dipyridylium ion) and diquat (1,1' - ethylene - 2,2' - bipyridylium ion) are bipyridylium quaternary ammonium salts with a high degree of herbicidal activity. The phytotoxicity of diquat was first reported by Brian, Homer, Stubbs & Jones (1958). Subsequently, Homer, Mees & Tomlinson (1960) investigated the herbicidal properties of a range of related compounds and reported the high activity of paraquat.

Diquat is manufactured as its dibromide salt while paraquat is manufactured as its diiodide or dimethyl sulphate salts. According to Homer et al. (1960), the herbicidal activity of bipyridylium herbicides is associated solely with the organic cations. Since their discovery, paraquat and diquat have been used extensively for weed control in both terrestrial and aquatic environments. As herbicides they are extremely fast-acting but are rather non-selective in their action. The past quarter century or so has seen the emergence of a long list of chemical weapons for use against weeds. More recently, concern has arisen about the long term consequences arising from the widespread use of herbicides. However, the bipyridylium herbicides are outstanding in that they are rendered biologically inactive on contact with soils owing to strong adsorption on soil colloids. The degradation of both herbicides is believed to be brought about partly by photochemical means and partly by the activities of microorganisms.

Due perhaps largely to the low application rates at which paraquat and diquat are used and to the speed with which they are inactivated on contact with soil, the use of these herbicides appears not to result in undesirable side effects on the environment.

At recommended field rates, paraquat and diquat appear to have no appreciable influence on the general microbial activity contributing towards soil fertility (Calderbank, 1968). In aquatic ecosystems paraquat and diquat do not persist for long periods and the bulk of the available evidence suggests that these herbicides offer little hazard to aquatic animals (Calderbank, 1968 & Mullison, 1970). Bipyridylium herbicides are certainly potentially lethal to wild animals and man if administered in large enough doses. In recent years, a number of human deaths both accidental and intentional, have resulted from the ingestion of these chemicals. However, since the residues of paraquat and diquat generally found in food crops are either immeasurable or very low (Calderbank, 1968), it is considered unlikely that bipyridylium herbicides will give rise to toxic symptoms in man under normal conditions.

Homer & Tomlinson (1959) and Homer et al. (1960) showed that bipyridylium compounds having herbicidal properties could be converted upon reduction to stable, water soluble free radicals by the uptake of a single electron and that the reduction was freely reversible in the presence of molecular oxygen. It was therefore postulated that the herbicidal activity of bipyridylium herbicides involved reduction within plants to their free-radical forms. Subsequent physiological and biochemical investigations have confirmed and extended this original hypothesis. Mees (1960) demonstrated that although diquat-treatment caused death in darkness, it was more effective in the light. Subsequent workers using paraquat or diquat also observed higher herbicidal activity in the light (Boon, 1964; Blackburn & Weldon, 1965; Merkle et al, 1965; Brian, 1967, Sue-Fei

Tsay, Jhy-Mei & Lynd, 1970; Harris & Dodge, 1972b). Mees (1960) also showed that the rate of herbicidal activity was proportional to incident light intensity. He further showed that the damage caused by diquat was more rapid in green than in etiolated shoots.

Such findings pointed to the importance of photosynthesis as the main source of reducing power leading towards the formation of bipyridylium free radicals. Mees (1960) also showed that oxygen was necessary for the rapid action of diquat in the light. He obtained an almost immediate inhibition of herbicidal activity by the removal of oxygen and showed that the toxicity of diquat could be increased by raising the oxygen tension.

Originally it was thought that the bipyridylium radicals formed by the reduction of the herbicide cations within the plant were responsible for bringing about the death of treated plants. However, Mees (1960) suggested that hydrogen peroxide may be formed within the plants during reoxidation of the free radical by molecular oxygen. An investigation by Davenport (1963) has in fact provided indirect evidence for the formation of hydrogen peroxide by isolated chloroplasts upon illumination in the presence of diquat.

According to the current scheme accounting for the mode of action of bipyridylium herbicides the herbicide cation readily accepts a single electron from a suitable source of reducing power and is thus converted to its free radical form. In the presence of molecular oxygen herbicide free radicals are then thought to undergo rapid reoxidation to the original cations and at the same time hydrogen peroxide is formed. The hydrogen peroxide itself, or toxic radicals derived from it, may then attack the cells in which they are formed

and so bring about the rapid death of the plant. The regenerated herbicide cations may undergo continuous reduction and reoxidation within the plant thereby, leading to a build-up of hydrogen peroxide.

Owing to the influence of light on the speed with which toxic symptoms develop, photosynthesis is thought to be the prime source of reducing power for the bipyridylium herbicides. In the dark however, photosynthetic light reactions are no longer available for the provision of reducing power and it is believed that respiration may be involved. This mode of action theory is supported by the findings of a variety of workers and of work reported here.

At the onset of the present investigations (Oct. 1969) little was known about the effects of paraquat or diquat on plants at the ultrastructural level. Baldwin, Dodge & Harris (1968) briefly described some of their ultrastructural findings concerning paraquat-treated flax cotyledons but published no micrographs. One of the major purposes of the present work was to obtain information concerning this aspect of herbicidal activity. Since this work was begun, a small number of reports in this area of research have emerged.

Firstly, Baur, Bovey, Baur & el Seify (1969) published micrographs showing the breakdown of fine structure of mesquite mesophyll cells following treatment with paraquat. Following this, Stokes, Turner & Markus (1970) published an account of the ultrastructural changes in diquat-treated cells of Chlorella vulgaris. Dodge (1971) and Harris & Dodge (1972a) have since published micrographs showing the effect of paraquat on the ultrastructure of flax cotyledon leaf cells and recently Dodge & Lawes (1974) described the changes occurring in flax cotyledon leaf cells after treatment with diquat.

All of the investigations reported so far have dealt with the effects of treatment on plants with eukaryotic cellular organizations. In the work reported here the effects of both paraquat and diquat on plants with both eukaryotic and prokaryotic cellular organizations are investigated.

DUCKWEED STUDIES

INTRODUCTION

The duckweeds are small, free-floating monocotyledonous water plants belonging to the family Lemnaceae. These plants have no leaves but have a flat green floating blade or frond which is regarded as a modified stem performing leaf functions (Willis, 1948). Duckweeds occur mainly in still or slightly moving water and many species have an almost world-wide distribution Although capable of sexual reproduction, duckweeds reproduce most commonly by vegetative means. Mother fronds give rise to vegetatively produced daughter fronds which in turn may form their own daughter fronds before becoming detached from the original parent frond. The resulting collections of attached fronds are referred to as colonies.

The fronds of the duckweeds have very little vascular tissue (Hillman, 1961) and are composed largely of chlorenchymatous cells with large gas-filled intercellular spaces which aid buoancy. In some species e.g. Lemna gibba, these spaces may be relatively large. Duckweeds belonging to the genera Lemna and Spirodela have one and two or more adventitous roots per frond. The lengths of the roots also vary from species to species and with the environmental conditions (Hillman, 1961). These roots are considered to be of little importance in the uptake of nutrients from the medium since such materials can readily enter the fronds through the lower epidermis. Evidence for the greater importance of the lower epidermis as an absorbing surface has come from investigations in which the undersides of fronds have been covered with lanolin (Gorham, 1941; Blackman & Robertson-Cunninghame, 1955).

Duckweeds are eaten by a variety of birds and fish but are of little or no economic importance. However, under suitable conditions, especially in the presence of a rich supply of nutrient salts, duckweeds may multiply rapidly

and cover the surface of large bodies of water. Such heavy growths reduce gas exchange between the air and the water beneath and also interfere with the penetration of light, thereby setting up conditions unfavourable for rooted plants and many aquatic animals.

While the Lemnaceae may be of little economic importance, they have a number of characteristics which make them useful research tools in many areas of plant physiology. Firstly, due to their small size duckweeds can be grown or treated in large numbers in a minimum of space. Their small size also makes the control of environmental conditions more simple. Also, the modest dimensions of duckweeds together with their relative structural simplicity may facilitate many physiological investigations. For example the duckweed plant may be used *in toto* in gas exchange studies employing conventional manometric techniques. The effects of externally applied chemicals on the more advanced angiosperms are often greatly dependent upon the extent of translocation within the plants which in turn may vary greatly under different environmental conditions. By using the duckweeds such complications are largely avoided. Thirdly, genetic variability between test plants can be eliminated by using fronds from a single duckweed clone since reproduction is usually achieved by vegetative means.

The useful characteristics of the Lemnaceae in general have been exploited by workers in a variety of fields of study. Much of this work has been reviewed by Hillman (1961). Duckweeds have been used in herbicide research where a variety of different chemicals, including paraquat and diquat have been tested. These plants were found to have a high sensitivity to both paraquat and diquat and this has led to the use of the duckweeds in bioassay tests for the bipyridylium herbicides (Funderburk & Lawrence, 1963; Blackburn & Weldon, 1965; Damanakis, 1970).

An investigation of the available literature suggests that the Lemnaceae have received scant attention from electron microscopists. DeKock & Innes (1970) demonstrated the ultrastructural disorganization in cells of Lemna minor following treatment with amitrole. There would appear to be no reports of similar studies with duckweeds treated with bipyridylium herbicides.

The first major problem associated with the investigations carried out with the duckweeds was to obtain a good reproducible fixation procedure for this type of tissue. Once this was obtained, duckweed plants and the techniques of electron microscopy were used to study the effects of paraquat and diquat on eukaryotic photosynthetic cells. In addition these plants were used to investigate the effects of paraquat and diquat on various aspects of plant physiology.

I(A) MATERIALS AND METHODS

A. Plant Material

The higher plants investigated all belong to the family Lemnaceae. These were *Spirodela oligorrhiza* (Kurz) Hegelm., *Lemna gibba* L. and *Lemna minor* L. The duckweeds *S. oligorrhiza* and *L. gibba* were propagated from cultures kindly donated by Dr. P. C. DeKock of the Macaulay Institute for Soil Research, Aberdeen. The *L. minor* was initially obtained from a fresh water aquarium.

B. Chemicals

The two bipyridylium herbicides which have been used in this work are paraquat (1,1' - dimethyl - 4,4' - bipyridylium cation) and diquat (1,1' - ethylene - 2,2' - bipyridylium cation). Pure samples of both herbicides were kindly donated by Plant Protection Ltd., Jealott's Hill Research Station, Bracknell, Berks. The paraquat was supplied as paraquat dichloride and the diquat as diquat dibromide monohydrate. The herbicide stock solutions used throughout this work were made up in distilled water and in many experiments the herbicides were supplied to the plants in concentrations calculated in ppm. of the salts. However, where the effects of paraquat and diquat were to be compared in the same experiment, concentrations were calculated in ppm. of the cation.

1.0 ppm. paraquat (salt) = 3.9×10^{-6} M paraquat (salt)

1.0 ppm. paraquat (cation) = 5.4×10^{-6} M paraquat (cation)

1.0 ppm. diquat (salt) = 2.9×10^{-6} M diquat (salt)

1.0 ppm. diquat (cation) = 5.4×10^{-6} M diquat (cation)

Unless otherwise stated, all other chemicals were obtained from British Drug Houses, Poole, Dorset, England.

C. Culture Conditions

After initial trials on a variety of culture media under differing conditions, the duckweeds S. oligorrhiza, L. gibba and L. minor were finally grown in 250 ml. Erlenmeyer flasks containing 100 ml. Hutner's medium (Hutner, 1953) which had been diluted 1 in 3 with distilled water and adjusted to pH 5.6. Stock cultures were maintained at room temperature ($22 \pm 2^{o}C$) under continuous illumination supplied by an array of Crompton 80W white fluorescent lamps. The light intensity at culture level was 3500 lux. Under these conditions growth was rapid, a doubling of frond number being achieved approximately every 2 days with S. oligorrhiza.

In order to inhibit algal contamination of these cultures, two procedures were employed throughout. Firstly, the bottom portions of the Erlenmeyer flasks were painted black up to the surface of the culture solution. Secondly, cultures were routinely washed in Buchner funnels held for 1-2 minutes under cold, running water prior to sub-culturing. Together, these methods proved to be highly effective in producing duckweed fronds which were free from unwanted algae.

D. Electron Microscopy

For purposes of uniformity, the duckweed tissues which were processed for electron microscopy were always selected from the middle portions across the breadth of the fronds. In this way, both the youngest and the oldest cells were omitted from study.

A variety of fixation schedules was initially employed with tissue from untreated fronds of S. oligorrhiza, L. gibba and L. minor.

1. Permanganate Fixation

In attempts to achieve adequate fixation using potassium permanganate

the following procedures were carried out at different times after first carefully cutting the frond tissue into approximately 1 mm 2 segments in a little of the fixative using a sharp razor blade

(i) 2% aqueous potassium permanganate (Mollenhauer, 1959) at room temperature (22 $\pm$ 2^{o}C) for a) 30 minutes and b) 60 minutes.

(ii) 1.2% aqueous potassium permanganate at room temperature for a) 15 minutes, b) 30 minutes, c) 45 minutes and d) 60 minutes.

Procedures (i) and (ii) were repeated employing vacuum infiltration with the fixative for the first 15 minutes.

(iii) 1% aqueous potassium permanganate at room temperature for a) 5 minutes, b) 10 minutes, c) 15 minutes, d) 20 minutes, e) 30 minutes and f) 60 minutes.

(iv) 2% aqueous potassium permanganate at 4^{o}C for 60 minutes.

(v) 2% aqueous potassium permanganate at room temperature for 60 minutes without prior cutting into 1 mm squares.

2. <u>Glutaraldehyde/$KMnO_4$ Fixation</u>

1 mm^2 portions of frond tissue were initially fixed for 2 hours at room temperature with 3% glutaraldehyde in 0.05M sodium cacodylate buffer, pH7.2. After washing in 3 changes of fresh buffer for 2 hours, the tissue was post-fixed in 1% aqueous potassium permanganate for a) 5 minutes, b) 10 minutes and c) 15 minutes.

3. <u>Glutaraldehyde/osmium tetroxide Fixation</u>

(i) 1 mm^2 segments of frond tissue were fixed with 3% gluteraldehyde in 0.05M sodium cacodylate buffer, pH7.2 for 2 hours at room temperature. In order to overcome the reluctance of the

tissue to remain submerged during fixation, the procedure was carried out in completely filled screw top bottles placed on a rolling machine. After this period of gluteraldehyde fixation, the tissue segments remained submerged throughout all subsequent treatment without further help. Following washing in 3 changes of fresh buffer over a 2 hour period, the tissues were post-fixed with 1% osmium tetroxide in 0.05M sodium cacodylate buffer, pH7.2 for 2 hours at room temperature.

(ii) The procedure as in (i) above was repeated employing vacuum infiltration with the glutaraldehyde for the first 15 minutes.

(iii) The procedure as in (i) above was repeated with the fixatives buffered with 0.05M potassium phosphate buffer, pH 6.8

All subsequent fixation schedules were employed with S. oligorrhiza only.

(iv) In attempts to improve the degree of tissue preservation, 1 Mm^2 segments of S. oligorrhiza were subjected to the following fixation regimes. In all cases, both the glutaraldehyde and the osmium tetroxide were made up in 0.05M potassium phosphate buffer, pH6.8. Gluteraldehyde fixation was carried out in completely filled screw cap bottles placed on a rolling machine.

3% glutaraldehyde	Buffer	1% osmium tetroxide
a) 2h. at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$
b) 2h. at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$	4h. at $22\pm2^{o}C$
c) overnight at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$
d) overnight at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$	4h. at $22\pm2^{o}C$
e) 2h. at $4^{o}C$	2h. at $4^{o}C$	2h. at $4^{o}C$
f) 2h. at $4^{o}C$	2h. at $4^{o}C$	4h. at $4^{o}C$
6% glutaraldehyde	**Buffer**	**1% osmium tetroxide**
g) 2h. at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$
h) 2h. at $22\pm2^{o}C$	2h. at $22\pm2^{o}C$	4h. at $22\pm2^{o}C$

(v) In a further attempt to improve fixation, fronds were initially sectioned across their breadth in such a way as to produce comparatively large blocks of tissue with dimensions of up to 3.0 x 1.5 mm. Fixation with 3% glutaraldehyde in 0.05 M potassium phosphate buffer pH6.8 was carried out in completely filled capped bottles on a rolling machine for 1.5 hours at room temperature. The tissue blocks were then sliced into smaller pieces of dimensions up to 1.0 x 1.5 mm and fixation was continued as before for a further 0.5 hour. After washing in fresh buffer for 2 hours with 3 changes the tissue segments were post-fixed with 1% osmium tetroxide in the same buffer for 3 hours at room temperature.

On completion of the fixation period, permanganate-fixed tissues

were washed for 1 hour in 3 changes of distilled water. Glutaraldehyde/ osmium tetroxide-fixed material was washed for the same length of time with 3 changes of the appropriate buffer.

Subsequently, fixed material was dehydrated through the following ethanol series:

25% ethanol	30 minutes
50% "	"
75% "	"
90% "	"
95% "	"
Absolute ethanol	"
Absolute ethanol	overnight
Absolute ethanol	30 minutes

After this dehydration schedule, the specimens were transferred to propylene oxide for 30 minutes with 2 changes. The specimens were then infiltrated with either

(a) Araldite obtained from Ciba-Geigy (U.K.) Ltd., Duxford, Cambridge, England.

or (b) Taab Embedding Resin obtained from Taab Laboratories, Emmer Green, Reading, England.

These embedding media were prepared as follows: -

<u>Ciba Araldite</u>

Araldite CY 212	20 ml.
Hardener 964 B	20 ml.
Accelerator DY 064	0.6 ml.
Dibutyl phthalate	2 ml.

Mixing was facilitated by placing both the Araldite and the Hardener separately in an oven at 60°C for 15 minutes prior to making up the medium. After addition of the four components, the mixture was stirred vigorously with a glass rod for 10 minutes. The air bubbles which developed during this procedure were removed by placing the mixture in an oven at 60°C for a few minutes (Glauert, 1965)

Taab Embedding Resin

Resin	25 ml.
DDSA	12.5 ml.
MNA	12.5 ml.
DMP-30	1 ml.

This resin was mixed as above.

Sufficient of these resin mixtures was added to the specimens in propylene oxide contained in uncapped glass bottles to give a propylene oxide: resin ratio of 3:1. After thorough mixing, the specimens were left to stand in this mixture in a fume cupboard at room temperature for 48 hours to allow the propylene oxide to evaporate and the resin to slowly increase to full concentration (Juniper, Cox, Gilchrist and Williams, 1970). The specimens were then transferred to fresh resin and left to soak at room temperature for a further 24 hours to ensure complete infiltration.

After infiltration was complete, the tissue segments were withdrawn with a Pasteur pipette and excess resin was removed from them by lightly dabbing them onto lens tissue. With the aid of a needle the segments were quickly transferred onto the surface of Araldite-filled gelatin capsules (size 00) or Taab capsules filled with Taab resin. The resin contained within the capsules was always prepared immediately before use.

The tissue segments were left to sink to the bottom of the capsules and kept overnight at room temperature. Finally, the resins were polymerised by placing the capsules in an oven at 60°C for 48 hours.

Silver-grey sections (600-700 Å thickness) were cut on an LKB "Ultratome" III using glass knives and picked up on fine mesh (200 meshes/inch) copper grids either uncoated or coated with formvar (Pease, 1964). The sections on the grids were then stained in 2% aqueous uranyl acetate (glutaraldehyde/osmuim tetroxide-fixed tissue only) and lead citrate (Reynolds, 1963). Observations were carried out using an AEI EM6B electron microscope which was operated at an accelerating voltage of 60 kV.

E. Herbicide Treatment

Of the various fixation schedules employed for the higher plant material, the best degree of tissue preservation was achieved with glutaraldehyde followed by osmium tetroxide as described in 3 (v) above. Using this method of fixation, investigations were made into the effects of various treatments on the ultrastructure of S. oligorrhiza. To this end, young, actively-growing 3-frond colonies of S. oligorrhiza were placed in covered Petri dishes containing 40 ml. 1/3 strength Hutner's medium (pH 5.6) incorporating the desired concentrations of paraquat or diquat. Control colonies were placed in nutrient solution alone. Light-treated cultures were incubated at room temperature with a light intensity of 3500 lux. In experiments designed to investigate the effects of herbicide treatment in the dark, cultures were enclosed in black polythene bags immediately after inoculation. Colonies were removed at intervals and processed for eventual examination in the electron microscope.

F. Cytochemical Localization of Catalase Activity

The localization of catalase activity in S. oligorrhiza was investigated using the electron cytochemical reagent diaminobenzidine (DAB). Segments of frond tissue were initially fixed with 3% glutaraldehyde in 0.05M potassium phosphate buffer at pH 6.8 as described in 3(v) above. After washing for 20 minutes in 4 changes of fresh buffer, the frond segments were incubated with the incubation medium of Novikoff & Goldfischer (1968) as described by Frederick & Newcomb (1969). The medium, which was always freshly prepared, had the following composition:-

(i) 10 mg DAB (3,3' - diaminobenzidine)

(ii) 5 ml. 0.05 M propanediol buffer (2-amino-2-methyl-1,3-propanediol) at pH 10.0

(iii) 0.1 ml. 3% hydrogen peroxide

The medium was filtered and the pH was adjusted to 9.0 prior to the addition of the frond segments.

As a variation in the procedure, 0.1 ml. 1% hydrogen peroxide was used instead of (iii) above. Incubations were also carried out in the DAB medium minus hydrogen peroxide. All incubations were carried out in corked vials with occasional agitation, for 25 and 50 minutes at 37°C.

Following the incubations, tissues were washed for 20 minutes with 4 changes of fresh 0.05 M potassium phosphate buffer at pH 6.8 and then post-fixed with 1% osmium tetroxide in the same buffer for 3 hours at room temperature. All subsequent processing was carried out as described for all glutaraldehyde/osmium tetroxide-fixed tissue.

RESULTS

In any ultrastructural study it is obviously of great importance to achieve a high standard of tissue preservation. In investigations into the ultrastructural changes following treatment with potentially damaging chemicals such as diquat or paraquat proper fixation is doubly important. Failure to achieve consistently good cellular preservation makes it impossible to state with certainty that a given ultrastructural abnormality has been caused by a particular treatment.

The ultrastructural preservation of duckweed tissue proved to be extraordinarily difficult. The results of many of the fixation trials which were carried out are now described.

FIXATION TRIALS

1. Potassium permanganate fixation

2% aqueous $KMnO_4$ was first employed with a fixation period of 60 min. Figs. 1 and 2 show the typical appearance of cells of *L. gibba* fixed thus. Damage to membranous structures was substantial. The chloroplast envelope showed damage which ranged from small to very extensive breaks. Those parts of the chloroplast envelopes which were preserved intact often followed a rather undulating course imparting to the chloroplasts an irregular shape. Considerable damage to the internal structure of the chloroplasts was also evident. Varying degrees of intrathylakoidal swelling were found both in the grana and in the stroma thylakoids. Although preservation of the fine structure of chloroplasts was, generally very poor, chloroplasts exhibiting only slight signs of fixation damage were occasionally observed. One such chloroplast is shown in Fig. 3.

Fixation of both the plasma membrane and tonoplast, like that of the chloroplast envelope, appeared to be incomplete. Gaps, large and small, were found to occur frequently along the lengths of both membranes. The intermittent preservation of the chloroplast envelope, plasma membrane and tonoplast are illustrated in greater detail in Figs. 4, 5 and 6. These micrographs also reveal varying degrees of intrathylakoidal swelling. Preservation of mitochondria was also incomplete. Breaks in the outer membrane frequently observed (Fig. 2)

When the fixation time was cut to 30 min. with 2% $KMnO_4$, similar results were obtained (Figs. 7, 8 and 9). Again the degree of tissue preservation was not uniform in the samples. In general, however, damage to the chloroplasts was slightly less severe although still unacceptable. Both intrathylakoidal swelling and rupture of the chloroplast envelope were less noticeable than in the previous treatment. Nonetheless, some chloroplasts were more typical of tissue fixed for the longer period (Fig 9). The shorter fixation period resulted in no apparent improvement in the preservation of either the plasma membrane or the tonoplast. Similarly, although preservation of mitochondrial membrane structure was consistently better than for other membranous structures, the shorter fixation period afforded no noticeable improvement over the 60 minute samples.

Since the shorter fixation period resulted in only a slight reduction in the extent of tissue damage, the concentration of $KMnO_4$ fixative was next reduced to 1.2%. After 15 min. exposure to 1.2% $KMnO_4$ very little tissue preservation was achieved (Figs. 10 and 11). Cells appeared empty apart from the occasional chloroplast which was generally in a state of severe disruption (Fig. 10). The chloroplast shown in Fig. 11,

although reasonably well-preserved, is not representative of these organelles after short fixation. However, despite the fair degree of preservation in this chloroplast, little remains of either the plasma membrane, the tonoplast or indeed the cytoplasm of the cell.

Fixation in 1.2% KMnO4 for 30 min. greatly improved tissue preservation (Figs. 12, 13 and 14). Most of the chloroplasts were lens shaped and had their limiting membranes intact although some had rather irregular outlines and occasional breaks in their envelopes (Fig. 14). Intrathylakoidal swelling was seldom pronounced but there was a variable tendency for the thylakoids to assume a wavy appearance in both granal and intergranal regions. Once again, however, the plasma membrane and the tonoplast were poorly preserved (Figs. 12 and 13) but the mitrochondrial membranes appeared generally to be intact.

Fixation periods longer than 30 min. appeared to have an adverse effect on tissue preservation. The chloroplast envelopes of duckweed tissue exposed to 1.2% KMnO4 for 45 min. were frequently ruptured in more than one place and intrathylakoidal swelling was conspicuous (Fig. 15). Again the preservation of the plasma membrane and the tonoplast was very poor while, in addition, breaks in the outer membrane of the mitochondria were frequently observed. Tissue fixed for a period of 60 min. gave similar results (Figs. 16, 17 and 18).

No improvement in the degree of tissue preservation with KMnO4 was achieved by initial vacuum infiltration of the fixative or by carrying out the fixation at $4^{o}C$.

In a further attempt to overcome the apparent sensitivity of duckweed tissue to KMnO4, whole fronds were fixed without prior cutting into small segments. Tissue preservation was variable although generally better than

that which was obtained when segments were fixed with the same fixitive for the same length of time. Fig. 19 shows the best results obtained by fixing entire fronds. The fixation, while good by previous standards, was not without fault. Small breaks were often observed in chloroplast envelopes and a slight amount of intrathylakoidal swelling was evident in most chloroplasts. Preservation of the plasma membrane and the tonoplast was improved but still poor.

Throughout all the attempts to achieve adequate tissue preservation by fixation with $KMnO_4$, a noticeable feature was the frequent lack of uniformity in the appearance of cells and their contents following any single fixation regime. This variation occurred not only between different cells in the same section but also between different parts of the same cell.

2. Glutaraldehyde/$KMnO_4$ fixation

Improved preservation of chloroplasts was obtained when duckweed tissue was fixed initially with buffered glutaraldehyde prior to a brief post-fixation with 1% $KMnO_4$. Intrathylakoidal swelling was never observed in tissue fixed thus but even the best preserved parts of the cells revealed some fixation damage (Fig. 20). Chloroplasts often possessed a slightly irregular shape and, in certain places, slight swellings were observed between the paired membranes of the envelope. Small breaks were found in the envelopes of some chloroplasts but much less frequently than the swelling just described.

Not all chloroplasts, however, were preserved to the same degree. In a number, the envelope and the chloroplast contents had become widely separated (Fig. 21). This figure also shows poor preservation of both the plasma membrane and the tonoplast. However, micrographs in which

considerable portions of these structures had been preserved intact were often obtained (Figs. 22 and 23).

The degree of preservation of the mitochondria was also inconsistent. While many of these organelles appeared to be intact after fixation, it was not uncommon to find mitochondria with visible membrane damage (Figs. 20 and 21).

3. Gluteraldehyde/OsO4 fixation

Approximately 1 mm^2 segments of duckweed frond tissue were double-fixed with gluteraldehyde and osmium tetroxide using cacodylate buffer, as described in Materials and Methods (14). The preservation of fine structure which was obtained in this way was superior to anything achieved using KMnO4 with or without gluteraldehyde pre-fixation. Despite this, serious faults still remained. Fixation of the mitochondria and the lamellar system of the chloroplasts was good (Figs. 24 and 25). However, as these micrographs also show, swelling apart of the paired membranes of the chloroplast envelope was noticeable in certain regions. Indeed in some regions the outer part of the envelope was no longer present.

Preservation of the plasma membrane was variable. Where it remained intact it was not closely appressed to the cell wall but followed instead a rather undulating course at a short distance from the latter (Figs. 24 and 25). Similarly, preservation of the tonoplast varied even in different parts of the same section. Breaks in the tonoplast were frequently observed (Fig. 24). The rather low concentration of ribosomes found in the cytoplasm of these cells (Fig. 24) may be due to loss of cytoplasimic material through either the damaged plasma membrane or the damaged tonoplast.

The use of phosphate as opposed to cacodylate as fixative buffer resulted in slightly better preservation of the chloroplast envelope.

As before, slight swelling was found between the two membranes of the envelope, the outer membrane often having a rather wavy appearance (Fig. 26) However, actual breaks in the envelope were seldom seen. Preservation of all other cell structures was otherwise similar to that obtained when using cacodylate buffer. Damage to the plasma membrane and the tonoplast was once again variable. In some tissue, the damage was localised and slight (Fig. 26). Damage to the tonoplast did not always necessarily involve actual rupture of this structure. Fig. 27 shows a cell in which parts of the tonoplast have separated from the cytoplasm and engulfed within the vacuole. In much of the material examined, the plasma membrane was also seen to be separated from the cell wall by relatively long distances (Fig. 28). Damage to the plasma membrane and the tonoplast, while occurring frequently appeared to have little effect on the ultrastructure of the chloroplasts or other organelles although they did tend to be scattered loosely throughout the cell instead of being confined to the periphery (Fig 28). A sparsity of cytoplasmic ribosomes was also a characteristic of serious damage to the plasma membrane and the tonoplast.

Subsequent variations in fixation procedures with glutaraldehyde and osmium tetroxide as outlined in Materials & Methods (page 16) resulted in no significant improvement in the standard of ultrastructural preservation of S. oligorrhiza using 1 mm^2 segments of frond tissue. The preservation achieved by any one procedure was variable. Lengthening the fixation periods had no observable effect while fixation at $4^{o}C$ (Fig. 29) proved to be no better than at room temperature. Efforts to achieve consistently good preservation of all the cellular components by increasing the concentration of gluteraldehyde also met with failure (Figs. 30, 31 and 32).

These attempts to obtain adequate fixation of 1 mm^2 segments of duckweed

tissue using glutaraldehyde and osmium tetroxide were not only disappointing but also failed to provide any clues as to what steps should be taken to gain this required improvement. The initial cutting of the tissue into 1 mm^2 segments had been to ensure rapid penetration of the fixative into all parts of the specimen. Great care was always taken to minimize damage to the duckweed tissue during cutting. However, eventually it was suspected that, despite the precautions taken, some if not much of the damage visible in the fixed material may have occurred at this early stage as a result of the unavoidable physical disturbance involved. To test this hypothesis, frond tissue was initially cut into comparatively large segments (4 x 2 mm.) in the hope that the tissue in the centre of the segments would be unaffected by cutting stresses. Towards the end of the glutaraldehyde fixation period, small segments were cut from the centre of the large pieces. It was felt that damage caused by cutting at this stage would be minimal while the infiltration of fixatives and the other materials involved in the further preparation of the specimens for ultramicrotomy would be facilitated.

Examination of tissue processed in this way revealed a substantial improvement in the fixation. Preservation of detail at the ultrastructural level was not only improved but also far more consistent. The typical appearance of the contents of a cell of S. oligorrhiza is illustrated in Fig. 33. For the most part, both the plasma membrane and the tonoplast were preserved intact although in some cells a slight amount of very localized damage to one or both of these membranes was found. Slight damage to the tonoplast can be seen in Fig. 33, while similar localized damage to the plasma membrane is illustrated in Fig. 34. In addition to the isolated breaks, slight blistering of both membranes was occasionally

observed (Fig. 35). These cells always possessed a high concentration of cytoplasmic ribosomes, a finding which perhaps reflects the greatly improved preservation of the plasma membrane and tonoplast. Improvement was also obtained in the preservation of the chloroplast (Fig. 36). Even the most successful of previous fixation methods had frequently resulted in swelling between the two membranes of the chloroplast envelope. Such swelling was rarely observed in material fixed according to this method. Mitochondria (Fig. 37) and nuclei (Fig. 38) were also well preserved.

To summarize, this last fixation schedule gave adequate fixation of all cellular components except the plasma membrane and the tonoplast. Fixation of these two membranes, although imperfect, was nonetheless reasonably successful. Ideally of course, preservation of both membranes should have been complete at all times. Shortage of time however, precluded further experimentation and necessitated the use of the above method in subsequent investigations with S. oligorrhiza with the acceptance of its limitations.

TREATMENT WITH DIQUAT (10 ppm CATION) IN THE LIGHT

The following sequence describes the effects of treatment of young 3-frond colonies of *S. oligorrhiza* with 10 ppm diquat (cation) in the light (3500lux). In this investigation, 1 mm^2 segments of frond tissue were prepared and double-fixed with glutaraldehyde and osmium tetroxide. Stresses involved in the cutting of such small tissue segments may have caused damage to both the plasma membrane and the tonoplast. No firm conclusions regarding the fates of either membrane can therefore be reached from the following results.

The typical appearance of control material is shown in Figs. 39, 40 and 41. Starch grains were visible in most chloroplasts although numbers varied. A variable, but generally small number of plastoglobuli also occurred throughout the stroma in most chloroplasts.

4 hours treatment.

Treated fronds showed no outward signs of damage after exposure to this concentration of diquat for a period of 4 hours. Electron microscopical examination at this stage also failed to reveal signs of ultrastructural damage. Treated tissue appeared closely similar to control material although there was an apparent reduction in the amount of starch present in many chloroplasts (Figs. 42 and 43).

8 hours Treatment

After 8 hours exposure to diquat, fronds still remained normal in outward appearance. However, alterations in fine structure could be clearly recognised at this stage (Figs. 44, 45, 46 and 47). Most of the chloroplasts observed had a roughly circular shape unlike the normal flattened lens shape (Figs. 44 and 45). The internal membrane structure of the chloroplasts remained intact although the thylakoids displayed a

slight tendancy towards waviness particularly in the intergranal regions. Furthermore, the thylakoids were generally displaced towards the periphery of the chloroplasts. Starch grains were seldom seen but some chloroplasts possessed slightly more plastoglobuli than did control material (Fig. 44). Small electron dense areas, possibly also lipoidal in nature, were regularly found at short intervals along the chloroplast envelopes (Figs. 44, 45, 46 and 47). Although this finding may suggest some kind of damage to these membranes, actual breaks were not observed.

At this stage the appearance of the mitochondria was also noticeably altered. In untreated material the electron density of the mitochondrial matrix was closely similar to that of the chloroplast stroma (Fig. 39). However, after 8 hours diquat treatment a marked reduction in the electron density of the matrix of all mitochondria was evident (Figs. 44, 46 and 47). In addition, many mitochondria were observed to have irregular shapes such that adjacent mitochondria often fitted closely together very much like pieces in a jig-saw puzzle (Fig. 46).

Also evident at this stage were many irregularly shaped microbodies occurring close together in groups (Fig. 48). These structures were regularly found in the cytoplasm of untreated cells although their shape, like that of the mitochondria, was usually oval (Figs. 33, 36 and 40). These microbodies were never found to be bunched together in control material.

In some areas of treated cells neither the plasma membrane nor the tonoplast appeared to have sufferred damage although nearby chloroplasts and mitochondria were visibly altered (Fig. 47). Nonetheless, damage to both the plasma membrane (Fig. 46) and the tonoplast (Figs. 44 and 45) was usually evident at this stage. However, owing to the nature of the

fixation method employed, it is not possible to ascertain whether or not this damage was caused by the diquat treatment.

12 hours Treatment.

After exposure to diquat for 12 hours, treated fronds appeared very slightly lighter in colour than controls. The appearance of treated material at the electron microscope level was more variable than after 8 hours (Figs. 49 and 50). In much of the material examined, damage to the chloroplasts was more advanced than in tissue exposed to herbicide for only 8 hours. Fig. 49 illustrates the increase in the amount of electron dense material found at this stage both within the chloroplasts and along parts of the chloroplast envelopes. Within the chloroplasts, this material was confined to the regions occupied by the thylakoids. As Fig. 49 also shows, the thylakoids were frequently confined to an arch on one side of the chloroplast leaving a considerable proportion of the stroma free of any membranous components. Thylakoid structure was otherwise similar to that observed after 8 hours except for slight intrathylakoidal swelling found occasionally, particularly in the intergranal regions. In other treated material examined at this time, however, the chloroplasts appeared less severely damaged and were similar to those found after only 8 hours. (Fig. 50).

Mitochondria observed after 12 hours all revealed a considerable reduction in the electron density of the matrix but did not differ noticeably from those observed after only 8 hours (Fig. 50). Irregularly-shaped microbodies were commonly found packed close together at this time. Generally, these microbodies appeared similar to those observed after only 8 hours.

16 hours Treatment

Chlorosis of treated fronds was more evident after exposure to diquat for 16 hours. Similarly, ultrastructural damage was further advanced (Figs. 51 and 52). The occurrence of plastoglobuli and other electron dense areas within the chloroplasts was increased but starch grains were generally absent. Also, the arrangement of the thylakoids revealed considerable disorder while intrathylakoidal swelling was more marked than after 12 hours. The chloroplast shown in Fig. 51 is typical of those observed after 16 hours, although more severely damaged chloroplasts were occasionally observed (Fig. 52). Irrespective of the condition of the chloroplast the paired membranes of the chloroplast envelope could seldom be resolved. However, large breaks in the envelope were rare although points where it had become separated from the rest of the chloroplast were occasionally observed (Fig. 52). The mitochondria at this stage consisted of a collection of vesicle - like membrane fragments of various sizes contained within an ill-defined outer envelope (Fig. 51). Microbodies, on the other hand, appeared less severely damaged although all displayed a somewhat patchy reduction in the electron density of their contents (Fig. 52).

20 hours Treatment

After 20 hours treated fronds were badly chlorotic. A further deterioration of fine structure was also evident at this stage although considerable variation did exist (Figs. 53, 54 and 55). Once again, starch grains were seldom seen in any chloroplast irrespective of the degree of damage sustained. Plastoglobuli and other electron dense deposits were present but slightly less noticeably than in previous treatments despite the more advanced degree of tissue breakdown.

The different levels of chloroplast breakdown often observed within the same cell are illustrated in Fig. 53. In most chloroplasts the internal membrane structure was arched away from the part closest to the cell wall. However, the amount of intrathylakoidal swelling observed in individual chloroplasts varied. Many chloroplasts remained largely intact and closely resembled typical chloroplasts from the 16 hour treatment (Fig 54). Others were seen to have sustained considerably greater damage (Fig. 55). In such chloroplasts, intrathylakoidal swelling was very pronounced and the entire internal membrane system was totally disrupted. There was also a considerable reduction in the electron density of the stroma of the most badly affected chloroplasts and, in many, a sizeable portion of the outer envelope was no longer present.

Other organelles were rarely observed after 20 hours except where damage was least severe. However, when observed, microbodies once more appeared to be more resistant to complete disruption than did mitrochondria (Fig. 56).

Comparison of the effects of treatment in the light with diquat and paraquat on the ultrastructure of S. oligorrhiza

The following sequence compares the ultrastructural changes observed after different durations of treatment with diquat and paraquat at concentrations of 10 ppm (cation). In this, as in all subsequent ultrastructural investigations with S. oligorrhiza, tissue was double-fixed with glutaraldehyde and osmium tetroxide using large (approximately 4 x 2 mm.) segments for the initial glutaraldehyde fixation (Materials and Methods, page 16).

The outward appearance of treated fronds throughout this period is summarized in Pl. 1 No symptoms were visible in any fronds after 6 hours. Chlorosis was noted after 12 hours in diquat-treated fronds. Paraquat-treated fronds also became chlorotic but only after longer treatment. Chlorosis became more marked as the duration of the treatment increased but, at any one time, chlorosis was always more advanced in diquat-treated fronds.

After 18 hours, tiny droplets of liquid had begun to appear on the surface of fronds treated with diquat. This 'sweating' subsequently became more noticeable. Such a condition was not evident in paraquat-treated fronds until the end of the treatment period.

Electron microscopical investigations showed that these external symptoms of damage induced by both herbicides were preceded by ultrastructural changes. The appearance of control material at the start of the experiment is shown in Figs. 57 and 58.

6 hours Treatment

After exposure to diquat for 6 hours only a relatively small amount of the frond tissue examined appeared to have remained undamaged at the ultrastructural level (Fig. 59). Most of the treated tissue examined revealed

PLATE 1

The external appearance of fronds of S. oligorrhiza during treatment in the light with diquat or paraquat

DURATION OF TREATMENT	APPEARANCE OF FRONDS	
	DIQUAT (10 ppm cation)	PARAQUAT (10 ppm cation)
6 h.	Normal	Normal
12 h.	Faint chlorosis	Normal
18 h.	Medium chlorosis Slight 'sweating'	Faint chlorosis
24 h.	Severe chlorosis Moderate 'Sweating'	Medium chlorosis Slight 'Sweating'

that a number of changes had already taken place (Figs. 60, 61 and 62). Figs. 60 and 61 illustrate the typical appearance of the chloroplasts at this stage. Starch grains were generally absent and, although there was no uniformity of shape, the chloroplasts generally had a more rounded appearance than controls. Complete rupture of the chloroplasts was never evident at this stage although breaks in the outer membrane of the chloroplast envelope were frequently observed (Fig. 60). A reduction in the electron density of the loculi of the thylakoids was observed and in many chloroplasts the arrangement of the thylakoids was irregular (Fig. 61). However there was no appreciable build-up of plastoglobuli and other electron dense deposits similar to that previously observed at the early stages of cellular damage with diquat.

Damage was not confined to chloroplasts. Fig. 62 reveals considerable changes in both the mitochondria and the microbodies. The mitochondria remained generally intact although most of their matrix substance appeared to have been lost. As Fig. 62 also shows, many of the microbodies had suffered considerable damage to their limiting membranes. However, damage to both types of organelle at this stage was generally less severe than just described.

Despite the alterations in fine structure listed above, both the plasma membrane and the tonoplast remained unbroken. Occasional blistering of the tonoplast was observed (Fig. 62) but no more frequently than in control material.

Following treatment with paraquat for 6 hours, no visible evidence of ultrastructural damage was observed (Fig. 63).

12 hours Treatment

After exposure to diquat for 12 hours, intrathylakoidal swelling was

evident in the chloroplasts. The extent to which this swelling had occurred, however, varied from very slight (Fig. 64) to moderate (Fig. 65). These micrographs also indicate that many chloroplast envelopes had sustained variable amounts of damage. The appearance of the mitochondria and the microbodies at this stage was more consistent. Most mitochondria appeared similar to those observed after 6 hours in those portions of the cytoplasm which suffered the greatest amount of damage (Figs. 66, 67 and 68). Despite the loss of most of their matrix material, they remained largely intact, although in some cases parts of the outer membrane could not be resolved. The shapes of the mitochondria differed little from controls. Microbodies generally appeared in clusters rather than singly but showed little sign of internal damage (Figs. 66 and 68). However, as with the mitochondria, areas where no outer membrane could be detected were often found.

Damage to both the plasma membrane and the tonoplast was also visible at this stage. Breaks in these membranes were observed in all cells examined although the extent to which damage had occurred was variable (Figs. 64, 65, 67 and 68). Despite these changes, a high concentration of ribosomes was always found in the cytoplasm.

Ultrastructural damage was clearly visible in paraquat-treated fronds after 12 hours (Figs. 69, 70, 71 and 72). The degree of damage sustained was, however, less than that observed in diquat-treated fronds after the same period. The chloroplasts were generally intact but had assumed a somewhat rounded appearance. A reduction in the electron density of the thylakoid loculi was evident in some chloroplasts (Fig. 69) as it was in diquat-treated fronds after only 6 hours. While intrathylakoidal swelling was observed in 12 hour diquat-treated fronds, no such swelling was found in tissue exposed to paraquat for the same length of time. The arrangement of the thylakoids

was however irregular. In some chloroplasts the thylakoid system was arched to one side (Fig. 69) while in others the thylakoids displayed uncharacteristic undulations through the stroma (Figs. 70 and 71).

While damage to the chloroplasts in paraquat-treated fronds was less advanced than the corresponding diquat-treated tissue, no such retardation was observed in the degeneration of other cellular components. The mitochondria and microbodies appeared similar to those observed in 12 hour diquat-treated tissue (Figs. 69 and 72). Similarly, variable amounts of damage to both the plasma membrane and tonoplast were observed in all cells examined at this stage (Figs. 69, 70, 71 and 72).

18 hours Treatment

After exposure to diquat for 18 hours the degenerative changes in the chloroplasts had advanced considerably (Figs. 73, 74, 75 and 76). The appearance of chloroplasts varied but all displayed a reduction in the electron density of the stroma. Intrathylakoidal swelling was greater and the arrangement of the thylakoid system more disorganized. Most chloroplasts had also sustained considerable damage to their outer envelope, many of them being devoid of their envelope over the greater part of their surface (Fig. 75). Isolated fragments of the thylakoid system derived from disintegrated chloroplasts were observed in many cells (Fig. 76). There was no evidence of an accumulation of plastoglobuli.

Mitochondria at this stage had lost almost all of their matrix material the remaining membranous portions often showing signs of degenerative changes (Fig. 74). However, microbodies still appeared to retain most of their internal substance (Fig. 74).

Both the plasma membrane and the tonoplast were totally disrupted by this time (Figs. 73, 74, 75 and 76) and the contents of the cytoplasm occurred

scattered throughout the cell. As these micrographs also reveal, ribosomes were scarce in the cytoplasm at this stage.

Paraquat-treated tissue again appeared less badly affected at this stage. In some tissue, the chloroplasts were similar to those exposed to paraquat for only 12 hours (Figs. 77 and 78). However, other chloroplasts had undergone further degeneration (Figs. 79 and 80). A reduction in the electron density of the stroma was observed together with varying degrees of intrathylakoidal swelling.

The mitochondria and microbodies were also less badly damaged than those in diquat-treated tissue at the same stage (Figs. 77, 78 and 79). Damage to both the plasma membrane and the tonoplast, although variable, was only slightly increased after 18 hours. In contrast to the situation in diquat-treated tissue at this time, large areas where these membranes were still intact were commonly observed (Figs. 77, 78, 79 and 80). Also, as these micrographs show, there remained a plentiful supply of ribosomes in the cytoplasm of 18 hour paraquat-treated tissue.

24 hours Treatment

Examination of diquat-treated tissue after 24 hours revealed very few chloroplasts which had escaped complete disruption (Fig. 81). Figs. 82 and 83 illustrate the typical appearance of these organelles at this stage. The majority of chloroplasts had lost a considerable proportion of their outer envelopes leaving behind a highly disorganized mass of swollen membranes and loosely scattered remains of the stroma. Mitochondria and microbodies had deteriorated to such an extent that they could no longer be identified with certainty. All that remained of these organelles were irregularly-shaped membranous shells (Fig. 82).

Intrathylakoidal swelling was evident in all chloroplasts of paraquat-

treated tissue after 24 hours (Figs. 84, 85, 86 and 87). Both the shape of the chloroplasts and the arrangement of their thylakoids were grossly irregular. However, most of these organelles did remain intact although a small proportion had sustained sufficient damage to their outer envelopes to enable fragments of the thylakoid system to become detached (Figs. 86 and 86). While chloroplast disintegration was less advanced, mitochondria and microbodies appeared similar at this stage to those observed in tissue treated with diquat for the same length of time (Fig. 87). Again, so little of their fine structure remained as to make positive identification difficult.

Other cellular components appeared similar to those observed in tissue treated with diquat for the same length of time. While large portions of both the plasma membrane and the tonoplast remained intact in paraquat-treated tissue after 18 hours, very little of either was present after 24 hours. A marked reduction in the number of cytoplasmic ribosomes was also evident at this time.

The effects of prolonged exposure of S. oligorrhiza to diquat and paraquat in the dark.

Control fronds and fronds in the presence of either diquat or paraquat at concentrations of 10 ppm (cation) were examined after a period of 140 hours in complete darkness. An accumulation of plastoglobuli was evident in the chloroplasts of control fronds (Figs. 88 and 89). Starch grains were absent but otherwise the ultrastructure of the control chloroplasts was normal. Many of the mitochondria observed in control material at this time possessed internal membranous structures not normally found in fronds growing under continuous illumination (Figs. 88 and 90). Other cellular components appeared unaffected by the prolonged darkness.

Ultrastructural examination of treated fronds revealed that damage was caused by both diquat (Figs. 91, 92, 93 and 94) and paraquat (Figs. 95, 96, 97 and 98) during the period of darkness. The degree of damage caused by both herbicides was variable. Many chloroplasts showed little sign of injury (Figs. 91 and 95) and closely resembled the chloroplasts of control material, being devoid of starch grains but containing many plastoglobuli. In others the outer envelopes had sustained variable degrees of damage although the internal thylakoid systems appeared largely unaffected (Figs. 92 and 96). Often, parts of the envelopes could not be discerned (Fig. 92) while in other cases swelling between the paired membranes of the envelope was evident (Fig 96). The most seriously affected chloroplasts were swollen and revealed a considerable reduction in the electron density of the stroma (Figs. 93 and 97). As these micrographs also show, the thylakoid system, although intact, was often arched to one side of the chloroplast. In addition, a slight amount of intrathylakoidal swelling was observed in occasional chloroplasts (Fig. 97).

In treated cells which had sustained the least amount of ultrastructural damage the mitochondria appeared normal (Fig. 91). However, in the great majority of micrographs examined the mitochondria were visibly altered (Figs. 94 and 98). A considerable reduction in the electron density of the matrix compared with controls was observed. Some mitochondria had also become very irregular in outline (Fig. 98). Unusual membranous structures similar to those observed within control mitochondria were also common in tissue exposed to either herbicide (Figs. 94 and 98).

Damage to both the plasma membrane and the tonoplast was variable. In tissue apparently least affected by exposure to herbicide in darkness, the condition of both membranes was similar to that observed in control material (Figs. 91 and 95). In these cells the plasma membrane was often separated from the cell wall by relatively large distances. However, examination of control material revealed a similar finding (Fig. 88). In other treated cells, the tonoplast had become separated from the cytoplasm in the region of the chloroplasts and displayed varying degrees of invagination into the vacuole (Figs. 92 and 96). Disruption of parts of the plasma membrane was also generally evident in such cells. In more seriously affected cells there was a corresponding deterioration in the condition of both membranes. In those cells which had sustained the greatest amount of damage after 140 hours little remained of either the plasma membrane or the tonoplast except in small localized areas (Fig. 97).

The effects of short and long-term exposure of S. oligorrhiza to large doses of diquat in the light and in darkness

1 hour in the light in the presence of 500 ppm diquat (salt)

Fig. 99 shows a chloroplast which appears undamaged after the exposure of fronds to 500 ppm diquat (salt) in the light for 1 hour. However, almost all chloroplasts observed at this time did reveal obvious signs of structural damage (Figs. 100, 101 and 102). Intrathylakoidal swelling varied from slight (Fig. 100) to severe (Figs. 101 and 102). In addition there was a pronounced tendancy for the stroma material to round off into membrane-bound areas separated by large, irregularly-shaped spaces (Figs. 100, 101 and 102). Such changes were occasionally accompanied by damage to the chloroplast envelope (Fig. 100) but more often the envelopes appeared to remain undamaged (Figs. 101 and 102).

Neither the plasma membrane nor the tonoplast appeared to have suffered any structural damage during this time (Figs. 100 and 101) except occasionally in regions close to the most seriously damaged chloroplasts. Fig. 102 shows areas where the tonoplast has been ruptured.

Reduction in the electron density of the matrix was observed only rarely in certain mitochondria after 1 hour in the light (Fig. 103). Also at this stage a small proportion of the microbodies appeared to have lost part of their limiting membranes (Fig. 104). Otherwise, both mitochondria and microbodies appeared generally to be unaffected at this time despite visible damage to adjacent chloroplasts (Figs. 105 and 106).

1 hour in the dark in the presence of 500 ppm diquat (Salt)

Observation of tissue exposed to diquat for 1 hour in darkness revealed no evidence of any ultrastructural damage (Figs. 107, 108 and 109).

15 hours in the light in the presence of 500 ppm. diquat (salt)

Observations following exposure to diquat for 15 hours in the light revealed widespread ultrastructural damage (Figs. 110, 111, 112, 113 and 114). Disintegration of the plasma membrane and tonoplast was evident in all cells examined after this treatment. As a result, the distribution of cytoplasm and cytoplasmic ribosomes was patchy. The cytoplasm tended to be clumped together and densely staining in parts. The chloroplasts appeared unlike those observed after only 1 hour. Variable degrees of intrathylakoidal swelling were again evident but in addition there was a reduction in the electron density of the thylakoid membranes (Figs. 110, 111 and 112). Furthermore, the appearance of the stroma was altered. Figs. 110, 111 and 112 reveal a general increase in the electron density of the stroma. Small irregularly-spaced electron transparent areas can also be seen within the stroma material. Despite their grossly abnormal internal structure, most chloroplasts observed appeared still to be intact. However, chloroplasts which had lost considerable portions of their outer envelopes were observed occasionally (Fig. 113).

Ultrastructural damage to other cell organelles was sufficiently severe to make identification difficult. Fig. 114 shows structures which are probably the membranous remains of mitochondria and microbodies.

15 hours in the dark in the presence of 500 ppm diquat (salt)

Ultrastructural changes were also observed in tissue exposed to diquat for 15 hours in darkness. The extent to which damage had occurred was, however, considerably less than in tissue treated for the same length of time in the light (Figs. 115, 116, 117, 118 and 119). Only very occasional chloroplasts showed evidence of intrathylakoidal swelling as a result of treatment and then only slight swelling was visible (Fig. 117). However,

the spatial arrangement of the thylakoids within the chloroplasts was often altered (Figs. 115 and 116). The general outline of chloroplasts of treated fronds was also irregular at this time (Figs. 115, 116 and 117). In addition, large areas of the outer envelope could not be resolved in most chloroplasts. Despite these changes to the chloroplasts there was no evidence of a loss of material from within the organelles.

The condition of both the plasma membrane and the tonoplast was variable. Often these membranes could not be properly resolved yet the cytoplasmic contents showed no tendancy to become loosely dispersed throughout the cells. Indeed, the cytoplasm of treated cells at this time appeared to be more concentrated than in normal untreated cells. Areas where gaps had arisen between the tonoplast and the cytoplasm were often visible (Figs. 115 and 118). In addition, rounded membranous fragments were commonly observed between the cytoplasm and the cell walls, indicating damage to the plasma membranes (Figs. 115, 116 and 119). Such structures were also found in untreated tissue although to a much lesser degree.

The mitochondria and microbodies were still largely intact after 15 hours but their appearance differed from that of untreated material. All the mitochondria observed had suffered a considerable reduction in the electron density of their matrix material and many had become irregularly shaped (Figs. 115, 116, 117, 118 and 119). No reduction in the electron density was observed within the microbodies. However, their normal rounded appearance had become altered and there was a tendancy for these organelles to pack tightly against one another (Figs. 116 and 118).

Cytochemical localization of catalase activity

Incubation of glutaraldehyde-fixed frond segments of S. oligorrhiza in a medium containing diamino-benzidine (DAB) resulted in the production of an electron-opaque material within the microbodies (Figs. 120 and 121). This product is thought to be osmium black formed by the reaction of oxidized DAB and osmium tetroxide (Frederick, S.E. & Newcomb E.H., 1969) There was no evidence of any appreciable deposition of a similar precipitate within the mitochondria, chloroplasts or any other cellular organelles or indeed in the cytoplasm itself (Figs. 120, 121, 122 and 123). Electron-dense deposits were however often observed in the cell walls (Figs. 122 and 123). As will be discussed later, it is believed that such electron-dense deposits are indicators of either catalase or peroxidase activity.

The incubation of gluteraldehyde-fixed frond tissue in a medium containing both DAB and hydrogen peroxide resulted in the production of granular deposits of even greater electron density (Figs. 124 and 125). Apart from the deposits in the cell walls, the bulk of the electron-opaque material was centred once more within the microbodies. However, when hydrogen peroxide was included in the incubation medium, tissue preservation was poor and this situation was not improved by reducing either the concentration of hydrogen peroxide or the duration of the incubation period. As a result of the tissue damage which was almost certainly caused by the hydrogen peroxide in the incubation medium, the plasma membrane, tonoplast and all other limiting membranes were frequently observed to be ruptured. As Figs. 124 and 125 show, electron-dense deposits were also visible in the cytoplasm. However, careful examination of the electron micrographs from such experiments indicate that the microbody membranes were generally ruptured. It would thus appear that the presence of electron-dense deposits in the cytoplasm does not necessarily indicate the existence

of a significant amount of cytoplasmic catalase. As yet, this problem remains unsettled. It should be noted however, that, as in the previous experiments, no accumulation of electron-dense deposits could be observed within the chloroplasts.

The ultrastructure of senescing fronds of S. oligorrhiza

It has been suggested that many of the ultrastructural changes observed in fronds treated with diquat or paraquat are similar to those normally associated with senescence. In order to allow a closer comparison, a brief study was made of the alterations in the fine structure of senescing fronds of S. oligorrhiza. On the basis of colour, 7 week-old cultures of S. oligorrhiza contained fronds at different stages of senescence. Such cultures contained pale green, yellow and even white fronds.

Electron microscopical examinations of the pale green fronds revealed that the plasma membrane and the tonoplast were damaged to some extent in all cells (Figs. 126 and 127). Despite occasional breaks, large portions of the plasma membrane normally remained close to the cell wall (Fig. 126). On the other hand, the tonoplast was totally disrupted being reduced to many twisted fragments (Fig. 127). The micrographs also show a considerable reduction in the concentration of cytoplasmic ribosomes. Mitochondria and microbodies were still present and had retained much if not all of their internal substance (Fig. 127). However, little detail could be resolved within the mitochondria.

Slight swelling between the membranes of the chloroplast envelopes was often observed and, in places, the envelope could not be discerned (Figs. 126 and 127). However, as the micrographs also show chloroplasts remained largely intact. Within the chloroplasts the most noticeable feature was the accumulation of starch grains. In addition, plasteglobuli were more prominent than in the chloroplasts of young, actively-growing fronds. However, the thylakoid system of pale green fronds appeared to have undergone no changes at this stage.

Examination of tissue taken from yellow fronds revealed a further

deterioration in cellular fine structure (Figs. 128 and 129). The appearance of both the plasma membrane and the tonoplast was as observed in pale green fronds. However, cytoplasmic ribosomes were extremely scarce. Mitochondria and microbodies were less numerous than in younger tissue and many of these organelles possessed insufficient internal detail to enable the two types to be positively distinguished.

Chloroplast ultrastructure showed a marked deterioration. Most chloroplasts had lost considerable portions of their envelopes. The accumulation of starch in particular and the increase in size of the plastoglobuli were so pronounced that most chloroplasts had become grossly irregular in outline. As a further consequence of this, onlya small portion of the original thylakoid system remained. Where found, the thylakoids generally appeared relatively undamaged although slight swelling was occasionally observed (Figs. 128).

Ultrastructural examination of tissue taken from white fronds revealed cells which were almost totally devoid of any remnant of protoplasm (Figs. 130, 131 and 132). Mostly, all that remained within the cells were small membranous fragments lying close to the cell wall (Figs. 130 and 131). The only other structures observed within the cells were oval bodies which were never observed in younger tissue (Fig. 132). These however, were only found in a small proportion of the cells examined.

I(B) MATERIALS AND METHODS

A. Growth Studies

Experiments to investigate the effects of various concentrations of paraquat and diquat on the growth of S. oligorrhiza were carried out in covered Petridishes. Each Petri dish contained 40 ml. ½ strength Hutner's medium into which was incorporated the desired concentration of either herbicide. Control cultures received nutrient solution alone. Concentrations of both paraquat and diquat ranging from 5.0 to 0.0004 ppm (cation) were tested alongside controls. For every treatment 6 replicate cultures were examined. Due to the large number of cultures employed and the length of time required daily to assess growth, it was impossible to test the entire concentration range at one time. Instead, 2 experiments were performed in which the following concentration ranges were tested:

(i) 5.0 - 0.005 ppm (cation)

(ii) 0.05 - 0.0004 ppm (cation)

In each experiment cultures were inoculated at zero time with an identical number of actively-growing fronds. In order to ensure that all inocula had similar potentials for growth, precautionary measures were taken prior to inoculation. Fronds from actively-growing 2 week old cultures were bulked and washed thoroughly under cold running water. Subsequently, this material was sorted into different groups according to the number of fronds per colony. Further sorting ensured that all of the colonies in every one of the groups were as closely similar in appearance as possible. In this way, groups of virtually identical 3,4,5 and 6 - frond colonies were obtained and subsequently distributed equally among the various test solutions.

The initial number of fronds per culture at zero time in experiments (i) and (ii) were 43 and 42 respectively. In both experiments cultures were maintained at room temperature under continuous illumination at a light intensity of 3500 lux. The positions of the cultures in the test area were randomized every 24 hours to minimize the effects of any slight differences in light intensity. Growth was estimated throughout the following 7 days by counting the number of fronds per culture at 24 hour intervals. For this purpose, all out-growths of older fronds were counted as soon as they became visible. Furthermore, a regular visual assessment was made of the condition of fronds undergoing the various treatments and the number of fully chlorotic fronds per culture noted.

At the end of the 7 day period, the fronds from every culture were washed and then carefully blotted with filter paper to remove surplus water prior to fresh weight determination. Following this, the fronds were placed on pre-weighed planchets and dried to constant weight under an infra-red lamp.

B. Pigment Studies

Investigations into the effects of various treatments on the chlorophyll content of S. oligorrhiza were carried out at room temperature in covered Petri-dishes. Each dish contained 40 ml. ½ strength Hutner's medium (pH 5.6) into which was incorporated the desired concentration of the various test substances examined. In all experiments, the fronds added to the test solutions were taken from approximately 1 week old cultures.

Experience showed the chlorophyll content of the fronds to vary slightly from one batch to another despite closely similar appearances.

Thus immediately prior to every experiment, the stock cultures providing material for study were bulked in order to avoid the introduction of unnecessary variation at the start.

After washing under cold running water, approximately equal quantities of stock material (0.2–0.3g. fresh weight) were added to the test dishes. Valves for chlorophyll content determined at this stage were consistent for stock material used in any individual experiment, thus ensuring that at zero time plants in all test cultures had identical chlorophyll contents (on a mg./g. fresh weight basis).

Extraction of chlorophyll

The fresh weight of frond tissue present in the Petri dish cultures used in most of the investigations carried out was small (i.e. approximately 0.25 g.) In order to provide larger samples and so minimize the effects of any possible inefficiency in the extraction procedure, the fronds from groups of 3 cultures having undergone any particular test were initially bulked together. In some experiments 6 cultures were employed per treatment so that 2 samples were finally obtained for chlorophyll extraction while in others only 1 sample was possible. In the experiment conducted with cultures growing in Erlenmeyer flasks no such bulking was necessary owing to the much greater quantity of plant material which these flasks contained.

Each sample was initially washed briefly under cold running water and then blotted dry between two pieces of filter paper. Exactly 0.6 g. (fresh weight) of this material was subsequently transferred to a glass homogenizer containing 5.0 ml. 80% acetone. After homogenization for 4–5 minutes, the extract was centrifuged at a moderate speed for 5 minutes and the supernatant collected. The residue was then re-extracted

as before with a further two 5.0 ml. aliquots of 80% acetone. On completion of the extraction procedure, the 3 resulting supernatants were combined and made up to 25 ml. with 80% acetone. To avoid bleaching, chlorophyll extracts were kept darkened until optical density measurements were made.

Determination of chlorophyll

Optical densities of chlorophyll extracts were measured at 649 and 665 mm. in a Pye Unicam SP 800 A ultraviolet spectrophotometer. Valves for chlorophyll a, chlorophyll b and total chlorophyll were calculated using equations cited by Vernon (1960).

chlorophyll a = 11.63 (A665)-2.39 (A649) mg/l

Chlorophyll b = 20.11 (A649)-5.18 (A665) mg/l

Total Chlorophyll = 6.45 (A665)+17.72 (A649) mg/l

C. Manometric Studies

Prior to all manometric experiments with S. oligorrhiza, fronds from actively-growing stock cultures were bulked, washed under cold running water and blotted between pieces of filter paper.

(i) Gas exchange in the dark

In this series of experiments, all gas exchange measurements were made at 25 $\pm$ 0.5^{o}C using a Gilson respirometer. The same basic procedure was followed in all cases with modifications where necessary. Normally, manometric flasks without side-arms were employed. However, flasks with single side-arms were used in the first set of experiments in which the effects of a range of herbicide concentrations on oxygen uptake were investigated.

In all experiments the outer and inner compartments of the flasks contained 2.0 ml. $\frac{1}{3}$ strength Hutner's medium (pH 5.6) and 0.1 ml. 40%

KOH respectively. Filter paper "accordions" were also placed in the centre wells in order to increase the surface of the alkali. In one investigation, 0.1 ml. distilled water was added, in place of KOH solution, to the centre wells of an additional batch of manometric flasks in order to estimate the output of carbon dioxide. Exactly 100 mg. fresh weight of intact frond tissue was placed in the outer compartments of the flasks and the flasks were subsequently wrapped in 2 layers of aluminium foil to exclude light. As a further precautionary measure, the water bath of the respirometer was always covered with a double layer of light-proof black cloth. 0.5 ml. distilled water was added to the side-arms or outer compartments of those flasks serving as controls. Other flasks received 0.5 ml. of either paraquat or diquat. The concentrations of herbicides added to the flasks were arranged so as to achieve the desired final concentrations upon mixing.

In all experiments, oxygen uptake measurements commenced after a 20 minute equilibration period and were continued at intervals for a period of at least 5 hours. In flasks with side-arms, oxygen uptake measurements were made for the first hour before the contents of the side-arms were tipped into the main compartments of the flasks. The number of replicate flasks employed per treatment ranged from 4 to 6 depending upon the experiment concerned.

(ii) Gas exchange in the light

Measurements of oxygen exchange in the light were made at $25 \pm 0.5^{\circ}C$ using a Gilson respirometer, illumination being supplied by a built-in bank providing a light intensity of 3,000 lux at flask level. Manometric flasks with single side-arms were used throughout.

In all experiments exactly 100 mg. fresh weight of intact frond tissue was placed into the main compartments of flasks containing 2.0 ml.

Warburg buffer No. 9 (Umbreit, Burris & Stauffer, 1964). The side-arms of test flasks received 0.5 ml. of paraquat or added to the side-arms were arranged so as to achieve the desired final concentrations upon mixing. The side-arms of control flasks received 0.5 ml. distilled water. In some experiments duplicate control flasks with 0.1 ml. 40% KOH in the centre wells were double-wrapped in aluminium foil and enclosed in black polythene bags for the measurement of control dark respiration.

Following a 15 minute equilibration period, oxygen exchange measurements were begun. After 20 minutes, the contents of the side-arms were tipped into the outer compartments of the flasks and measurements were continued at frequent intervals for 3-4 hours depending upon the experiment in question.

In experiments comparing the effects of a range of diquat concentrations on oxygen exchange in the light, 2 replicate flasks were employed per treatment. 3 replicate flasks per treatment were used in experiments where the effects of paraquat and diquat on oxygen exchange were being compared.

RESULTS

A. GROWTH STUDIES

Effects of various concentrations of paraquat and diquat on the growth of S. oligorrhiza

Analysis of variance was carried out on the data collected according to Snedecor (1967). The differences between means were tested by Duncan's multiple range analysis ('D' test) (Duncan, 1955).

Experiment (i)

The daily frond number of cultures of S.oligorrhiza incorporating concentrations of paraquat and diquat in the range 5.0 - 0.005 ppm (cation) are summarized in Pl. 2. During the experimental period all the concentrations of both herbicides had an inhibitory influence on growth. There was no further increase in frond number after 24 hours in cultures exposed to 5.0 ppm paraquat. Paraquat at concentrations of 0.5 and 0.05 ppm completely inhibited frond multiplication after 48 and 96 hours respectively. The lowest concentration of paraquat used (0.005 ppm) still significantly reduced the rate of formation of new fronds although the inhibition was not complete.

Diquat at concentrations of 5.0, 0.5 and 0.05 ppm. completely inhibited further frond multiplication after 24 hours. The lowest concentration of diquat (0.005 ppm.) drastically interfered with growth, completely stopping the production of new fronds after 144 hours. At all of the concentrations below 5.0 ppm., diquat had a significantly greater inhibitory effect on frond multiplication than paraquat. There was no significant difference between the multiplication rate in paraquat and diquat-treated fronds at the highest concentration used (5.0 ppm).

Pl. 2 also lists the average fresh and dry weights of the cultures at the end of the treatment period. At this time all treated cultures had

PLATE 2 Growth of S. oligorrhiza in nutrient solution containing a range of concentrations of paraquat and diquat

SAMPLE		MEAN FROND NUMBER								MEAN FRESH WEIGHT	MEAN DRY WEIGHT
		0h.	24h.	48h.	72h.	96h.	120h	144h	168	g.	mg.
PARAQUAT	Control	43[a]	61[a]	95	140	194	258	365	470	0.4307	26.0
	0.005ppm	43[a]	59[b]	88	122	155	178	215	255	0.1207	9.1
	0.05 ppm	43[a]	58[b]	72	76	77	77	77	77	0.0297	1.9
	0.5 ppm	43[a]	56	59	59	59	59	59	59	0.0228[a]	1.4
	5.0 ppm	43[a]	48[c]	48[a]	48[a]	48[a]	48[a]	48[a]	48[a]	0.0212[a]	1.0[b]
DIQUAT	0.005ppm	43[a]	62[a]	75	80	83	87	93	93	0.0346	2.6
	0.05 ppm	43[a]	54	54	54	54	54	54	54	0.0259	1.2[a]
	0.5 ppm	43[a]	47[c]	47[a]	47[a]	47[a]	47[a]	47[a]	47[a]	0.0167	1.2[a]
	5.0 ppm	43[a]	47[c]	47[a]	47[a]	47[a]	47[a]	47[a]	47[a]	0.0132	1.0[b]

All values represent the mean of 6 replicates

'D' tests ($p = 0.001$). Common letter postscripts denote no significant differences between vertically-tabulated means.

considerably lower fresh and dry weights than controls. At all concentrations the diquat-treated cultures had the lower fresh weights. There was no significant difference between the dry weights of paraquat and diquat- treated cultures at the highest concentration employed (5.0 ppm). However, at all other concentrations the dry weight of diquat-treated cultures was significantly lower than that of cultures exposed to paraquat.

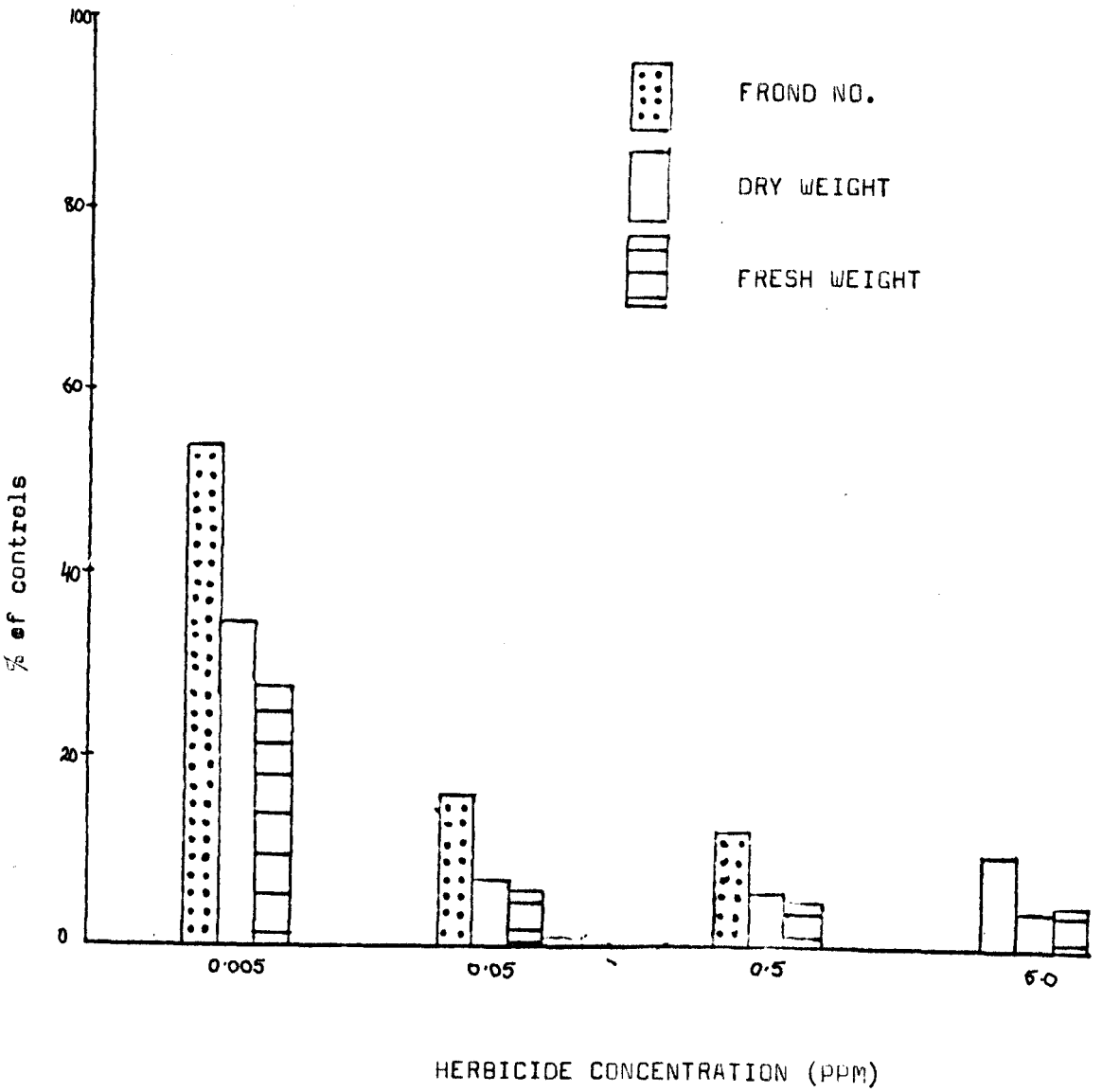
At the end of the treatment period, both the fresh and dry weights of treated cultures were more drastically reduced than frond number when compared with controls (pl. 3 and 4). However although Pl. 3 and 4 do show a relationship between herbicide concentration and inhibition of growth in terms of all three parameters measured, the difference between treatments was slight at the higher concentrations especially with diquat.

Experiment (ii)

Treatment of cultures of S. oligorrhiza for the same period of time with a range of concentrations of paraquat and diquat between 0.05 and 0.0004 ppm (cation) resulted in a greater spread of growth inhibition (pl. 5, 6 and 7) Paraquat treatment resulted in total inhibition of frond production only at the highest concentration used (0.05 ppm). Inhibition of frond production in the presence of 0.05 ppm paraquat was evident after only 24 hours and became increasingly marked with time, no new growth occurring after 96 hours. Frond multiplication continued throughout the treatment period at all lower paraquat concentrations although at all times the frond number of treated cultures was lower than in controls. As the duration of treatment increased the difference in frond number in cultures exposed to different paraquat concentrations became greater.

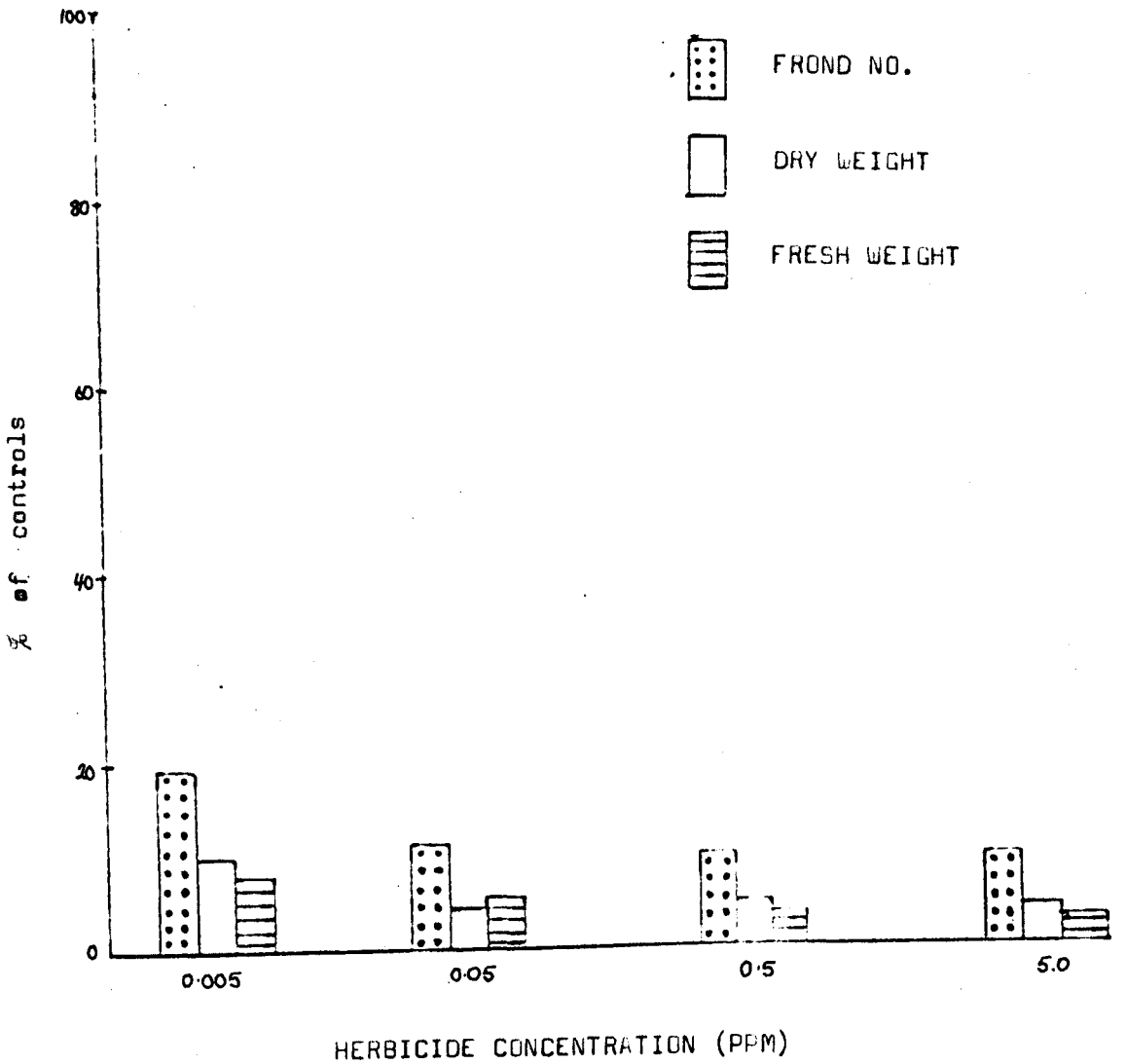
Complete inhibition of frond multiplication was achieved with diquat

PLATE 3 Growth of S. oligorrhiza in nutrient solution containing a range of concentrations of paraquat



All values represent the mean of 6 replicates

PLATE 4 Growth of S. oligorrhiza in nutrient solution containing a range of concentrations of diquat



All values represent the mean of 6 replicates

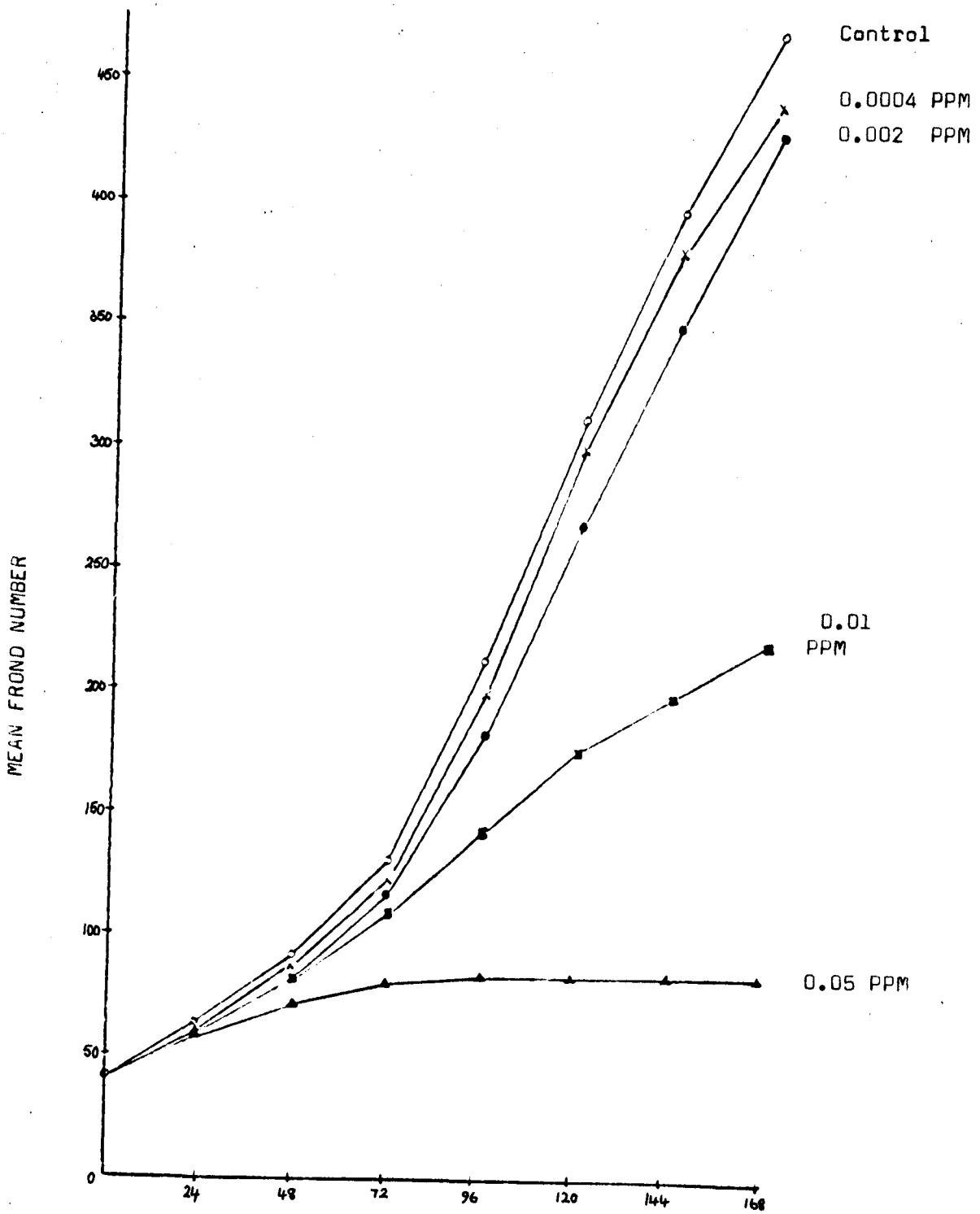
PLATE 5 Growth of S. oligorrhiza in nutrient solution containing a range of concentrations of paraquat and diquat

SAMPLE		MEAN FROND NUMBER								MEAN FRESH WEIGHT g.	MEAN DRY WEIGHT mg.
		0h.	24h.	48h.	72h.	96h.	120h.	144h.	168		
	Control	42[a]	63	91	130	212	311	396	470	0.4578	29.6
PARAQUAT	0.05ppm	42[a]	58[bc]	73	81	84[a]	84	84	84	0.0299	2.1
	0.01ppm	42[a]	58[bc]	82[c]	110	143	176	199	221	0.1306	10.1
	0.002ppm	42[a]	57[d]	83[bc]	118	182	270	349	427	0.4088	27.7
	0.0004ppm	42[a]	61[a]	87[a]	123[a]	200	300	379	441	0.4642	31.0
DIQUAT	0.05 ppm	42[a]	53	54	54	54	54	54	54	0.0203	1.4
	0.01 ppm	42[a]	56[d]	68	76	83[a]	87	87	87	0.0382	2.9
	0.002ppm	42[a]	59[b]	84[b]	113	154	201	246	337	0.2482	22.3
	0.0004ppm	42[a]	61[a]	86[a]	123[a]	195	277	355	456	0.3995	28.7

All values represent the mean of 6 replicates.

'D' tests ($P = 0.001$) Common letter postcripts denote no significant differences between vertically-tabulated means.

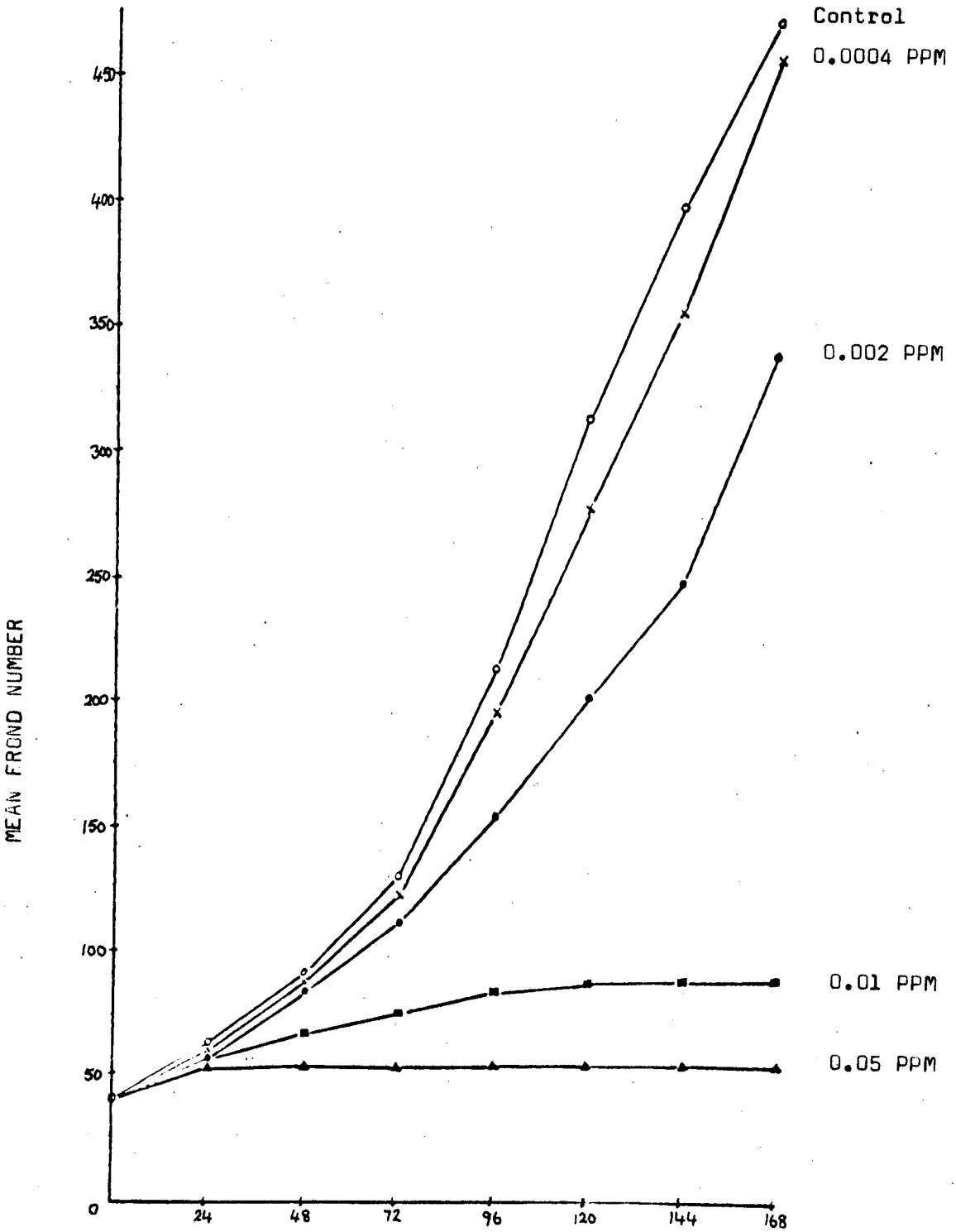
PLATE 6 The effect of a range of concentrations of paraquat on the growth of S. oligorrhiza



All values represent the mean of 6 replicates

P = 0.001 D = 1.12

PLATE 7 The effect of a range of concentrations of diaquat on the growth of S. oligorrhiza



All values represent the mean of 6 replicates

P = 0.001 D = 1.12

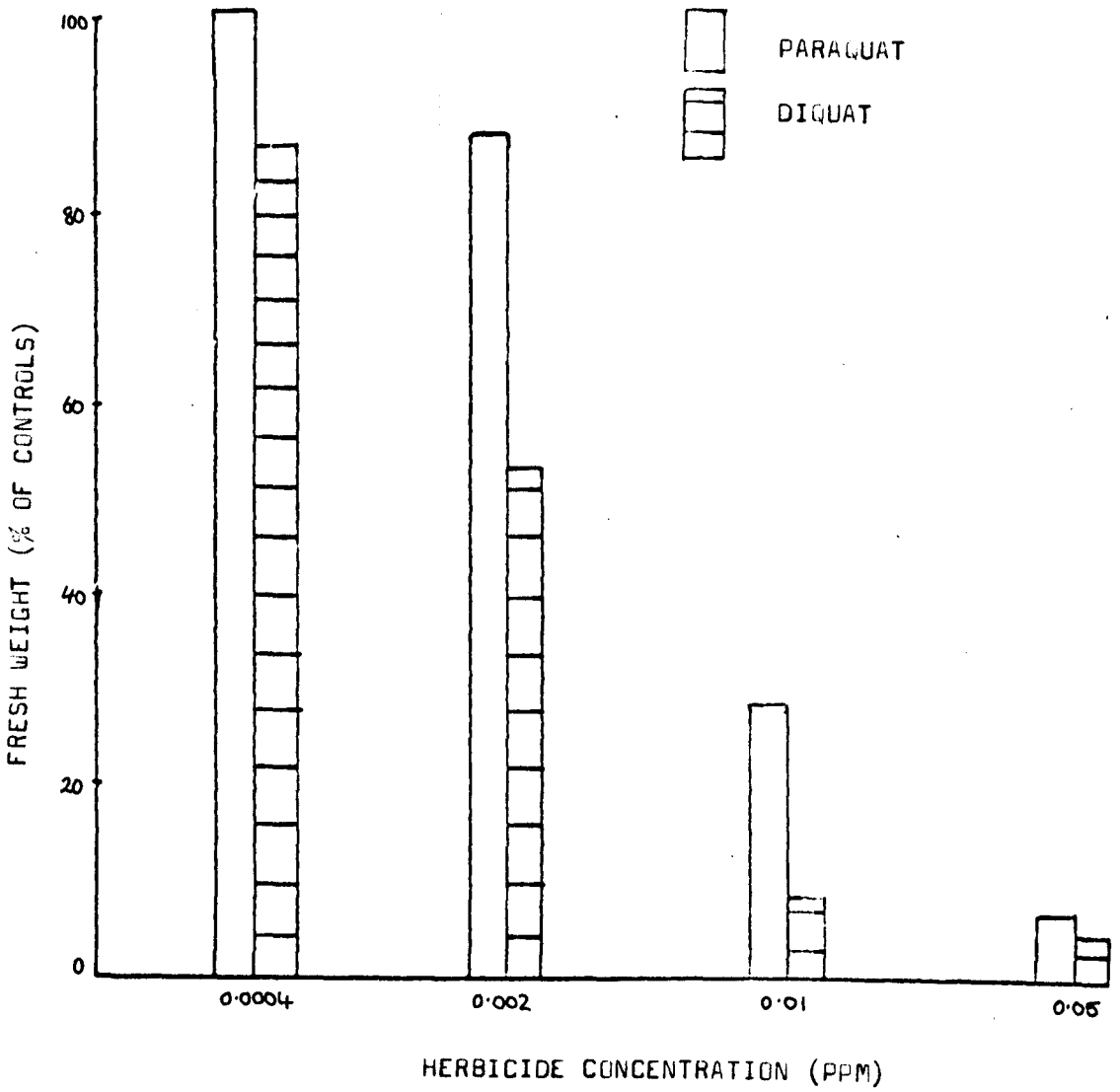
at concentrations of 0.05 and 0.01 ppm after 48 and 120 hours respectively. At the 2 lower concentrations of diquat tested, frond multiplication continued throughout the duration of treatment. For the first 48 hours there was a small but significant difference between the frond numbers in the presence of 0.002 and 0.0004 ppm. diquat. However, after this time the higher concentration had a more noticeably inhibitory effect. At these 2 concentrations there was a pronounced increase in the number of new fronds appearing compared with controls during the last 24 hours of the treatment period. This may be partly accounted for by the slight reduction in the frond multiplication rate which was measured in control cultures towards the end of the experimental period. However, despite this factor, Pl. 7 does clearly show an increase in the rate of frond production in treated cultures during the last 24 hours. This is especially evident at 0.002 ppm.

Apart from treatment with 0.0004 ppm paraquat, all treatments resulted in cultures with fresh and dry weights significantly lower than for controls (Pl. 5). At the end of the duration of treatment the fresh and dry weights of cultures exposed to 0.0004 ppm paraquat were slightly higher than controls, although the average frond number was lower.

The results also show that at all concentrations, diquat caused the greater reduction in both fresh weight (Pl. 8) and dry weight (Pl. 9). This difference was most evident at intermediate concentrations (0.002 and 0.01 ppm). Similarly, the data collected at the end of the experimental period show that, except at the lowest herbicide concentration (0.0004 ppm), frond numbers were always significantly lower in cultures exposed to diquat (Pl. 10).

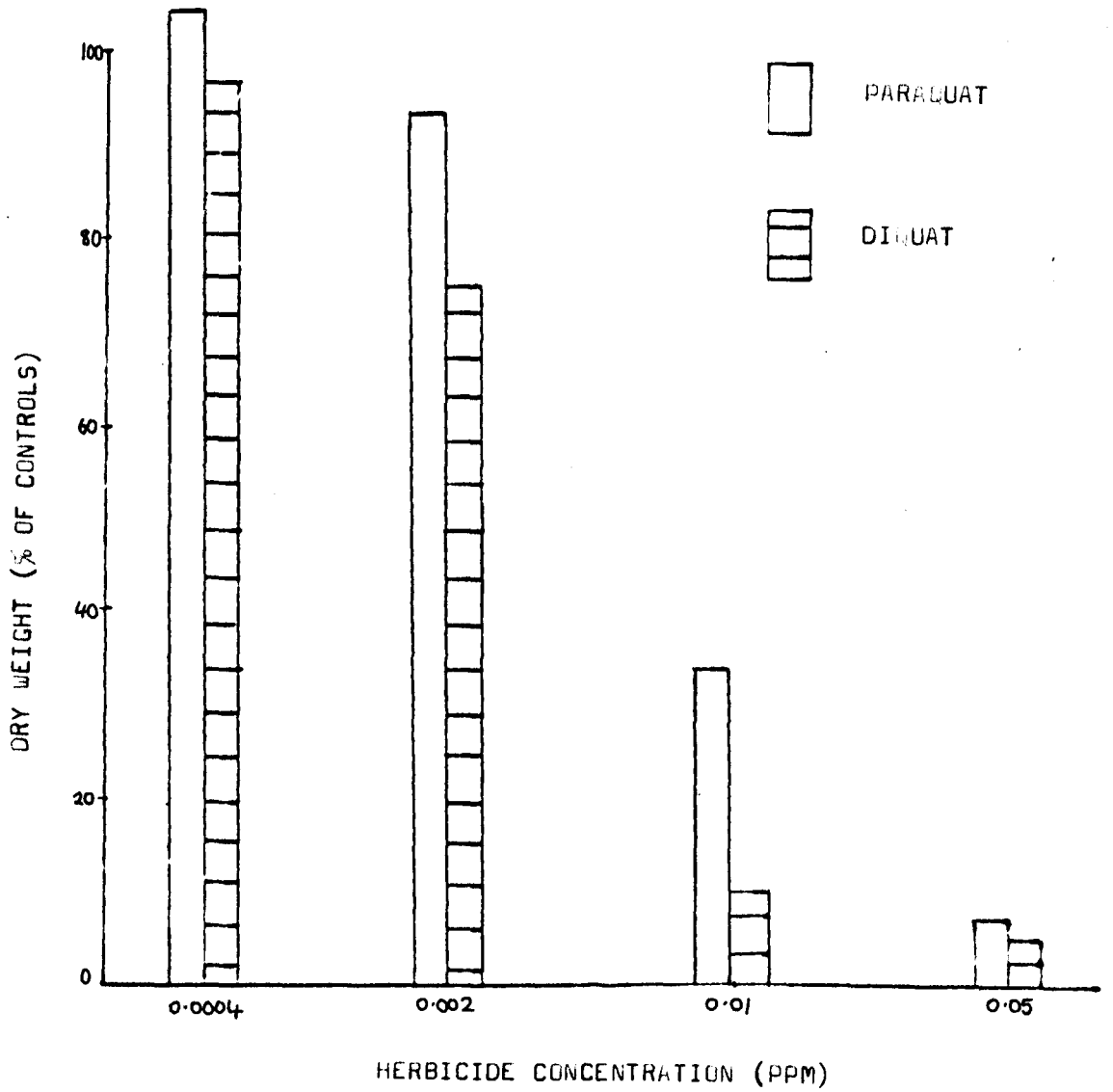
At all concentrations of paraquat and diquat tested, the fresh weight of treated cultures showed a greater reduction than did the dry weight (Pl. 11 and 12). As Pl. 11 and 12 also show, in cultures treated with the

PLATE 8 The effect of exposure to a range of concentrations of paraquat at 3500 lux on the fresh weight of S. oligorrhiza



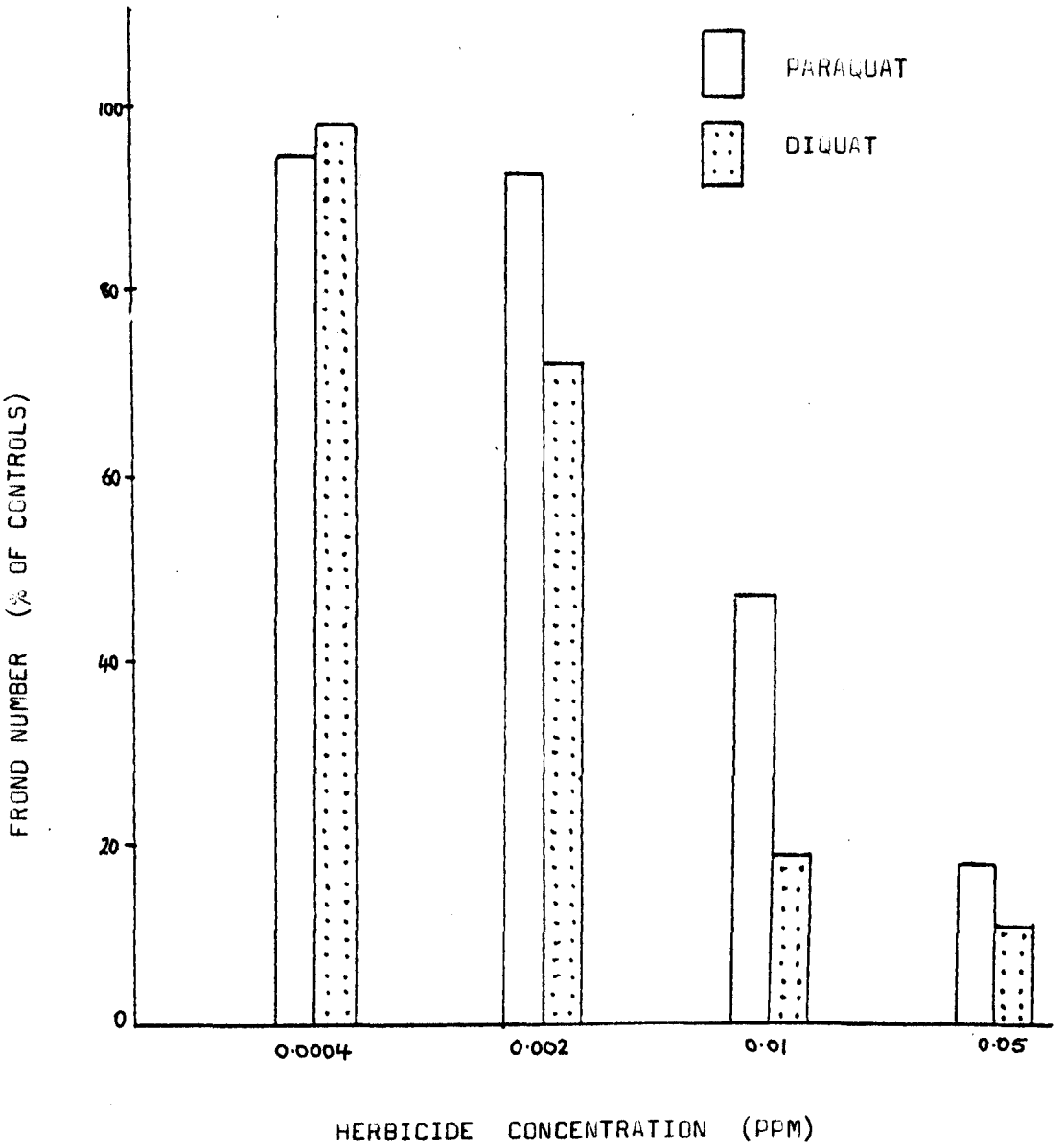
All values represent the mean of 6 replicates

Plate 9 The effect of exposure to a range of concentrations of paraquat and diquat at 3,500 lux on the dry weight of S. oligorrhiza



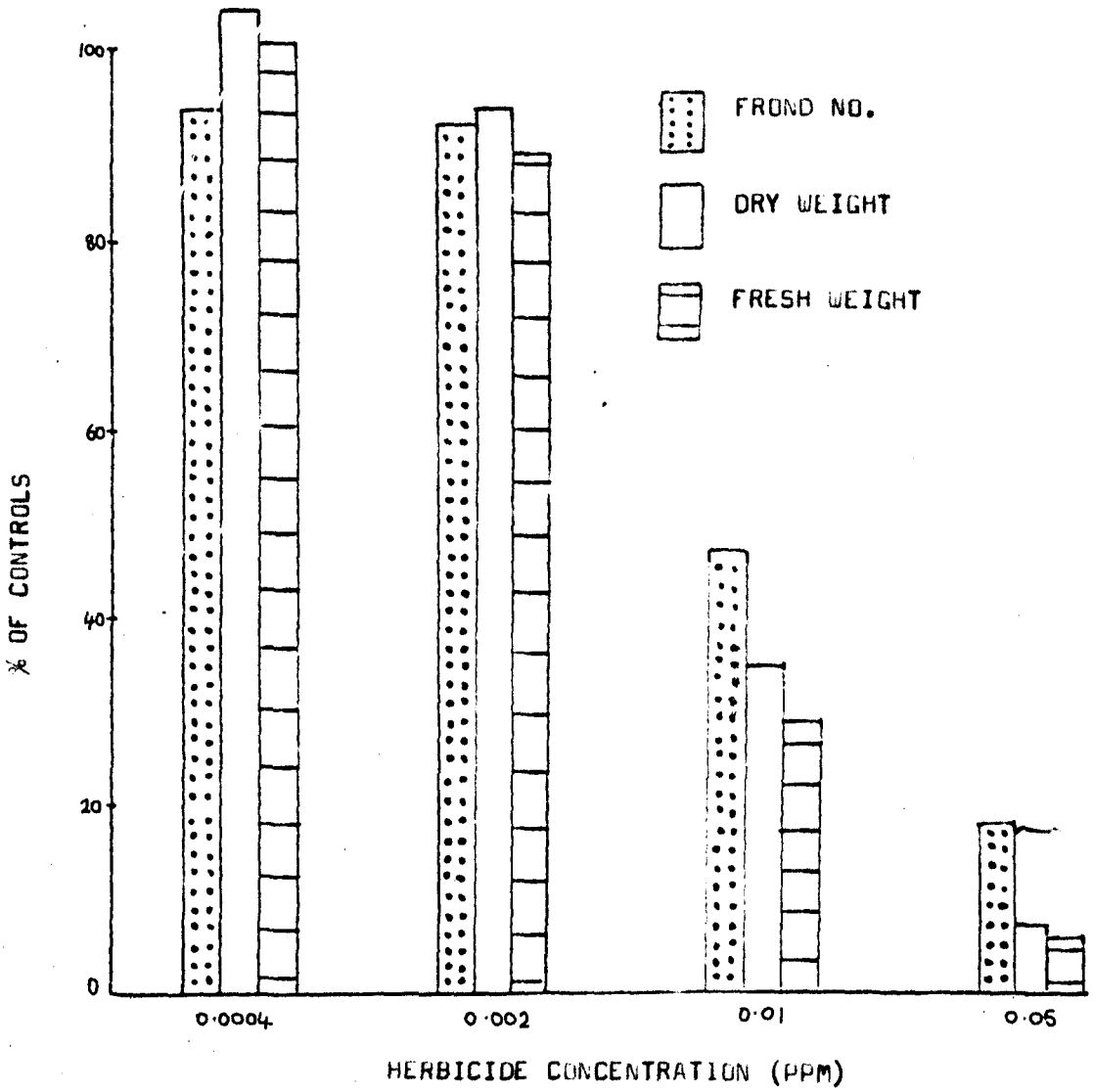
All values represent the mean of 6 replicates

Plate 10 The effect of exposure to a range of concentrations of Paraquat and diquat at 3500 lux on the frond number of S. oligorrhiza



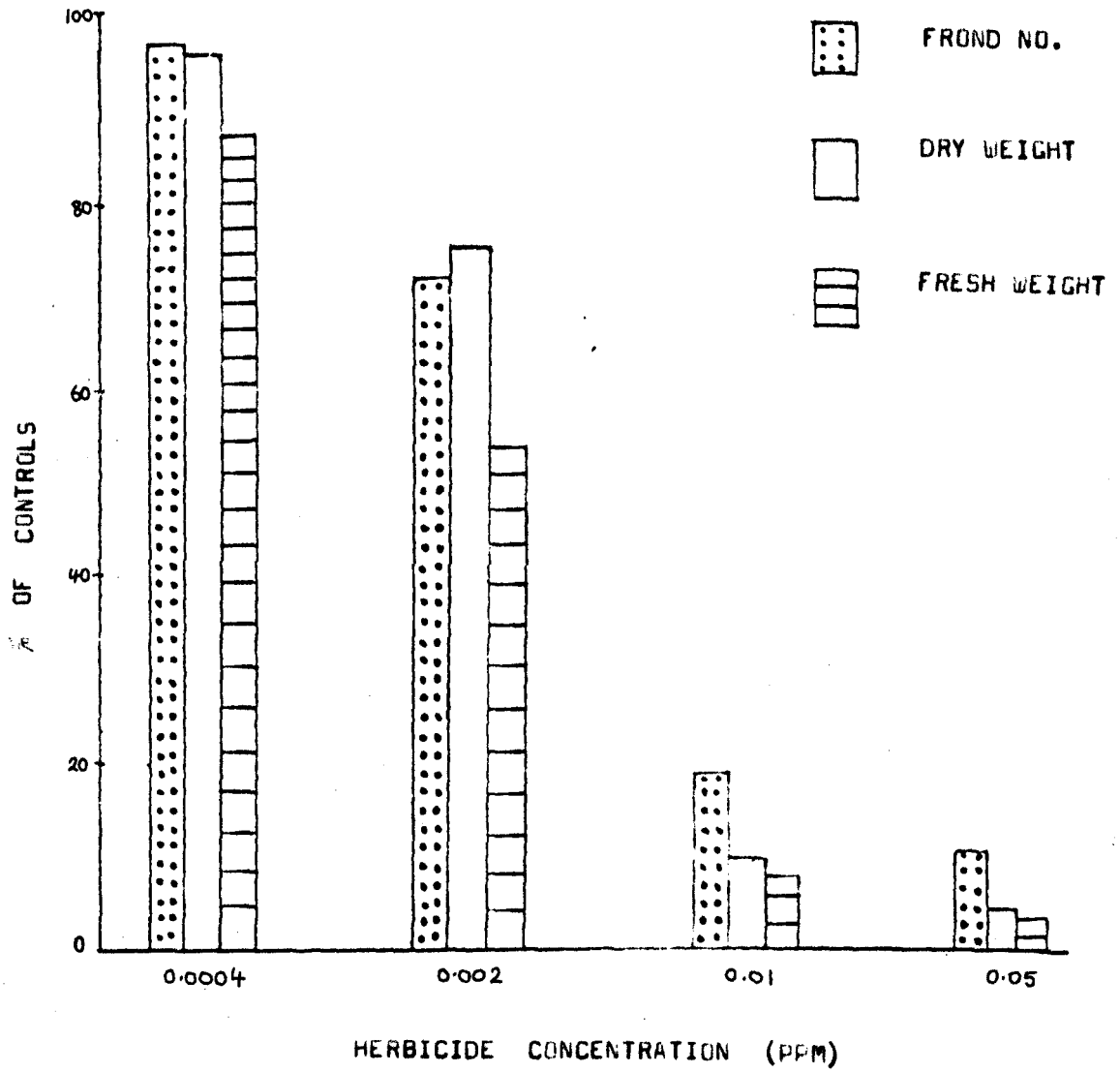
All values represent the mean of 6 replicates

Plate 11 Growth of S. oligorrhiza in nutrient solution containing a range of concentrations of paraquat



All values represent the mean of 6 replicates

Plate 12 Growth of S. oligorrhiza in nutrient solution containing a range of concentrations of diquat



All values represent the mean of 6 replicates

higher concentrations of both herbicides (0.05 and 0.01 ppm) both fresh and dry weights were reduced to a greater extent than frond number. However at the lower concentrations no consistent pattern was evident.

The effects of treatment with a range of concentrations of paraquat and diquat on the appearance of fronds of S. oligorrhiza

Treatment with both herbicides at many of the concentrations used in the two previous growth experiments greatly affected the appearance of the fronds of S. oligorrhiza during the experimental period. The most common symptom of toxicity was the development of chlorosis. Varying degrees of chlorosis were observed in fronds exposed to paraquat at concentrations of 0.005 ppm and upwards and to diquat at concentrations of 0.002 ppm and upwards. Every 24 hours the proportion of totally chlorotic fronds per culture was calculated. The results are shown in Pl. 13 and 14. These results show a clear relationship between the rate of chlorosis and herbicide concentration. In addition it is seen that chlorosis developed more rapidly in diquat-treated cultures.

In cultures undergoing many treatments chlorosis was accompanied by the exudation of numerous tiny droplets of liquid onto the uppermost surfaces of the fronds. This "sweating" was observed in fronds treated with paraquat and diquat at concentrations of 0.005 ppm and above. At all concentrations except the lowest used (0.0004 ppm) diquat treatment was more drastic as measured by the speed with which chlorosis and "sweating" developed. At the lowest concentration all fronds appeared unaffected.

Daughter fronds developed chlorosis more readily than mother fronds. In daughter fronds, chlorosis began in the regions closest to the mother frond (Pl. 15). In mother fronds, chlorosis generally began at the end

PLATE 13 Chlorosis of S. oligorrhiza in nutrient solution containing a range of concentrations of paraquat and diquat

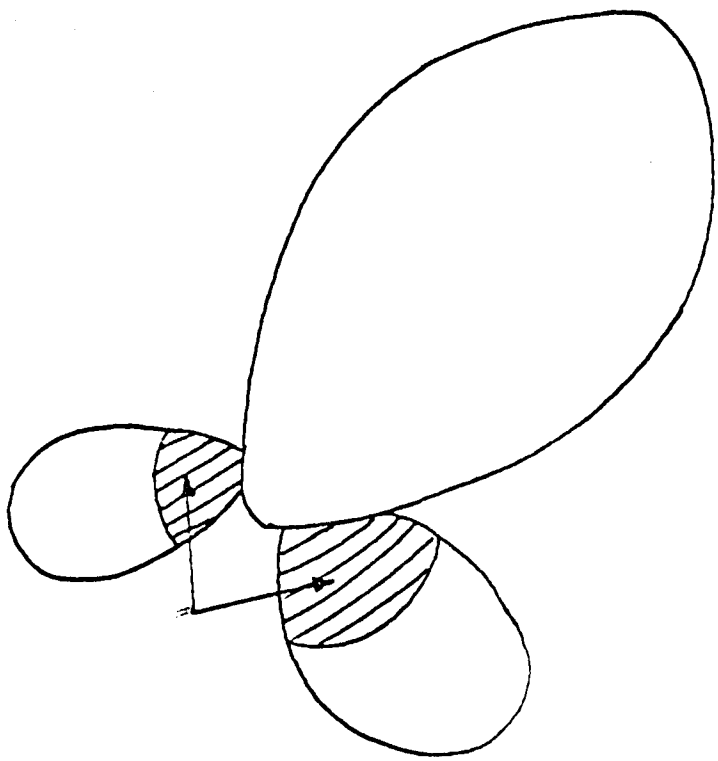
SAMPLE		% FULLY CHLOROTIC FRONDS							
		0h.	24h.	48h.	72h.	96h.	120h.	144h.	168h.
Control		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PARAQUAT	0.005ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.9	3.5
	0.05 ppm	0.0	0.0	0.0	30.3	79.2	87.0	94.8	98.7
	0.5 ppm	0.0	0.0	13.6	96.6	98.3	100.0	100.0	100.0
	5.0 ppm	0.0	0.0	85.4	100.0	100.0	100.0	100.0	100.0
DIQUAT	0.005ppm	0.0	0.0	0.0	0.0	2.4	13.8	22.6	30.1
	0.05 ppm	0.0	0.0	55.6	85.2	92.6	98.2	100.0	100.0
	0.5 ppm	0.0	2.1	100.0	100.0	100.0	100.0	100.0	100.0
	5.0 ppm	0.0	19.2	100.0	100.0	100.0	100.0	100.0	100.0

All values represent the mean of 6 replicates.

PLATE 14 Chlorosis of S. oligorrhiza in nutrient solution containing a range of concentrations of paraquat and diquat

SAMPLE		% FULLY CHLOROTIC FRONDS							
		0h.	24h.	48h.	72h.	96h.	120h.	144h.	168h.
CONTROL		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PARAQUAT	0.0004ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.002 ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.01 ppm	0.0	0.0	0.0	0.0	0.0	0.7	2.2	5.0
	0.05 ppm	0.0	0.0	0.0	3.1	15.8	74.7	87.2	93.2
DIQUAT	0.0004ppm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.002 ppm	0.0	0.0	0.0	0.0	0.0	5.3	16.7	16.3
	0.01 ppm	0.0	0.0	0.0	0.0	2.0	16.9	30.5	50.6
	0.05 ppm	0.0	0.0	23.8	73.0	96.3	100.0	100.0	100.0

Plate 15 A drawing of a 3-frond colony of S. oligorrhiza showing the regions where chlorosis usually started after treatment in light with bipyridylium herbicides (arrows)



furthest from the points of attachment of the daughter fronds. The last parts of fronds to become completely chlorotic were in areas of mother fronds close to the points from which the daughter fronds arise (Pl. 16). This was especially evident in fronds treated with the lower toxic concentrations of either herbicide.

At intermediate herbicide concentrations, fragmentation of treated colonies into individual fronds occurred after the early stages of chlorosis. With higher concentrations no such fragmentation occurred, even although all fronds may have become completely chlorotic. In many cases, herbicide treatment was also found to result in the roots breaking away from the fronds at their points of attachment. Once again however, this was not found to occur in fronds treated with the higher herbicide concentrations in which complete chlorosis took place rapidly.

At toxic concentrations of either herbicide below that resulting in fragmentation of the colonies, growth abnormalities of fronds accompanied chlorosis. These occurred mainly in the regions where daughter fronds are attached to the mother fronds (Pl. 17). Here the fronds became unusually narrow. Growth abnormalities of this were observed only in cultures treated with 0.01 ppm paraquat or 0.002 ppm. diquat.

Plate 16 A drawing of a colony of S. oligorrhiza showing the regions which were generally the last to become completely chlorotic (arrows)

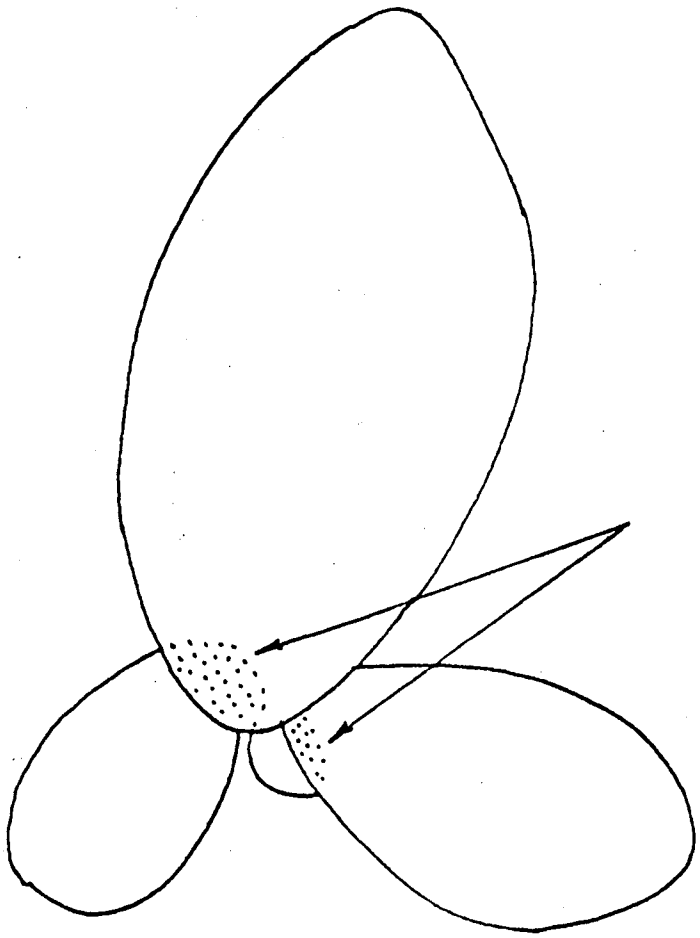
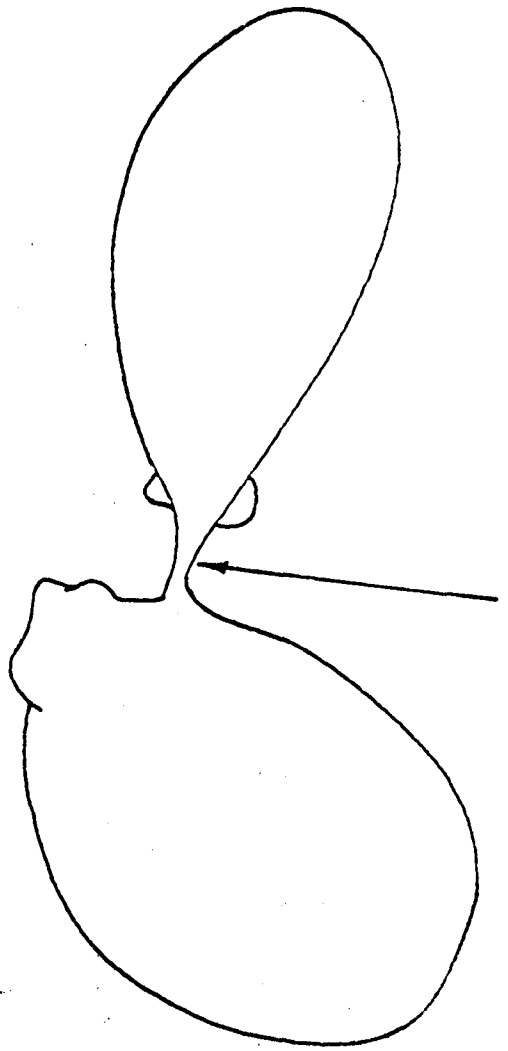


Plate 17 A drawing of a colony of S. oligorrhiza showing the type of growth abnormality (arrow) which often arose in the presence of low, toxic concentrations of paraquat or diquat



B. PIGMENT STUDIES

(i) Comparison of the effects of paraquat and diquat on chlorophyll content

Cultures of S. oligorrhiza were treated with paraquat or diquat at concentrations of 0.1, 1.0 and 10.0 ppm (cation) and placed at random beneath a light bank providing a light intensity of 3500 lux. A total of 6 cultures per treatment were employed. The plants were harvested after 24 hours and the chlorophyll extracted.

The results are shown in Pl. 18. At all concentrations tested both herbicides caused a reduction in total chlorophyll content. At all concentrations the reduction in total chlorophyll was greater in fronds treated with diquat (Pl. 19). All treatments, with the exception of exposure to 0.1 ppm paraquat, also resulted in a reduction of the chlorophyll a/chlorophyll 6 ratio (Pl. 18). The reduction of this ratio became more marked with increasing herbicide concentration and at all concentrations the ratio was lower in diquat-treated fronds (Pl. 20).

(ii) The effect of duration of treatment on chlorophyll content

Cultures were treated with diquat at a concentration of 1.0 ppm (salt) and illuminated with a light intensity of 3500 lux. Treated cultures and controls were harvested at various intervals and their chlorophyll contents determined.

The results are shown in Pl. 21. Untreated cultures showed a small rise in total chlorophyll after 6 hours due possibly to the transferal of the fronds onto fresh nutrient solution. After 6 hours the total chlorophyll content of control fronds steadied (Pl. 22). In diquat-treated cultures on the other hand, the total chlorpphyll content was almost unchanged after 6 hours. After this time the total chlorophyll content of treated fronds

PLATE 18 The effect of a range of concentrations of paraquat and diquat at 3500 lux on the chlorophyll content of S. oligorrhiza after 24 hours

	CONCENTRATION P.P.M. (Cation)	CHLOROPHYLL a (Mg/g. fresh wt.)	CHLOROPHYLL b (Mg/g. fresh wt.)	TOTAL CHLOROPHYLL (mg/g. fresh wt.)	CHLOROPHYLL a/b
	0.0	0.6119	0.1917	0.8036	3.19
		0.6111	0.1873	0.7984	3.26
PARAQUAT	0.1	0.5851	0.1739	0.7590	3.36
		0.5663	0.1763	0.7426	3.21
	1.0	0.4319	0.1719	0.6038	2.51
		0.4264	0.1660	0.5924	2.57
	10.0	0.2637	0.1325	0.3962	1.99
		0.2658	0.1268	0.3926	2.10
DIQUAT	0.1	0.5019	0.1788	0.6808	2.81
		0.5091	0.1768	0.6859	2.88
	1.0	0.2686	0.1434	0.4121	1.87
		0.2525	0.1233	0.3757	2.05
	10.0	0.0959	0.620	0.1579	1.55
		0.0959	0.0620	0.1579	1.55

Plate 19 Comparison of the effects of a range of concentrations of Paraquat and diquat on the chlorophyll content of S. oligorrhiza

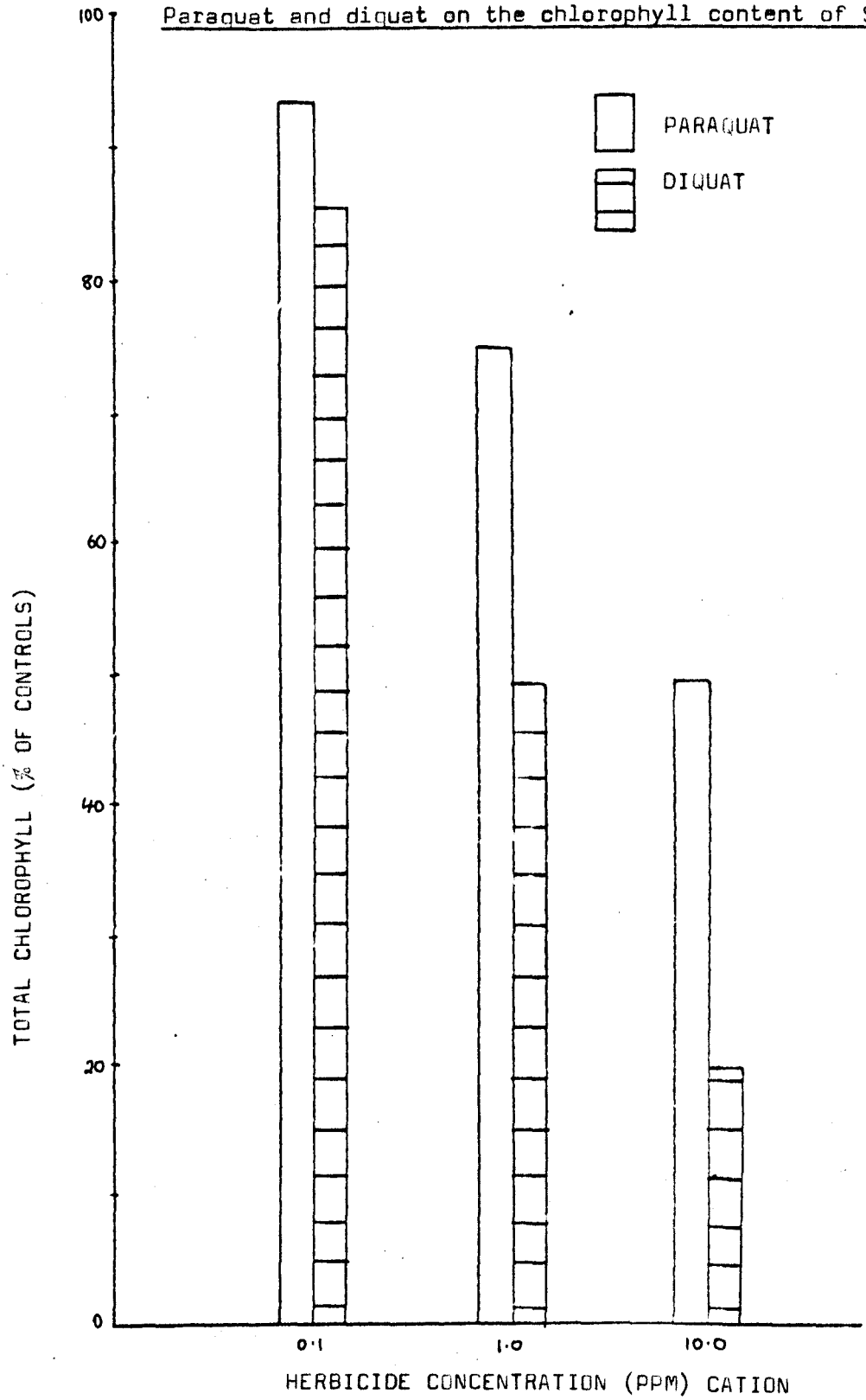


Plate 20 The effect of a range of concentrations of diquat on the chlorophyll a/b ratio in S. oligorrhiza

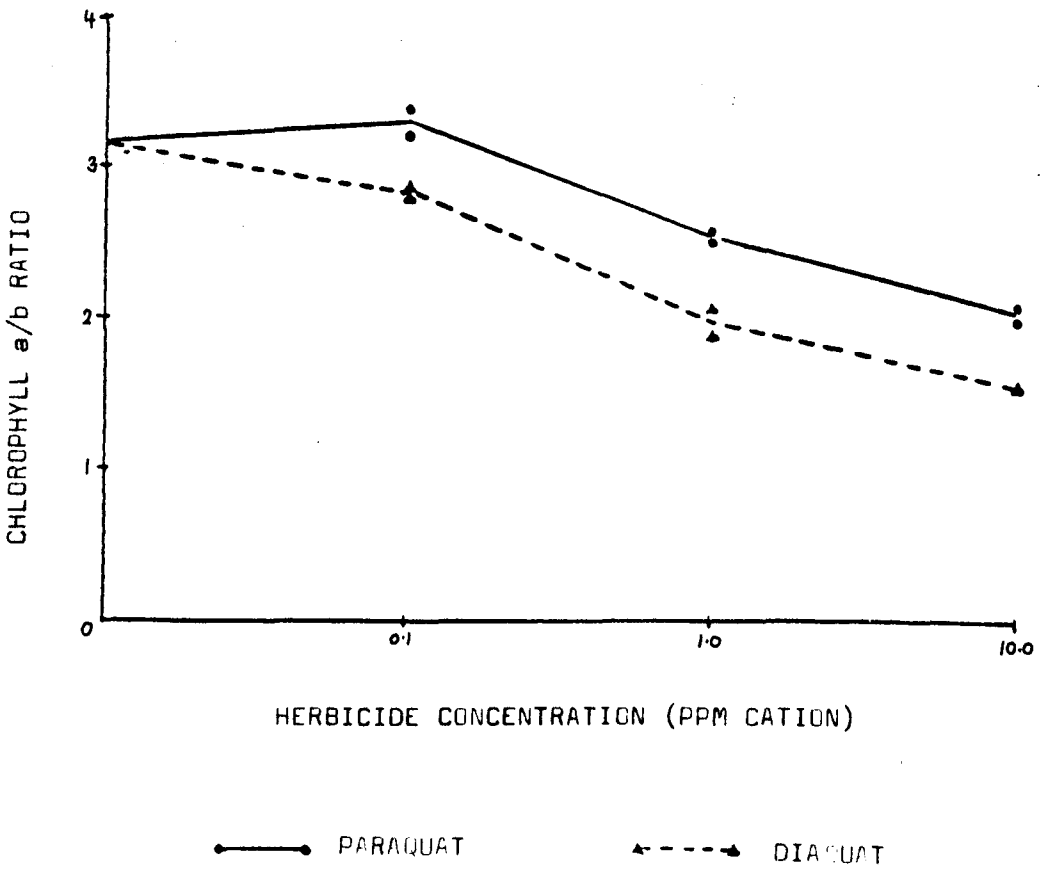
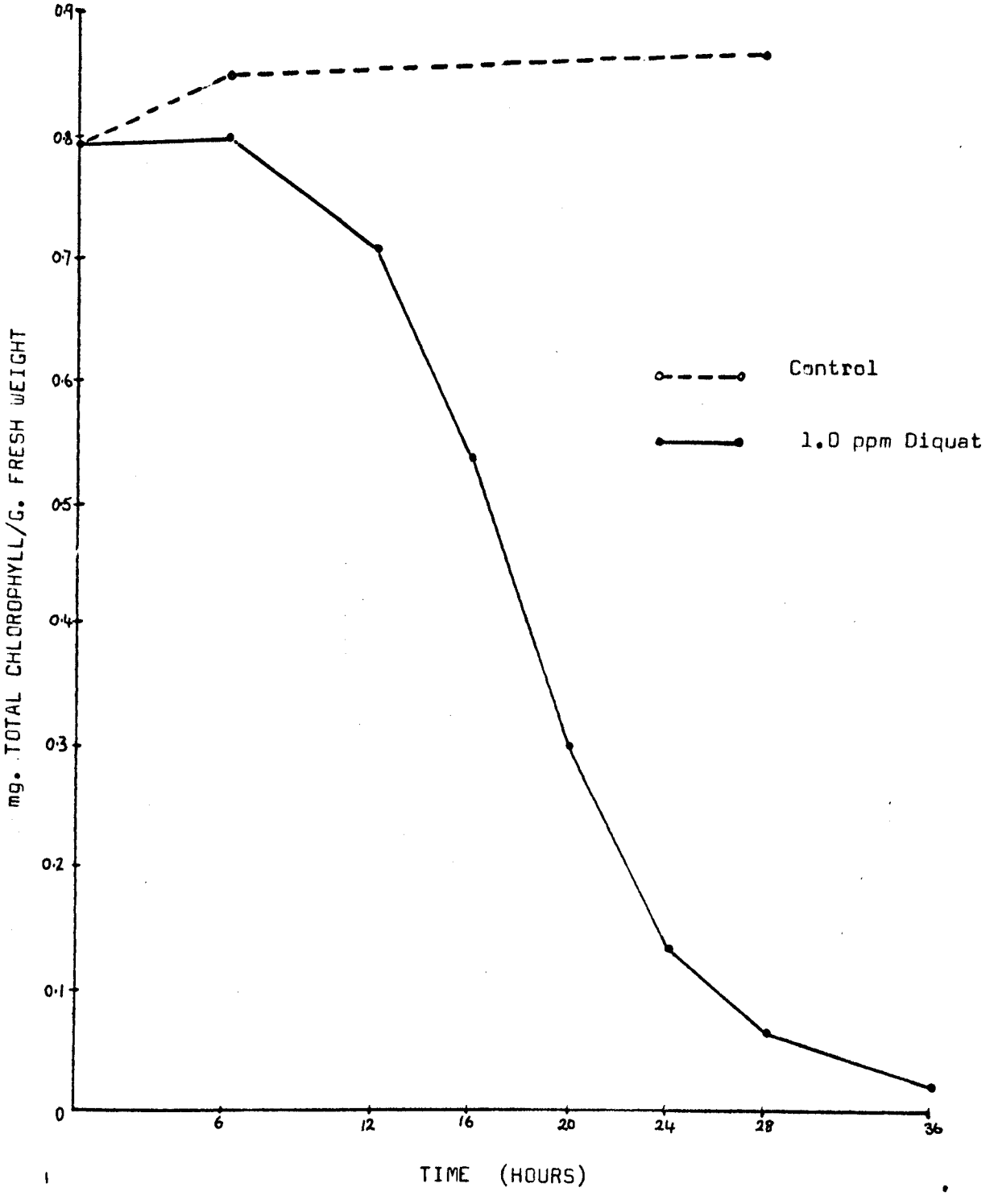


PLATE 21 The effect of exposure to 1.0 ppm diquat at 3500 lux on the chlorophyll content of S. oligorrhiza

TIME (h)	CHLOROPHYLL a (mg/g. fresh wt.)	CHLOROPHYLL b (mg/g. fresh wt.)	TOTAL CHLOROPHYLL (mg/g. fresh wt.)	CHLOROPHYLL a/b
		CONTROL		
0	0.5951	0.1992	0.7943	2.99
6	0.6362	0.2152	0.8514	2.96
28	0.6556	0.2115	0.8671	3.10
		1.0PPM DIQUAT		
0	0.5951	0.1992	0.7943	2.99
6	0.5972	0.2016	0.7988	2.96
12	0.5343	0.1740	0.7083	3.07
16	0.3878	0.1493	0.5371	2.60
20	0.2010	0.0985	0.2995	2.04
24	0.0867	0.0464	0.1331	1.87
28	0.0438	0.0214	0.0652	2.05
36	0.0171	0.0071	0.0242	2.41

Plate 22 The chlorophyll content of fronds of S. oligorrhiza at various intervals following treatment in the light with 1.0 ppm diquat (salt)



decreased rapidly. The loss of chlorophyll took place most rapidly between 12 and 24 hours. Subsequently, the rate of chlorophyll loss slowed down but nevertheless continued until the end of the experimental period. By this time (36 hours) the total chlorophyll content of treated fronds was only about 3% of the initial level.

During the time in which the loss of chlorophyll was occurring most rapidly in treated cultures, (12 - 24 hours) the chlorophyll a/ chlorophyll b ratio dropped (Pl. 23). No such reduction was measured in control cultures. However, after 24 hours in the presence of diquat the ratio steadily increased although it always remained below the initial value.

(iii) The effect of diquat on chlorophyll content at different light intensities

Control cultures and cultures treated with diquat at a concentration of 10 ppm (salt) were exposed to light intensities of 3500, 1300, 1000 and 650 lux. 3 cultures per treatment were employed. The reduction of light intensity was achieved by covering the cultures with different numbers of white paper tissues. Control and treated cultures were also incubated in complete darkness inside black polythene bags. Despite exposure to different conditions of illumination, all cultures were incubated together in the same area in order to prevent the introduction of unwanted variables. All cultures were harvested after 24 hours followed by chlorophyll extraction.

The chlorophyll contents of control and treated cultures of S. oligorrhiza after 24 hours at different light intensities are shown in Pl. 24. When the total contents of treated fronds are expressed as percentages of control valves it is seen that the loss of chlorophyll was greatly influenced by the incident light intensity (Pl. 25). The results show that within the limits

Plate 23 The effect of the duration of exposure to diquat on the Chlorophyll a/b ratio in S. oligorrhiza

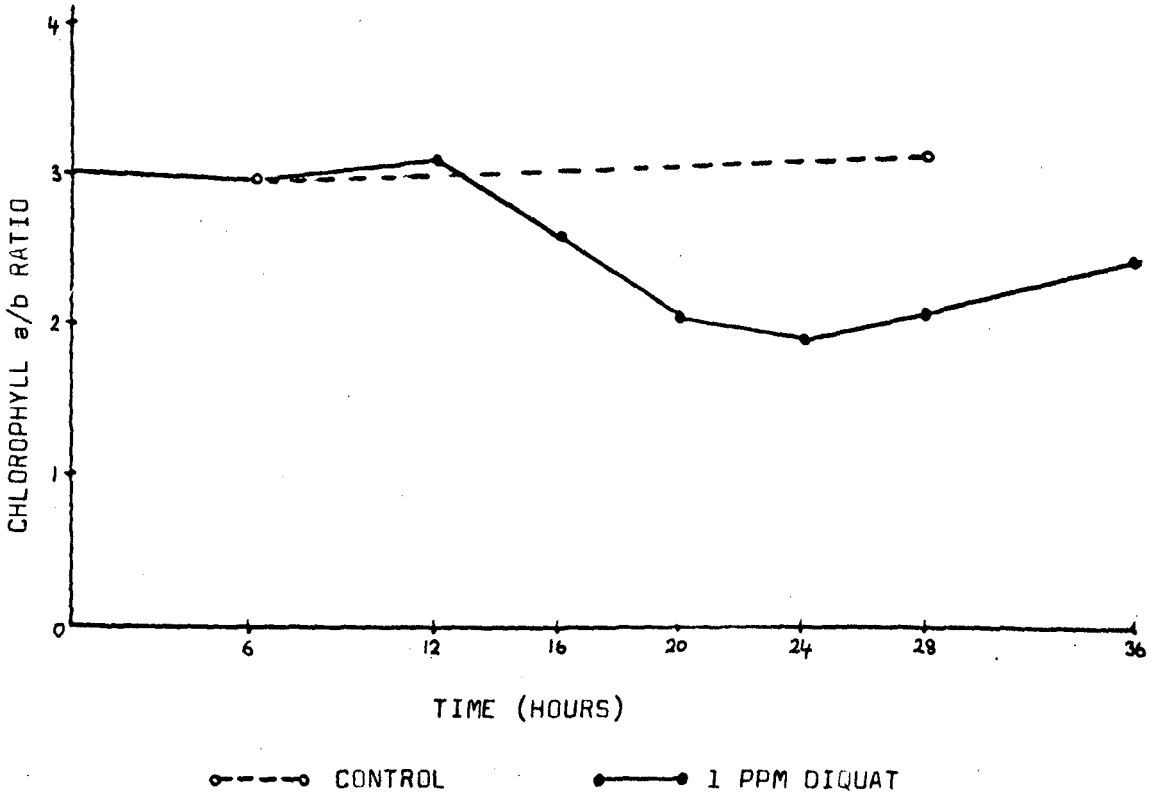
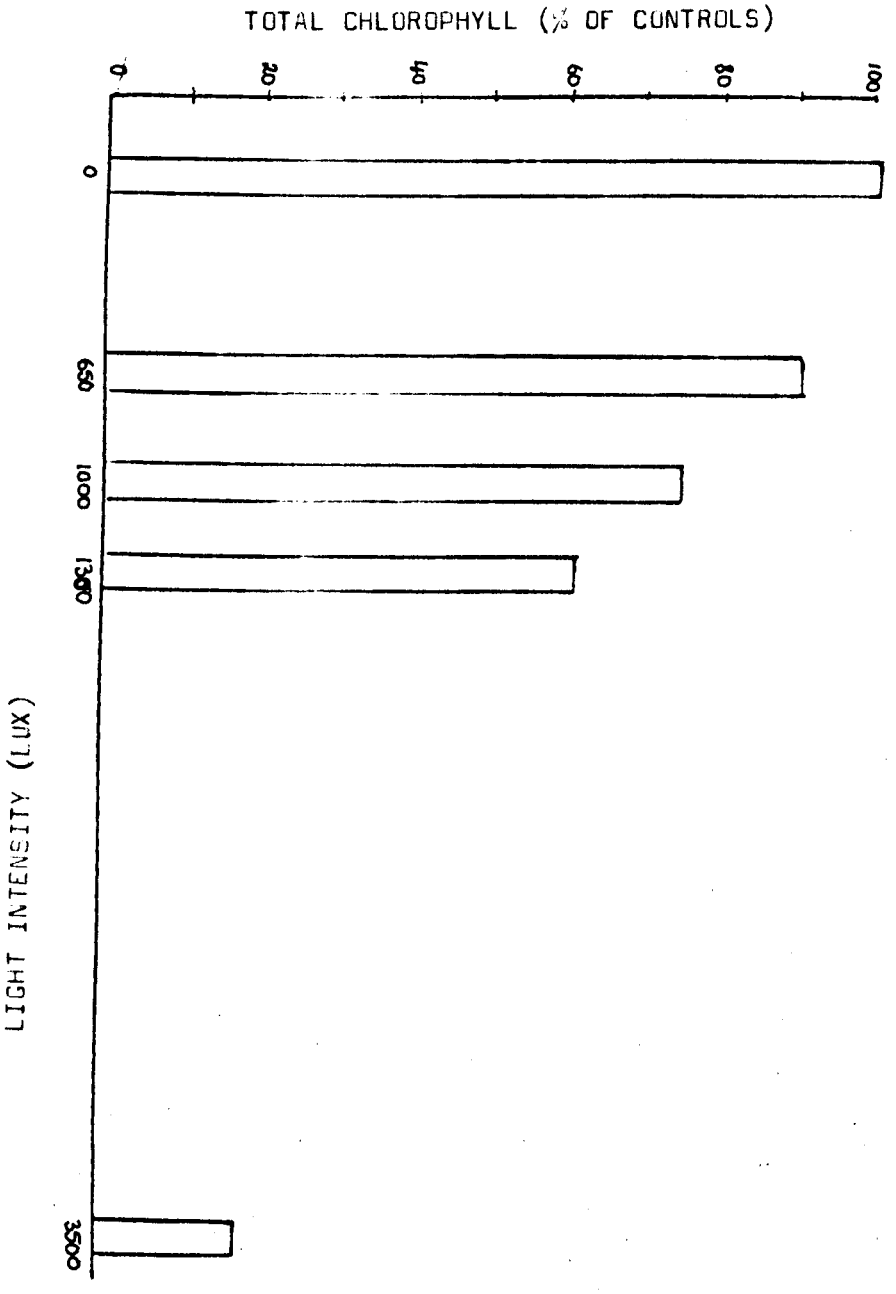


PLATE 24 The effect of exposure to 10.0 ppm diquat at a range of light intensities on the chlorophyll content of S. oligorrhiza after 24 hours.

LIGHT INTENSITY (Lux)	CHLOROPHYLL a (mg/g. fresh wt.)	CHLOROPHYLL b (mg/g. fresh wt.)	TOTAL CHLOROPHYLL (mg/g. fresh wt.)	CHLOROPHYLL A/b
		CONTROL		
3500	0.6987	0.2299	0.9286	3.04
1300	0.7055	0.2285	0.9340	3.09
1000	0.6938	0.2239	0.9177	3.10
650	0.6743	0.2130	0.8873	3.17
0 (Dark)	0.6624	0.2175	0.8799	3.05
		10.0 ppm DIQUAT		
3500	0.1044	0.0638	0.1683	1.64
1300	0.4012	0.1760	0.5772	2.28
1000	0.4998	0.1975	0.6973	2.53
650	0.5793	0.2324	0.8117	2.49
0 (Dark)	0.6673	0.2243	0.8915	2.89

Plate 25 The effect of exposure to 10.0 ppm diquat at a range of light intensities on the total chlorophyll content of S. oligorrhiza



Plants were harvested after exposure for 24 hours.

tested, the greater the light intensity the greater was the reduction in chlorophyll content. In complete darkness no loss of chlorophyll could be measured after 24 hours in the presence of diquat.

During the treatment period, light intensity had little effect on the chlorophyll a/chlorophyll b ratio in control cultures. A reduction of the chlorophyll a/Chlorophyll b ratio was measured in illuminated treated fronds at all light intensities. However, at lower levels of illumination the reduction of this ratio was less pronounced (Pl. 26)

(iv) The effect of an intervening dark period on the loss of chlorophyll in diquat-treated fronds

Control cultures of S. oligorrhiza and cultures treated with diquat at a concentration of 1.0 ppm (salt) were illuminated with a light intensity of 3500 lux. Control and treated cultures were harvested in groups of 3 at various times throughout the first 20 hours. The remaining cultures were then enclosed in black polythene bags to exclude light and left for a period of 16 hours. At the end of this interval, control and treated cultures were sampled and those cultures still remaining were returned to the light. Harvesting continued after a further 4 and 8 hours.

During the 20 hours prior to the intervening dark period, control fronds showed a slight increase in total chlorophyll content (Pl. 27 and 28). In the same period diquat-treated fronds suffered a slow but steady chlorophyll loss. Immediately following the 16 hour dark interval, the total chlorophyll content of control fronds was somewhat lower than at the end of the first period of illumination. However, on being returned to the light, control fronds slightly increased their chlorophyll content once more. During the dark interval the loss of chlorophyll in treated fronds was greatly

Plate 26 The effect of exposure to 10ppm diquat at a range of light intensities on the chlorophyll a/b ratio of S. oligorrhiza

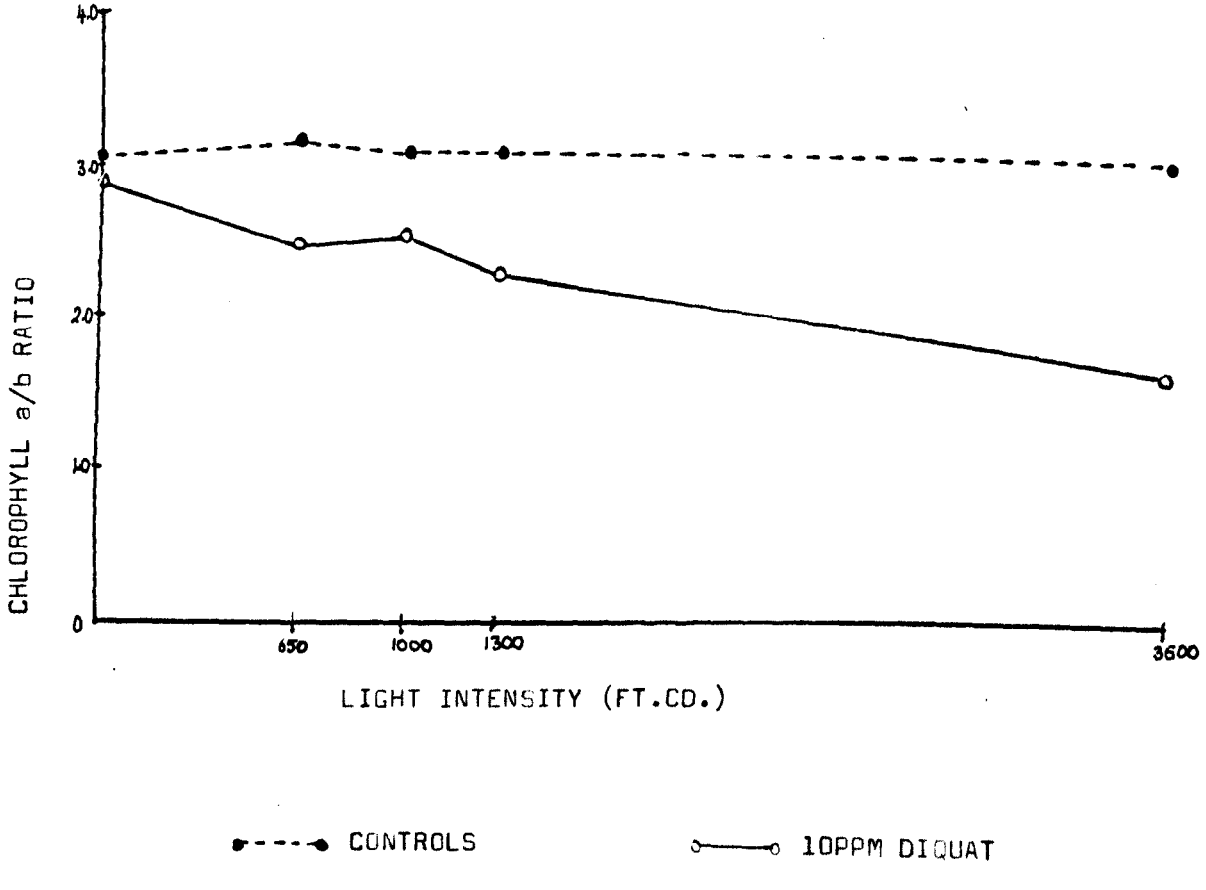


PLATE 27 The effect of an intervening dark period on the loss of chlorophyll in diquat-treated fronds of S. oligorrhiza

TREATMENT	CHLOROPHYLL a (mg/g. fresh wt.)	CHLOROPHYLL b (mg/g. fresh wt.)	TOTAL CHLOROPHYDD (mg/g. fresh wt.)	CHLOROPHYLL a/b
Control 0h.	0.6878	0.2088	0.8966	3.29
Control 12h	0.6824	0.2084	0.8908	3.27
Control 20h	0.7221	0.2307	0.9528	3.13
16 Hour dark interval				
Control 20h	0.6977	0.2187	0.9164	3.19
Control 28h	0.7043	0.2367	0.9410	2.98
1PPM Diquat 12h	0.6392	0.1914	0.8306	3.34
1PPM Diquat 16h	0.6186	0.1797	0.7983	3.44
1PPM Diquat 20h	0.5666	0.1819	0.7484	3.11
16 Hour Dark Interval				
1PPM Diquat 20h	0.5500	0.1912	0.7412	2.88
1PPM Diquat 24h	0.3396	0.1544	0.4940	2.20
1PPM Diquat 28h	0.2523	0.1295	0.3818	1.95

PLATE 28 The effect of an intervening dark period on the loss of chlorophyll in diquat-treated fronds of S. oligorrhiza

TIME IN LIGHT (Hr.)	TOTAL CHLOROPHYLL (% Initial Level)	
	1PPM DIQUAT	CONTROL
0	100.00	100.00
12	92.64	99.35
16	89.04	-
20	83.47	106.27
	16 hr. Dark Interval	
20	82.67	102.21
24	55.10	-
28	42.58	104.95

retarded. In fact, the reduction in chlorophyll content in treated fronds during this time was considerably less than in control fronds. However, upon the resumption of illumination the loss of chlorophyll in treated fronds was rapid. The interuption of chlorophyll loss resulting from the intervening dark period is strikingly demonstrated in Pl. 29.

(v) The effect of TTC on the loss of chlorophyll in diquat-treated fronds

Cultures containing diquat at concentrations of 0.1, 1.0 and 10.0 ppm (salt) were set up in addition to cultures containing the same concentrations of diquat plus 10.0 ppm TTC (2:3:5 - triphenyl-tetrazolium chloride). Control cultures with and without TTC were also prepared. In all, 6 cultures were randomly placed under a light bank providing a light intensity of 3500 lux at culture level. Cultures were harvested after a period of 24 hours followed by chlorophyll intraction.

The results are shown in Pl. 30. The presence of 10 ppm TTC caused a small reduction in the total chlorophyll content of control fronds. The results suggest that this reduction was due to the loss of chlorophyll a. However, at all concentrations of diquat tested, the presence of TTC retarded the loss of chlorophyll in diquat-treated cultures (Pl. 31). The results also show that the presence of TTC in treated cultures, lessened the reduction in the chlorophyll a/ chlorophyll b ratio (Pl. 32). In the presence of diquat alone, the chlorophyll a/ chlorophyll B ratio decreased steadily with increasing herbicide concentration. In the presence of TTC the ratio also decreased with increasing diquat concentration but did so to a lesser degree.

Plate 29 The effect of an intervening dark period on the loss of chlorophyll in diquat-treated fronds of S. oligorrhiza

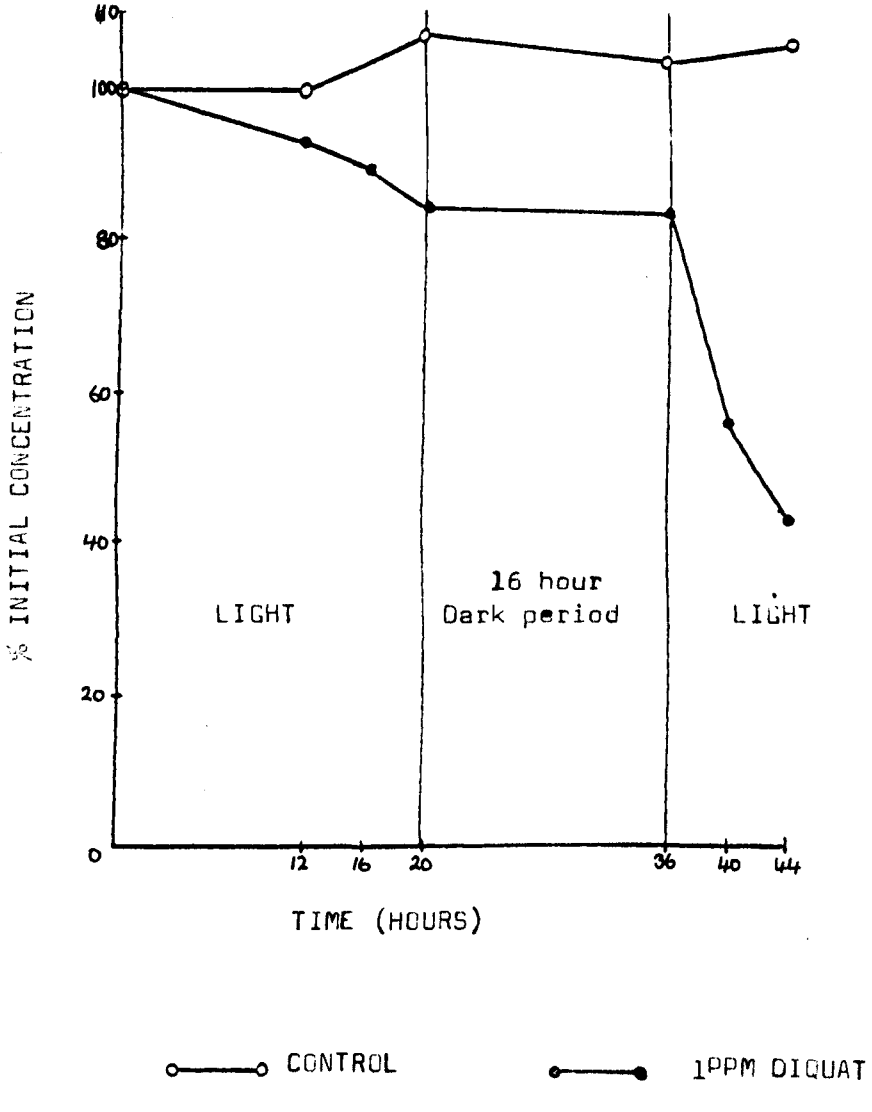
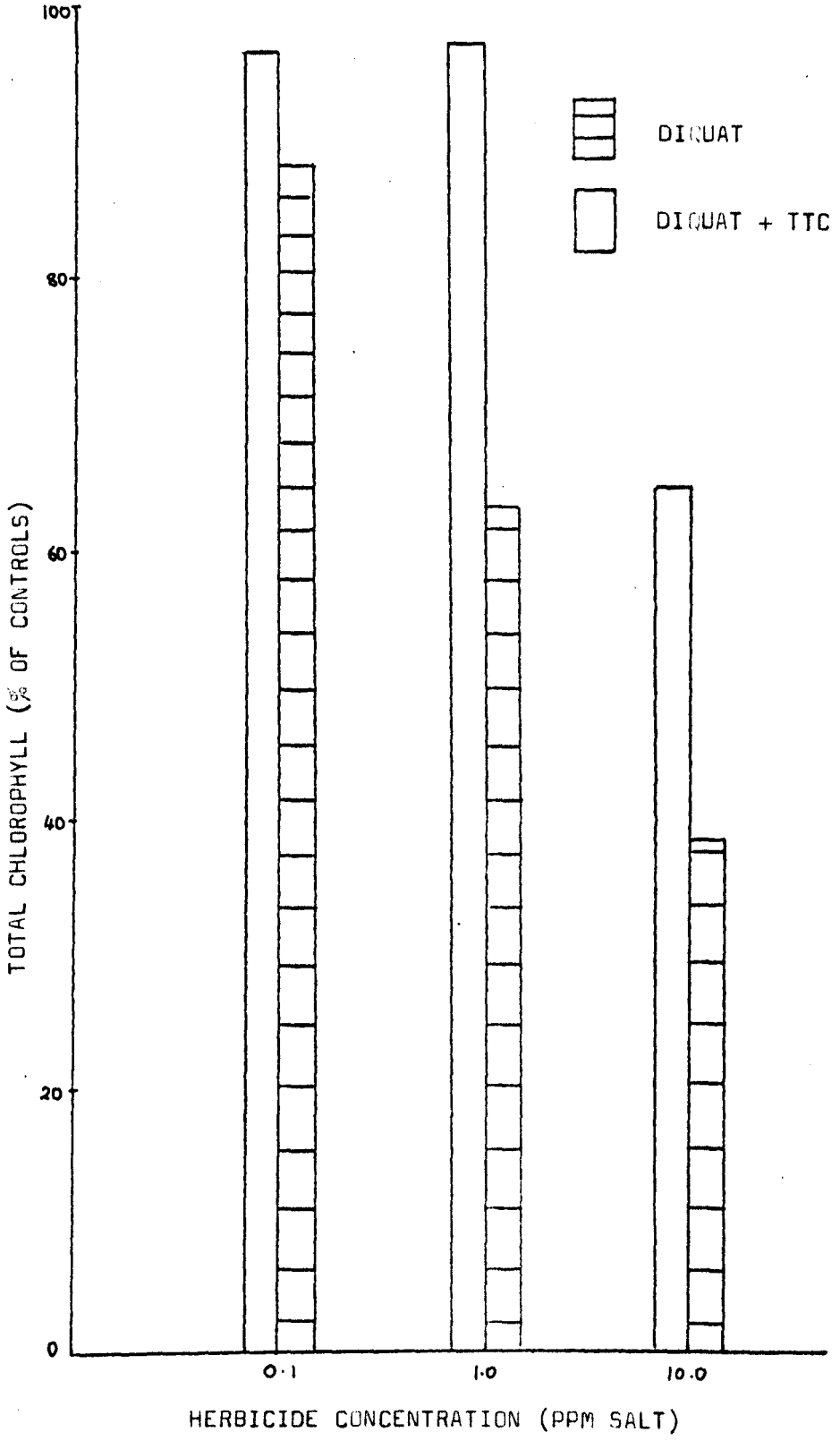


PLATE 30 A comparison of the effects of diquat and diquat + 10 ppm TTC (2:3:5 - triphenyl-tetrazolium chloride) on the chlorophyll content of S. oligorrhiza after 24 hours

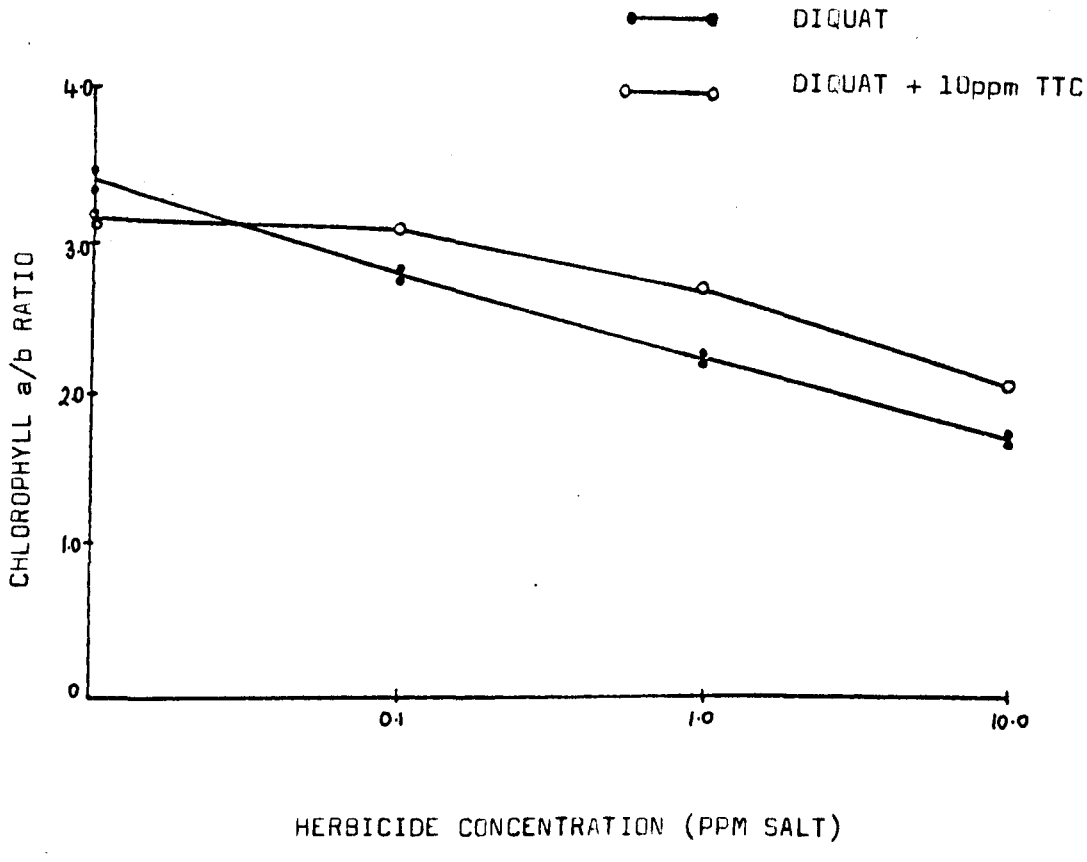
TREATMENT		CHLOROPHYLL a (mg/g. fresh wt.)	CHLOROPHYLL b (mg/g. fresh wt.)	TOTAL CHLOROPHYLL (mg/g. fresh wt.)	CHLOROPHYLL a/b
Control	1	0.6523	0.1880	0.8403	3.47
Control	2	0.6549	0.1952	0.8501	3.36
Control+TTC	1	0.6253	0.1953	0.8205	3.20
Control+TTC	2	0.6367	0.2033	0.8400	3.13
0.1PPM Diquat	1	0.5666	0.2024	0.7690	2.80
0.1PPM Diquat	2	0.5277	0.1935	0.7212	2.73
0.1PPM Diquat+TTC	1	0.6110	0.1992	0.8103	3.07
0.1PPM Diquat+TTC	2	0.5991	0.1950	0.7941	3.07
1.0PPM Diquat	1	0.3673	0.1650	0.5322	2.23
1.0PPM Diquat	2	0.3602	0.1669	0.5271	2.16
1.0PPM Diquat+TTC	1	0.5947	0.2208	0.8155	2.69
1.0PPM Diquat+TTC	2	0.5828	0.2166	0.7994	2.69
10.0PPM Diquat	1	0.2038	0.1211	0.3249	1.68
10.0PPM Diquat	2	0.1951	0.1190	0.3142	1.64
10.0PPM Diquat+TTC	1	0.3782	0.1886	0.5669	2.01
10.0PPM Diquat+TTC	2	0.3348	0.1663	0.5011	2.01

Plate 31 A comparison of the effects of diquat anddiquat + TTC (2:3:5 - Triphenyl-Tetrazolium Chloride) on the chlorophyll content of S. oligorrhiza after 24 hours



Values represent the mean of 2 replicates

Plate 32 A comparison of the effects of diquat and diquat + TTC (2:3:5 - triphenyl-tetrazolium chloride) on the chlorophyll a/b ratio of S. oligorrhiza after 24 hours.



(vi) The effect of culture age on chlorophyll content

In this experiment, fronds of S. oligorrhiza were grown in flask culture and maintained under continuous illumination at 3500 lux. Cultures were inoculated at day 0 with approximately equal numbers of young, actively-growing fronds and duplicate cultures were harvested after periods of 7, 14, 21, 28, 35, 42 and 49 days, followed by chlorophyll extraction.

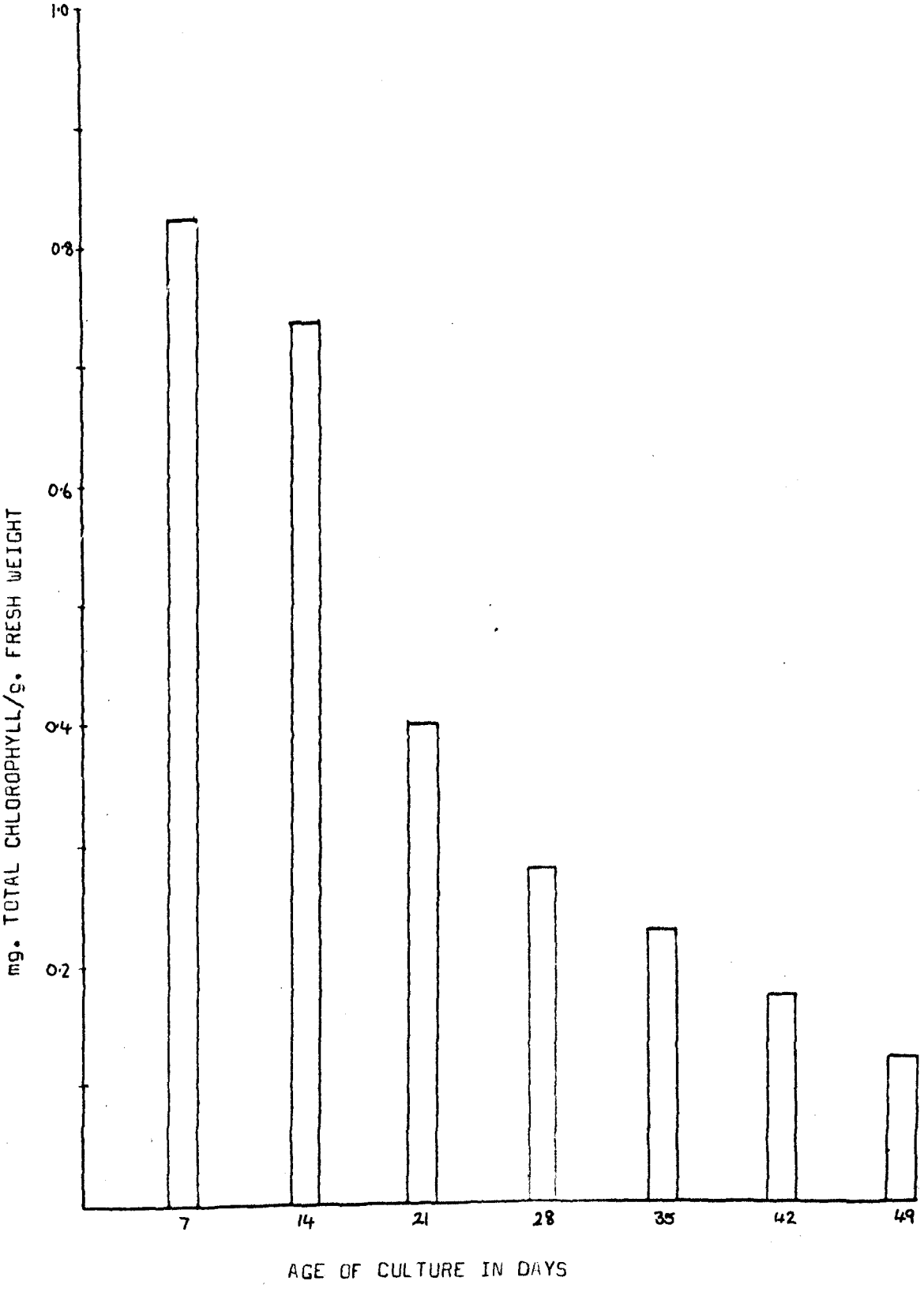
The chlorophyll contents of replicate cultures at different times are shown in Pl. 33. Pl. 34 shows the average values for total chlorophyll throughout the experimental period. The results show a clear reduction in total chlorophyll at all times investigated after 7 days. Loss of chlorophyll occurred most rapidly between 14 and 21 days. The chlorophyll content of the fronds after 21 days was approximately half of that in 7 day old cultures. After 21 days, chlorophyll loss continued but at a gradually diminishing rate.

Between 7 and 28 days the chlorophyll a/chlorophyll b ratio dropped, the most dramatic reduction occurring between 21 and 28 days (Pl. 35). However, after this time the ratio changed little.

PLATE 33 The effect of culture age on the chlorophyll content of *S. oligorrhiza*

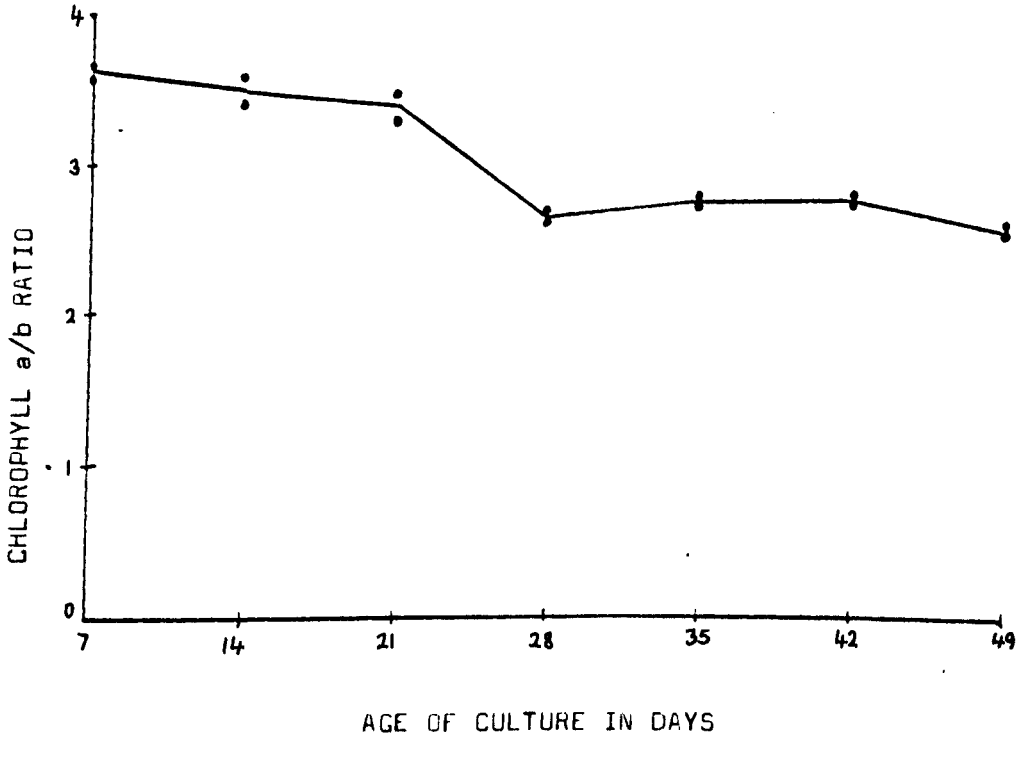
AGE OF CULTURE (Days)	CHLOROPHYLL a (mg/g. fresh wt.)	CHLOROPHYLL b (Mg/g. fresh wt.)	TOTAL CHLOROPHYLL (mg/g. fresh wt.)	CHLOROPHYLL a/b
7	0.6555	0.1840	0.8397	3.56
	0.6400	0.1759	0.8158	3.64
14	0.5779	0.1702	0.7481	3.40
	0.5715	0.1587	0.7302	3.60
21	0.3041	0.0922	0.3962	3.30
	0.3142	0.0905	0.4047	3.47
28	0.1950	0.0741	0.2691	2.63
	0.2114	0.0791	0.2906	2.67
35	0.1615	0.0595	0.2210	2.71
	0.1727	0.0621	0.2348	2.78
42	0.1268	0.0454	0.1722	2.79
	0.1287	0.0474	0.1762	2.72
49	0.0884	0.0349	0.1233	2.53
	0.0895	0.0344	0.1239	2.60

Plate 34 The effect of culture age on the chlorophyll content of *S. oligorrhiza*



Values represent the mean of 2 replicates

Plate 35 The effect of culture age on the chlorophyll a/b ratio in fronds of S. oligorrhiza



C. MANOMETRIC STUDIES

Following manometric investigations, analysis of variance was carried out on the data collected according to Snedecor (1967). The differences between the means were tested by Duncan's multiple range analysis ('D' test) (Duncan, 1955).

(i) The effect of diquat on oxygen uptake in the dark

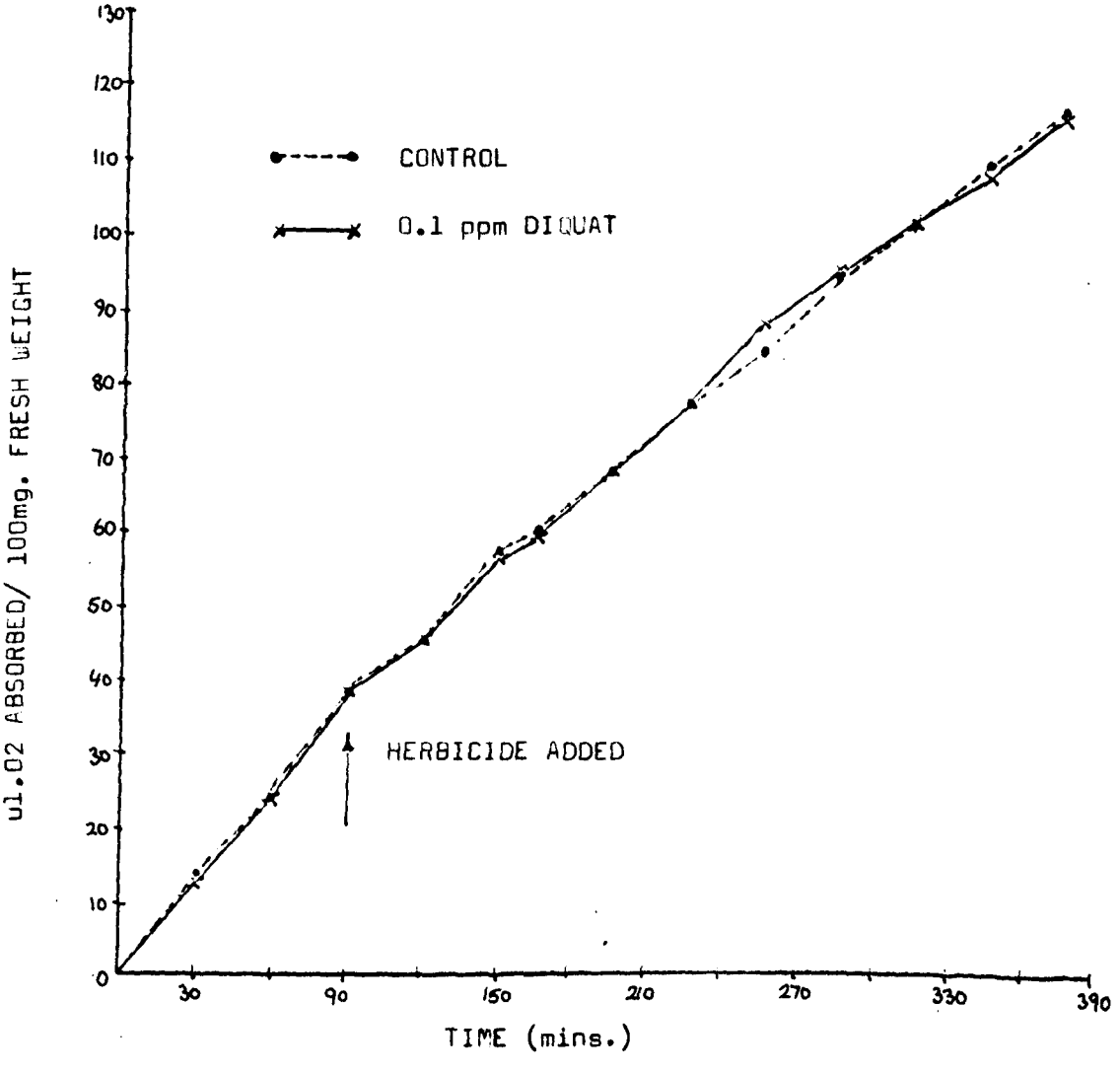
Treatment with diquat at a concentration of 0.1 ppm (salt) had no measurable effect on oxygen uptake by fronds of S. oligorrhiza in darkness during the treatment period (Pl. 36). However, all higher concentrations of diquat tested (1.0, 10.0 and 100.0 ppm salt) resulted in significant increases in oxygen uptake which were maintained throughout the duration of the experiments (Pl. 37, 38, and 39). Significant stimulations of oxygen uptake were evident at these concentrations 135 min. after the addition of herbicide. 1.0 ppm diquat (salt) resulted in a significant increase in oxygen uptake after only 105 min.

In all experiments the slopes of the graphs for cumulative oxygen uptake by test samples and controls remained approximately constant following the time at which the herbicide-induced stimulation of oxygen uptake became apparent. Calculation of the average rates of oxygen uptake per hour during the 5.5 hours gollowing the addition of diquat indicates that the stimulation of oxygen uptake increased with increasing herbicide concentration (Pl. 40).

(ii) Comparison of the effects of paraquat and diquat on oxygen uptake in the dark

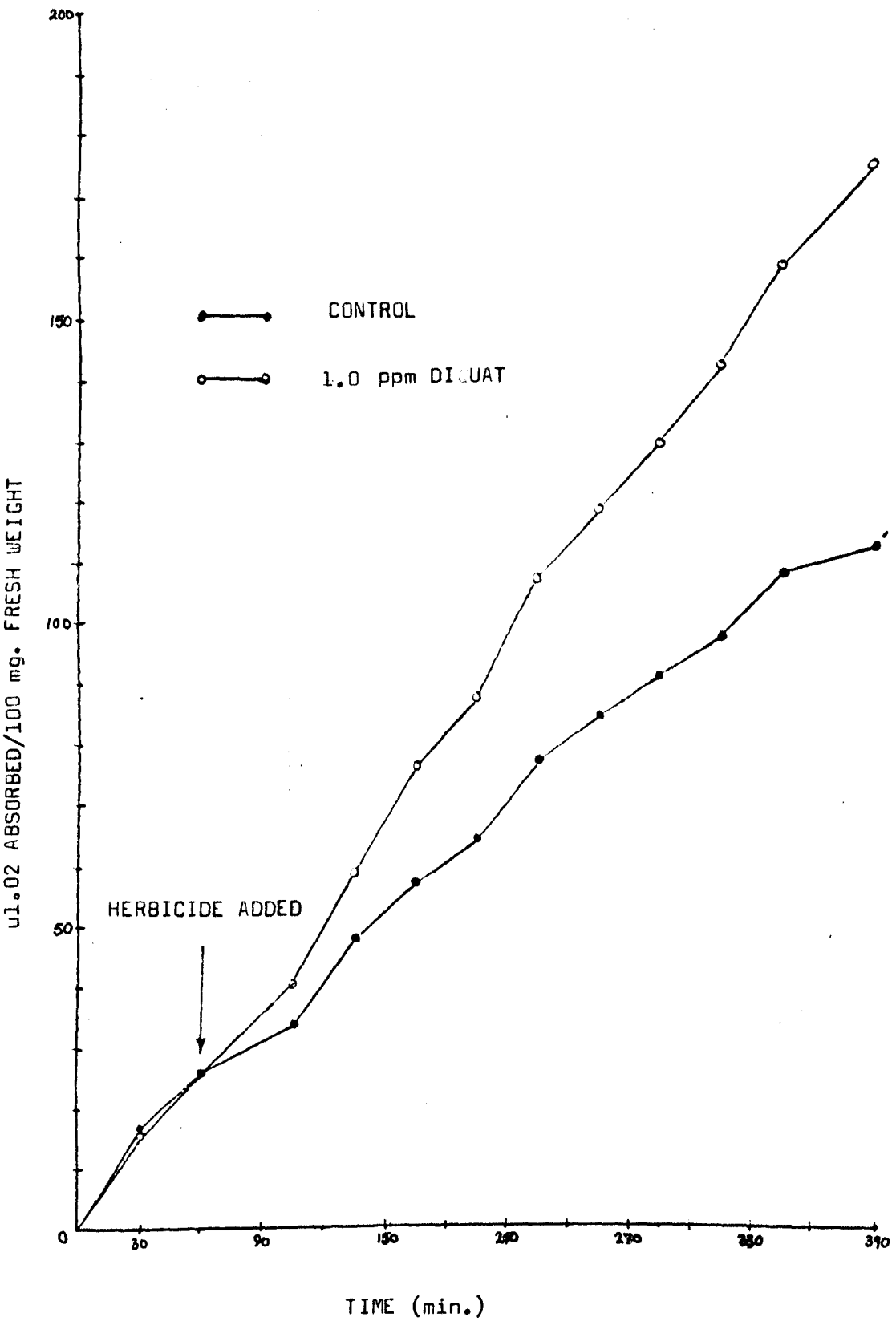
Both paraquat and diquat at concentrations of 10 ppm (cation) significantly increased the rate of oxygen uptake in darkness by S.

Plate 36 The effect of 0.1 diquat (salt) on oxygen uptake in the dark by fronds of S. oligorrhiza



All values represent the mean of 4 replicates

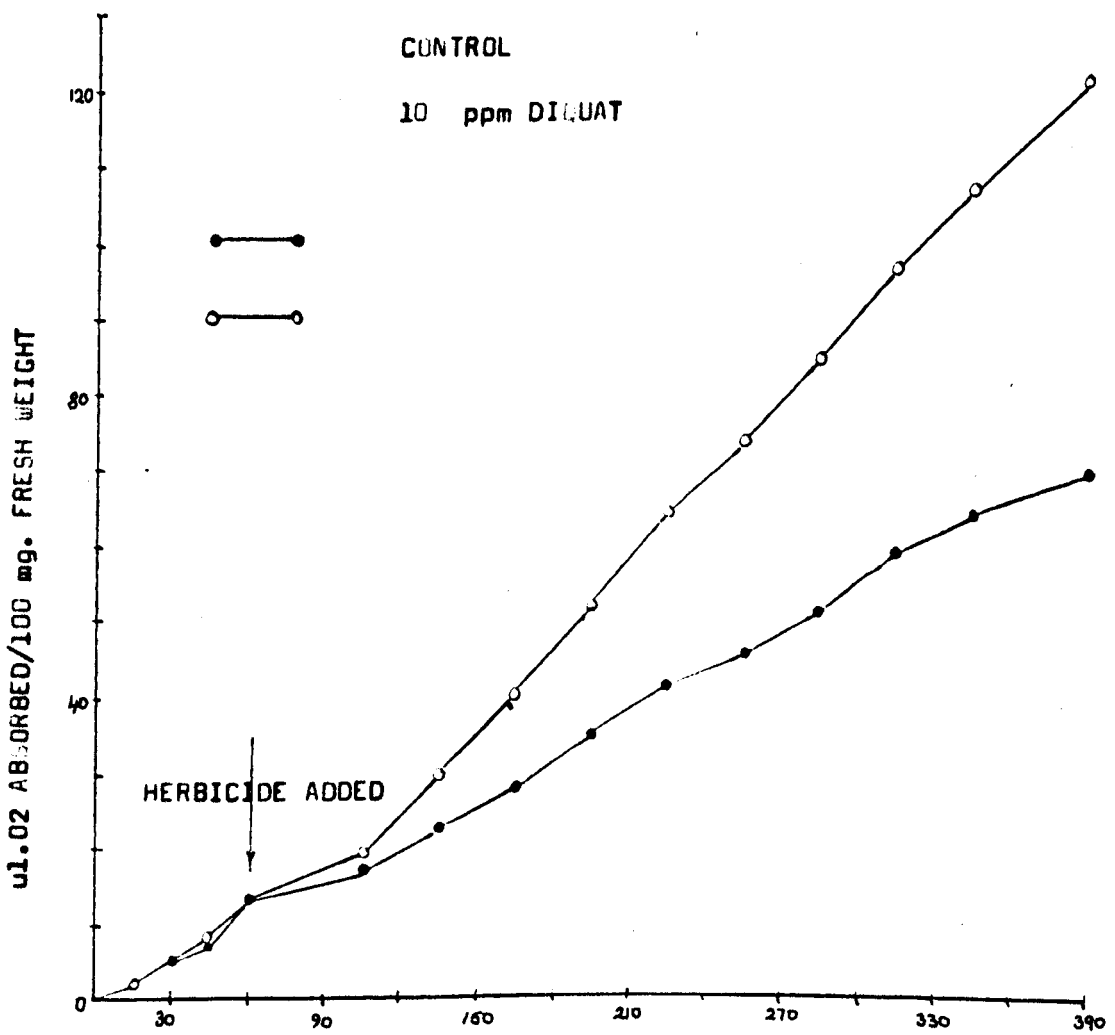
Plate 37 The effect of 1.0 ppm diquat (salt) on oxygen uptake in the dark by fronds of S. oligorrhiza



All values represent the mean of 4 replicates

P = 0.001 LSD = 17.6

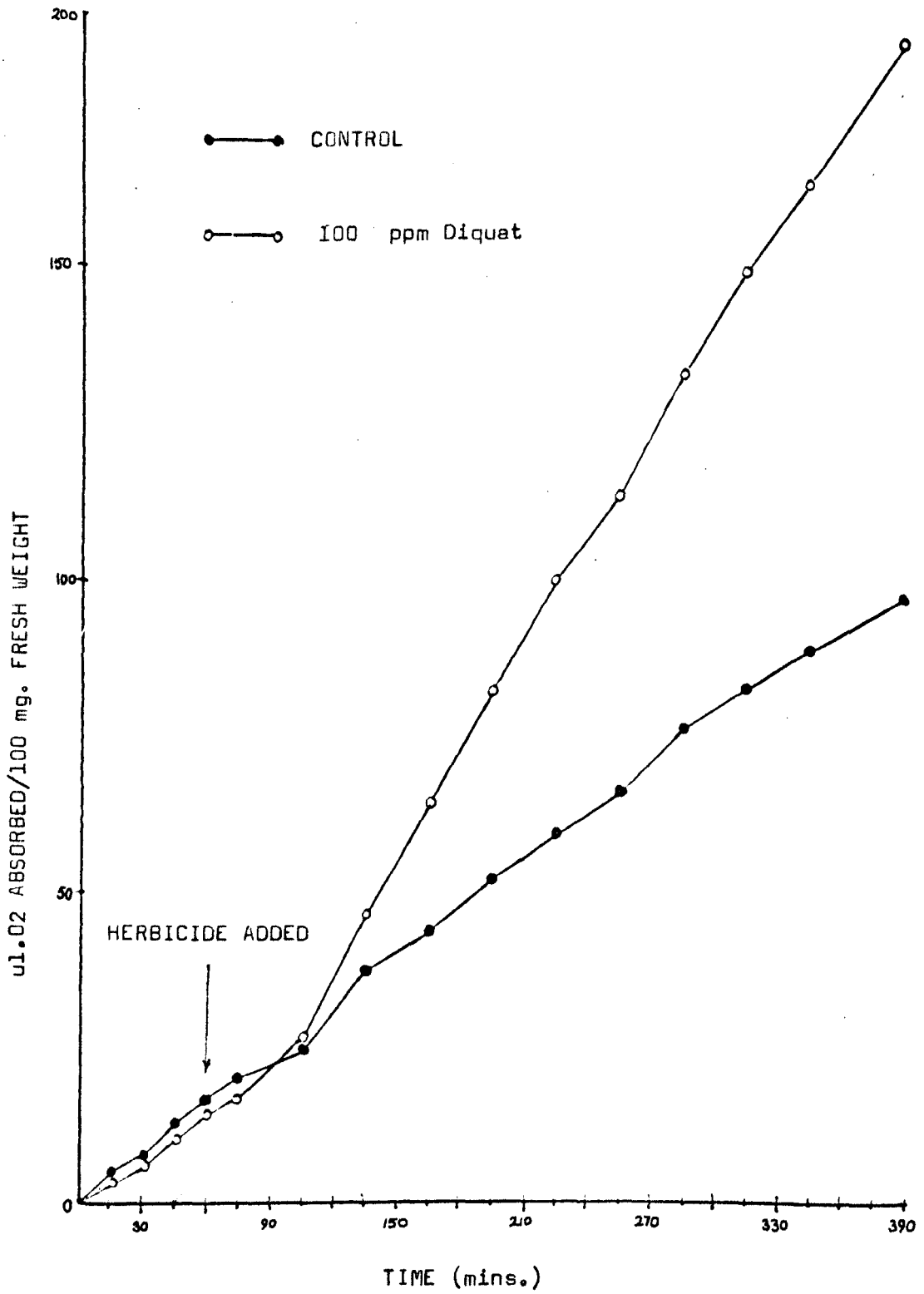
Plate 38 The effect of 10 ppm diquat (salt) on oxygen uptake in the dark by fronds of S. oligorrhiza



All values represent the mean of 4 replicates

P = 0.001 LSD = 13.0

Plate 39 The effect of 100 ppm Diquat (salt) on oxygen uptake in the dark by fronds of S. aligorrhiza



All values represent the mean of 5 replicates

P = 0.001 LSD = 21.6

PLATE 40 The average rates of osygen uptake in the dark expressed as a percentage of the control rate, by fronds of S. oligorrhiza in the presence of a range of concentrations of diquat.

CONCENTRATION (PPM)	%
1.0	172
10.0	191
100.0	215

oligorrhiza during a 5 hour period (Pl. 41 and 42). At all times the uptake of oxygen was more rapid in diquat-treated fronds. Paraquat and diquat increased the rate of oxygen uptake by 54 and 95% respectively after 1 hour. This stimulation was subsequently increased and, despite minor fluctuations in the rates of oxygen uptake by treated and control samples, the stimulatory effects of both herbicides were maintained throughout the 5 hour treatment period.

(iii) The effect of diquat, with and without a dark pre-treatment, on oxygen uptake in the dark

In this experiment, two batches of *S. oligorrhiza* were used. The plants in one batch were grown under continuous illumination (3500 lux) while the plants comprising the second batch had been kept in complete darkness for the previous 24 hours.

Oxygen uptake occurred in dark pre-treated control samples at a significantly slower rate than in control samples previously exposed to continuous illumination (Pl. 43). The average rate of oxygen uptake by dark pre-treated controls was only 9.3 ul./100 mg. fresh weight/hour compared with 18.1 ul./100 mg. fresh weight/hour by controls which did not receive the dark pre-treatment. Oxygen uptake by dark pre-treated fronds in the presence of 10 ppm diquat (salt) (22.9 ul./100 mg. fresh weight/hour) occurred at significantly greater rates than in either light or dark pre-treated controls but at a significantly slower rate than in diquat-treated light-grown fronds (39.2 ul./100 mg. fresh weight/hour). The reduction in oxygen uptake by diquat-treated fronds due to the dark pre-treatment (16 ul./100 mg. fresh weight/hour) was greater than the corresponding reduction in control oxygen uptake (8.8 ul./100 mg. fresh weight/hour).

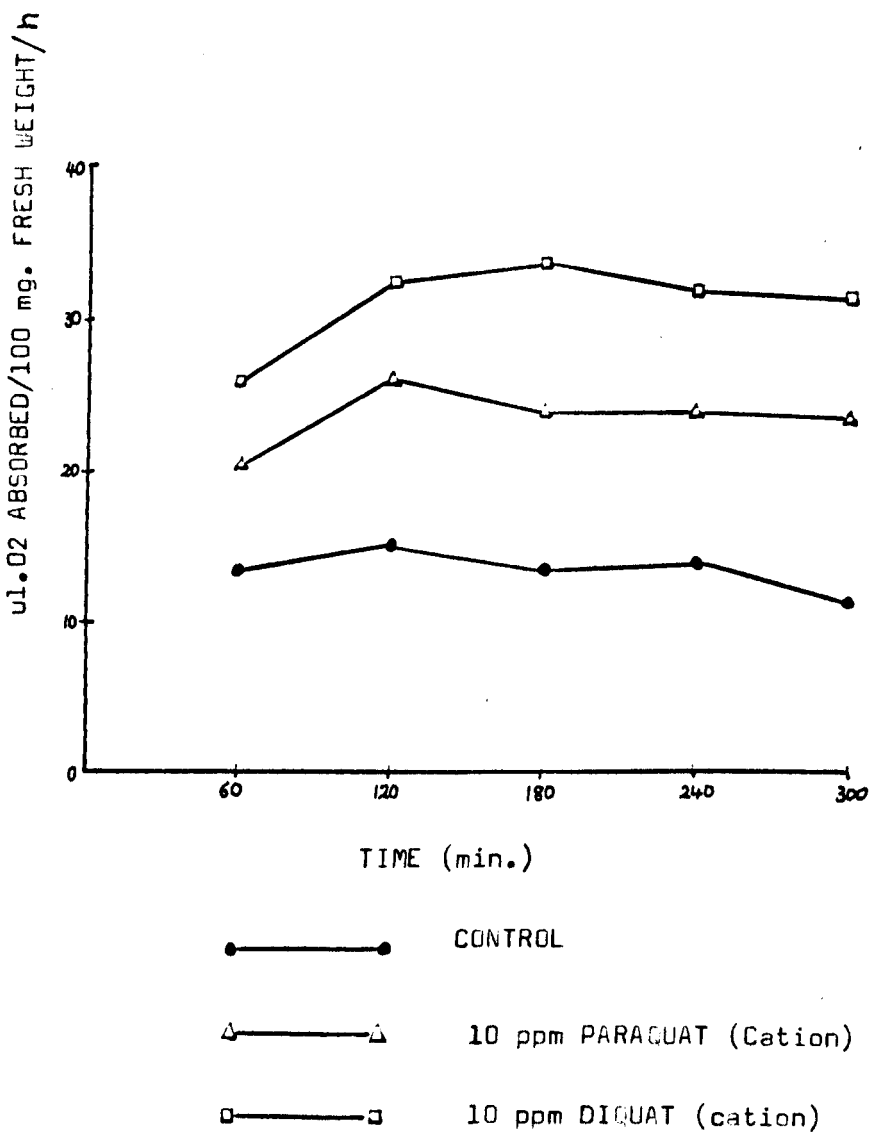
PLATE 41 A comparison of paraquat and diquat (10PPm cation) on the rate of oxygen uptake by fronds of S. oligorrhiza in the dark

TIME (min)	O_2 Uptake (ul/100 mg. fresh wt./hr.)				
SAMPLE	60	120	180	240	300
CONTROL	13.15	14.98	13.28	13.87	11.23
PARAQUAT	20.22	25.77	23.53	23.83	23.20
DIQUAT	25.67	32.32	33.50	31.65	31.37

All values represent the means of 6 replicates.

'D' tests ($P = 0.001$). All vertically-tabulated means are significantly different.

Plate 42 Comparison of the effects of paraquat and diquat on the rate of oxygen uptake in the dark by fronds of S. oligorrhiza



All values represent the mean of 6 replicates

P = 0.001 D = 3.47

PLATE 43 The effect of 10.0 ppm. diquat (salt) on oxygen uptake in the dark by fronds of S. oligorrhiza with and without a dark pre-treatment

TIME (Min.)	CUMULATIVE O_2 UPTAKE (ul./100 mg.^{2} fresh wt.)									
SAMPLE	30	60	90	120	150	180	210	240	270	300
CD	4.6	9.7	14.5	19.2	24.3	28.9	33.5	-	42.3	46.4
DD	9.8[a]	23.5[a]	33.2	44.7	57.4	69.0	80.3	-	101.5	114.3
CL	10.1[a]	22.5[a]	31.7	41.6	40.5	59.7	68.0	-	82.4	90.6
DL	19.2	40.7	67.6	80.5	101.2	120.5	140.0	-	175.9	195.9

CD = controls given a 24 h. dark pre-treatment

DD = diquat-treated after a 24h. dark pre-treatment

CL = controls grown in continuous light

DL = diquat-treated after growth in continuous light.

All values represent the mean of 6 replicates

'D' tests ($P = 0.001$). Common letter postscripts denote no significant differences between vertically-tabulated means.

(iv) The effect of diquat with and without added glucose, on oxygen uptake in the dark

Oxygen uptake by control fronds of S. oligorrhiza and fronds treated with 10.0 ppm diquat was significantly stimulated by the addition of 2% glucose to the culture medium (Pl. 44). During the 5 hour experimental period the average values for oxygen consumption by control fronds were 22.4 and 12.1 ul./100 mg. fresh weight/hour in the presence and absence of added glucose respectively. In the presence of glucose, the average rate of oxygen uptake by diquat-treated fronds was 44.6 ul/100 mg. fresh weight/hour compared with only 29.4 ul./100 mg. fresh weight/hour without added glucose. The increase in oxygen uptake by diquat-treated fronds due to the addition of glucose (15.2 ul./100 mg. fresh weight/hour) was greater than the corresponding increase in control oxygen uptake (10.3 ul./100 mg. fresh weight/hour).

(v) The effects of diquat on oxygen uptake and carbon dioxide output in the dark

Treatment of fronds of S. oligorrhiza with 10.0 ppm diquat (salt) in darkness resulted in a pronounced stimulation of both oxygen uptake and carbon dioxide evolution during the 5 hour experimental period (Pl. 45). By the end of this time both oxygen uptake and carbon dioxide output had been stimulated to the same extent, the R.Q. value for diquat-treated fronds (0.88) being unchanged from the control value (Pl. 46).

(vi) The effect of diquat on oxygen exchange in the light

The effects of diquat concentrations of 1.0 - 1,000 ppm (salt) on oxygen exchange in S. oligorrhiza in the light were compared in two separate

PLATE 44 The effect of 10.0 ppm diquat (salt) on oxygen uptake in the dark by S. oligorrhiza with and without added glucose

TIME (min.)	CUMULATIVE O_2 UPTAKE (ul./100 mg.^{2} fresh wt.)									
SAMPLE	30	60	90	120	150	180	210	240	270	300
C+GI	11.9[a]	22.2	32.9	43.7	54.1	66.7	78.3	88.9	-	112.0
D+GI	16.5	37.4	60.7	84.9	106.4	127.6	152.6	175.7	-	222.9
C	7.0	13.2	20.6	26.7	31.6	38.7	45.2	50.0	-	60.4
D	12.5[a]	29.5	41.6	61.8	76.2	87.9	105.0	118.6	-	146.8

C+GI = controls with 2% glucose

D+GI = diquat + 2% glucose

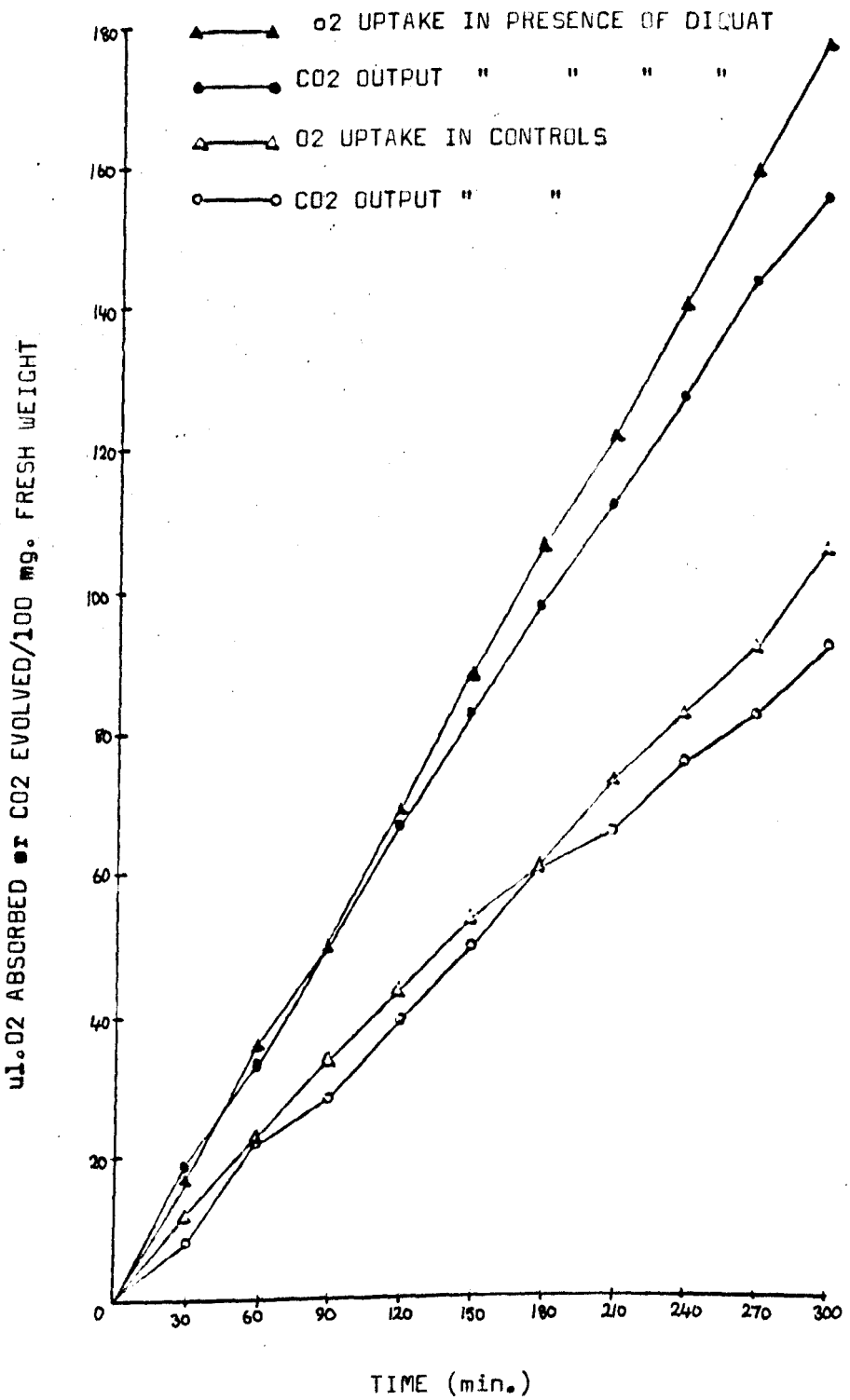
C = controls without added glucose

D = diquat without added glucose

All values represent the mean of 6 replicates.

'D' test ($P = 0.001$). Common letter postscripts denote no significant differences between vertically-tabulated means.

Plate 45 The effect of 10.0 ppm diquat (salt) on oxygen uptake and carbon dioxide output in the dark by fronds of S. oligorrhiza



All values represent the means of 3 replicates

PLATE 46 The effect of 10.0 ppm diquat (salt) on oxygen uptake and carbon dioxide output in the dark by S. oligorrhiza

TREATMENT	O_2 UPTAKE	CO_2 OUTPUT	R.Q.
	(ul./100 mg. fresh wt.)		
CONTROL	105.64	92.74	0.88
10PPm DIQUAT	177.19	155.50	0.88

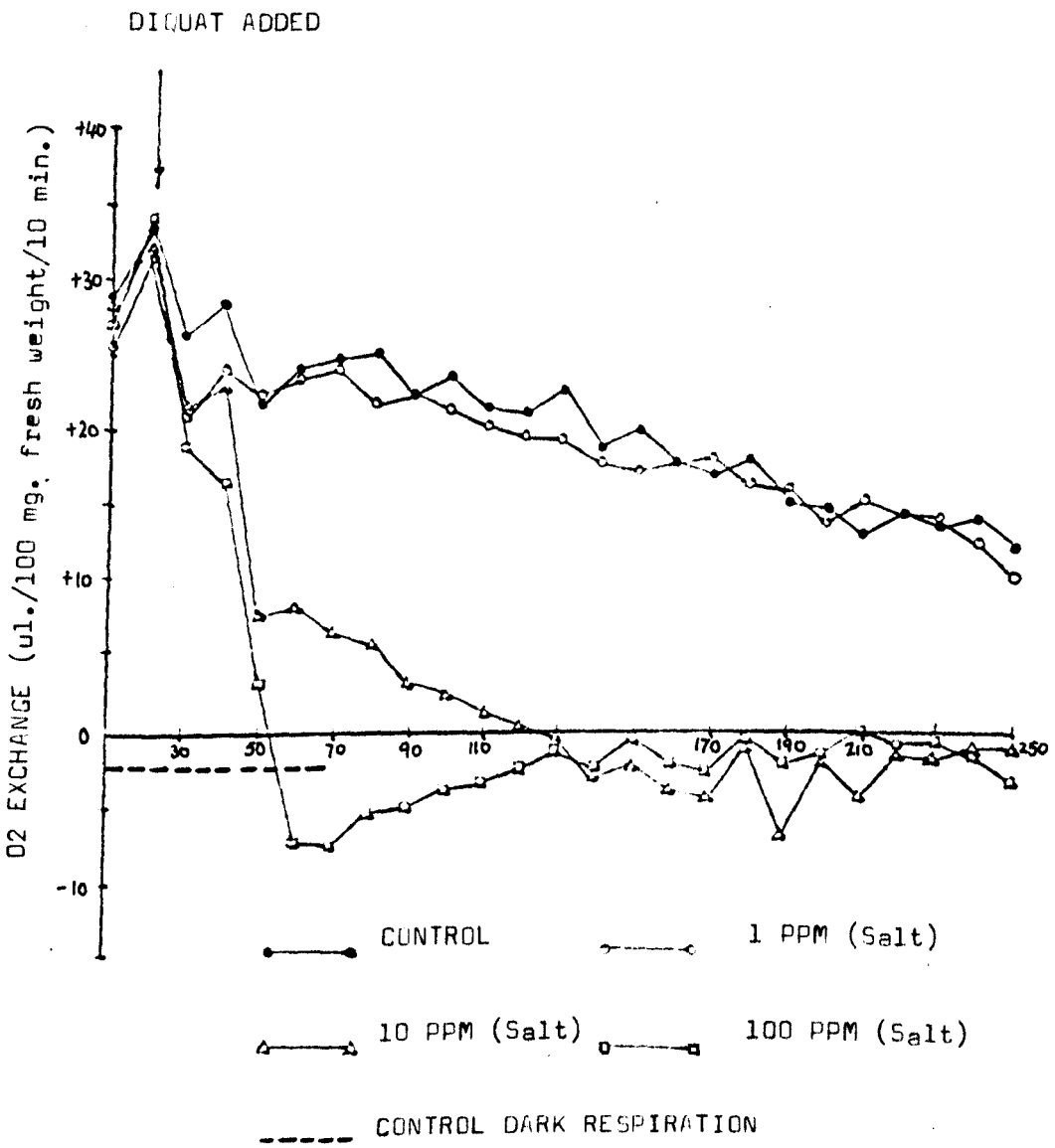
All values represent the mean of 3 replicates.

trials. Diquat at all concentrations tested above 1.0 ppm. (salt) had a pronounced effect on oxygen exchange. The results are illustrated graphically in Pl. 47 and 48. The rate of oxygen exchange in fronds exposed to 1.0 ppm. diquat did not differ significantly from control values over a period of 230 min. following the addition of herbicide (Pl. 47). However, the addition of higher concentrations of diquat resulted in a dramatic fall in oxygen output (apparent photosynthesis). The rate of apparent photosynthesis fell rapidly to zero, complete inhibition being reached more quickly the higher the diquat concentration. Complete inhibition of oxygen output was immediately followed in treated fronds by a prolonged period of oxygen uptake. The rate of oxygen uptake by treated fronds reached a peak soon after the complete inhibition of apparent photosynthesis. The size of the peak was a positive function of diquat concentration, but the rate at the peak of oxygen consumption was always greater than the rate of control dark respiration. After this time the rate of oxygen uptake was reduced and in some trials there was even a small transient net output of oxygen in treated fronds (Pl. 48). There then followed a second peak of oxygen uptake the magnitude of which was variable but generally slightly less than the initial one. After this peak, the rate of oxygen uptake was once again reduced to a low level which, apart from slight fluctuations generally remained a little below the level of control dark respiration.

(vii) Comparison of the effects of paraquat and diquat on oxygen exchange in the light

Oxygen exchange in fronds of S. oligorrhiza treated with paraquat or diquat at a concentration of 10.0 ppm. (cation) showed the same pattern

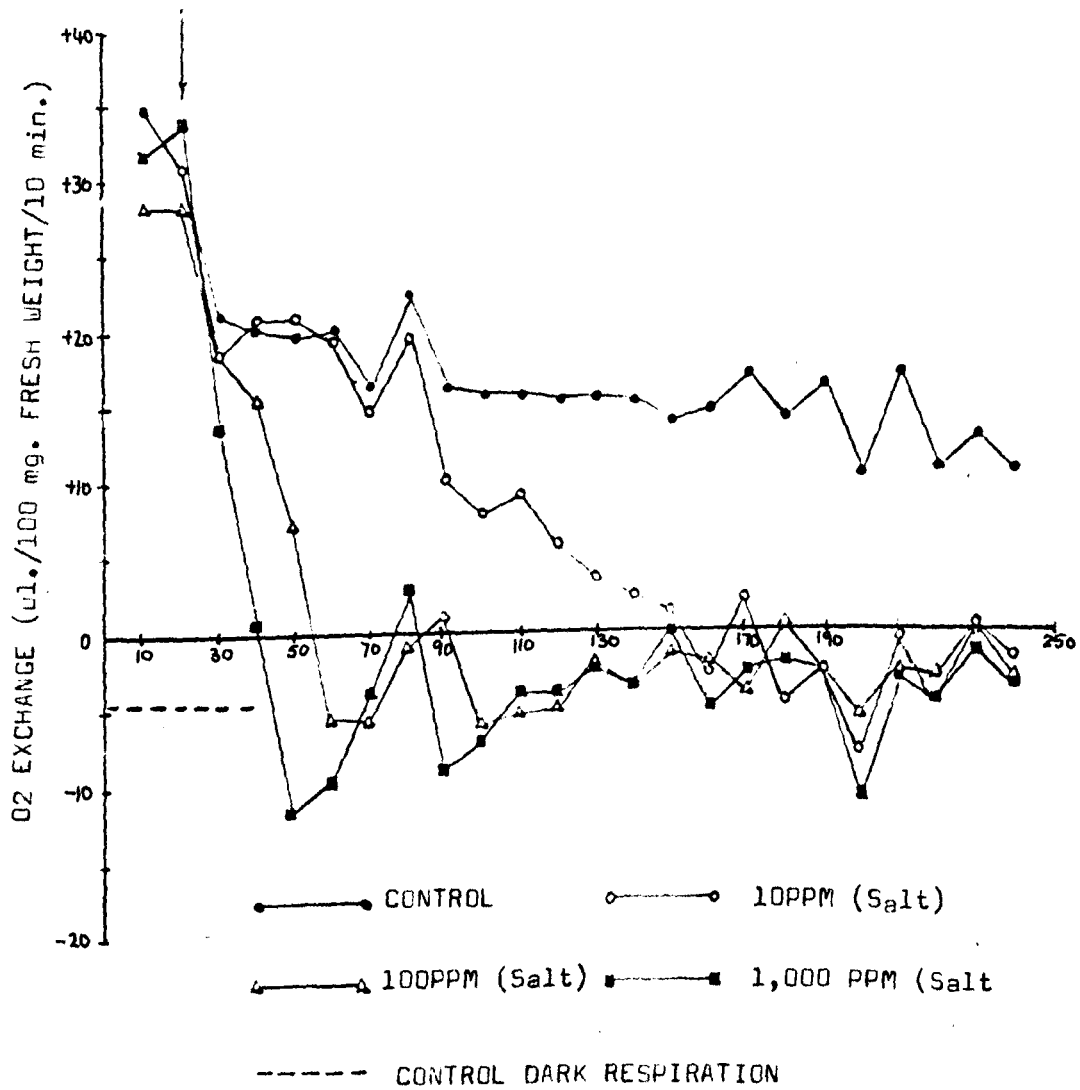
Plate 47 The effect of a range of concentrations of diquat on oxygen exchange in S. oligorrhiza in the light



P = 0.001 D = 1.3

All values represent the mean of 2 replicates

Plate 48 The effect of a range of concentrations of diquat on oxygen exchange in S. oligorrhiza in the light

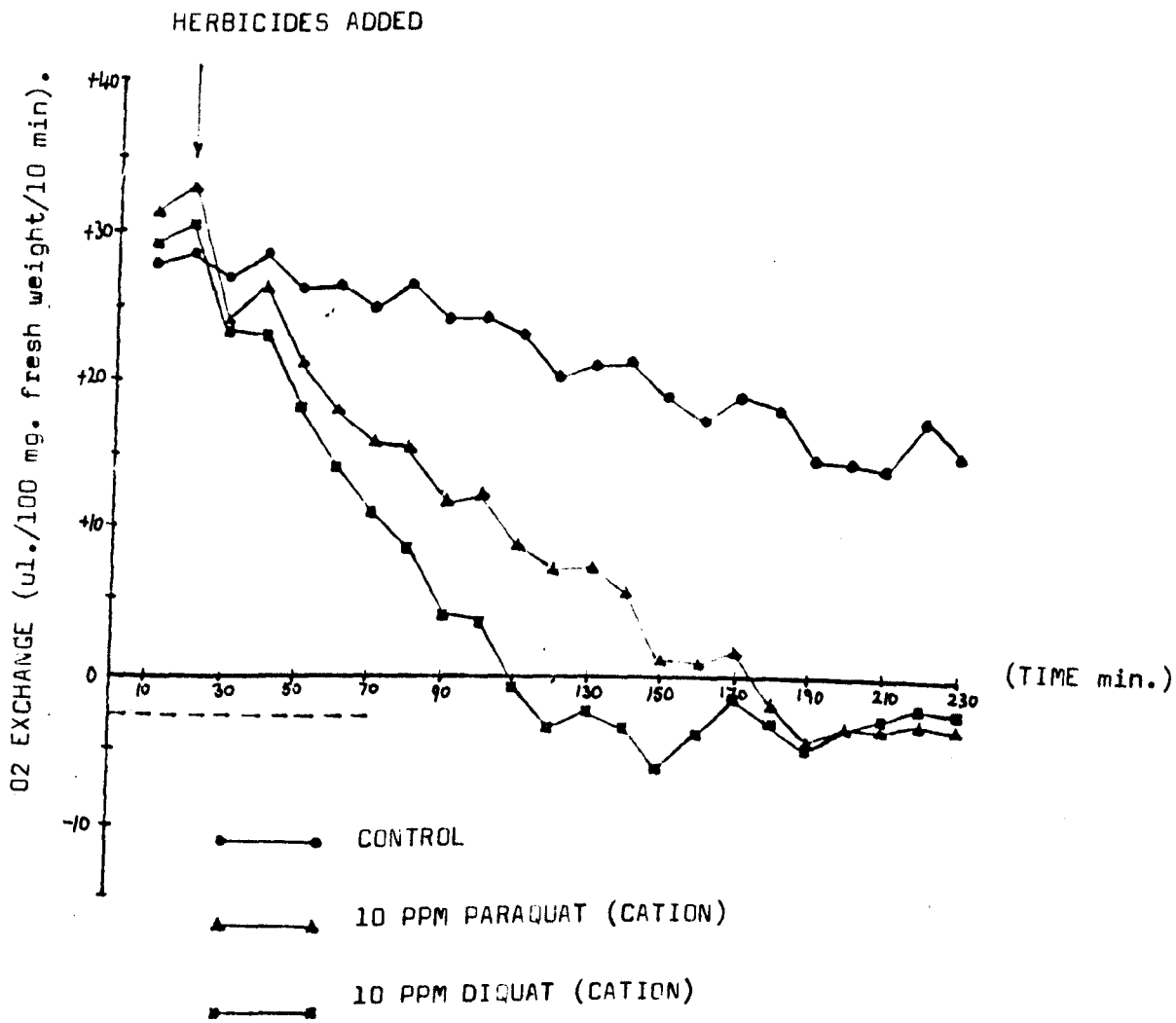


P = 0.001 D = 3.17

All values present the mean of 2 replicates

as described in section (vi) above. However, the rate of oxygen output in diquat-treated fronds decreased at a significantly more rapid rate than in fronds treated with paraquat (Pl. 49). The time required for the complete inhibition of apparent photosynthesis in treated fronds varied slightly on different occasions. However, oxygen output generally reached zero in diquat-treated fronds approximately 90 min. after the addition of the herbicide while paraquat-treated fronds required at least a further 60 min. before reaching the same stage. The peaks of oxygen uptake were similar in paraquat and diquat-treated fronds although the peak in diquat-treated fronds occurred even before the complete cessation of apparent photosynthesis in fronds treated with paraquat (Pl. 49)

Plate 49 Comparison of the effects of paraquat and diquat on oxygen exchange in S. oligorrhiza in the light



P = 0.001 D = 2.63

All values represent the mean of 3 replicates

V. DISCUSSION

ULTRASTRUCTURAL STUDIES

Exposure of fronds of S. oligorrhiza to diquat in the light resulted in visible alterations in cell untrastructure which preceded changes in their outward appearance. After 4 h. in the light (3500 lux) in the presence of 10 ppm diquat (cation) there was a reduction in the amount of starch within the chloroplasts but no evidence of ultrastructural damage. Subsequently, starch grains were seldom observed within these organelles. This effect on the starch content of chloroplasts agrees with the observations of other workers. Dodge & Lawes (1974) reported the complete disappearance of starch grains from the chloroplasts of detached flax cotyledons after 3 h. diquat treatment in the light (2500 lux). In these experiments the concentration of diquat used was 10^{-4}M (or 18.4 ppm cation). In an earlier ultrastructural study, Stokes et al. (1970) showed that in the green alga Chlorella vulgaris, although starch had increased in control cell chloroplasts, there was only a small quantity of starch remaining in those which had been exposed to 10^{-3}M diquat in the light (7500 lux). As in the present study, the effects on the starch content of these chloroplasts occurred in the absence of any visible ultrastructural damage.

After 6 h. diquat treatment, the chloroplasts, the mitochondria and the microbodies of S. oligorrhiza all exhibited varying signs of ultrastructural damage (Figs. 60 and 62). The ultrastructural changes in diquat treated fronds appeared to develop simultaneously in the chloroplasts and in the mitochondria. However, observations made after treatment for 1 hour, in the light with a massive dose of diquat (500 ppm salt) commonly revealed mitochondria which were apparently undamaged while almost all of the chloroplasts were visibly altered (Figs. 105 and 106). This would suggest

that the chloroplast could be the primary site of ultrastructural damage in diquat-treated fronds of S. oligorrhiza in the light.

The chloroplasts of fronds treated with low doses of diquat initially became swollen, causing a change from their normal flattened lens shape. Many chloroplasts began to assume a more spherical shape while others became irregular in outline. The alterations in the shape of the chloroplasts were generally accompanied by changes in the arrangement of the thylakoids. The thylakoid system in many cases being displaced towards the side of the chloroplast closest to the vacuole (Figs. 49, 53, 60 and 64). At the same time, the matrix of the mitochondria became less electron dense. In some cases, the shape of these organelles was somewhat irregular although they did appear to remain intact (Figs. 46 and 50).

These observations of the earliest signs of ultrastructural damage in S. oligorrhiza are in general agreement with the work of Dodge & Lawes (1974) on flax. These workers reported that the first sign of ultrastructural damage in the cotyledons treated in the light with diquat to be the swelling of the chloroplasts and the arching of the thylakoid system against the inner side of the chloroplast envelope. This was noted 6 hours after treatment had begun but, in this study, no other cell organelle appeared to have been affected and the mitochondria looked quite normal. Stokes et al. (1970) first observed ultrastructural damage in Chlorella after illumination for 5 hours in the presence of diquat. In this case the arrangement of the chloroplast lamellae appeared somewhat irregular and showed signs of buckling. Damage to the pyrenoid was also visible at this stage. However, the damage was not restricted to the chloroplast. Mitochondria and nuclei could no longer be seen in the cytoplasm from this time onwards. Thus the observations on Chlorella cannot be considered as providing evidence to support the view that

the chloroplasts are the sites of primary ultrastructural damage in diquat-treated tissue in the light.

Direct comparison of the development of the ultrastructural damage in S. oligorrhiza, flax and Chlorella is made somewhat difficult by the variations in herbicide concentration and light intensity used by the different workers. The present work with S. oligorrhiza involved a diquat concentration of 10 ppm (cation) while Dodge & Lawes (1974) treated flax and Stokes et al. (1970) treated Chlorella with diquat concentrations equivalent to 18.4 and 184 ppm (cation) respectively. The light intensity throughout the treatment of S. oligorrhiza was 3500 lux while Dodge and Lawes (1974) and Stokes et al. (1970) used light intensities of approximately 2500 lux. and 7500 lux respectively. However, it would appear that different organelles in different plant species may differ in their relative sensitivity to diquat-treatment in the light. In flax, the chloroplasts became swollen and the lamellar system was arched to one side while the mitochondria and other organelles appeared to remain undamaged even after 18 hours. (Dodge & Lawes, 1974). In S. oligorrhiza, the chloroplasts were similarly altered but the mitochondria also showed signs of damage. In Chlorella by the time early damage to the chloroplast was visible, the mitochondria and the nuclei were absent, presumably having been totally destroyed (Stokes et al., 1970)

The results of exposure of fronds of S. oligorrhiza to a massive dose of diquat (500 ppm salt or 1.45×10^{-3}M cation) in the light suggest that the concentration of herbicide employed has an effect on the pattern of early ultrastructural damage sustained by cells. As already mentioned, at this high concentration of diquat, the chloroplasts again showed the first signs of ultrastructural damage. Swelling of these organelles was observed

after 1 hour and this was generally accompanied by intrathylakoidal swelling and changes in the stroma. However, except in a few rare instances, the arching of the thylakoid system towards the inner side of the chloroplast was not noticeable. It is perhaps significant that, where the general displacement of the thylakoids was observed, it occurred in those chloroplasts revealing least sign of damage (Fig. 99) Furthermore, at this concentration, these changes in chloroplast structure appeared to have taken place without damage to the majority of the mitochondria and other organelles present or to any other cellular components.

Thepattern of early cellular disruption in S. oligorrhiza exposed to 10 ppm paraquat (cation) in the light (3500 lux) is less clear. Ultrastructural damage in paraquat-treated tissue was first observed after 12 hours and preceded any outwardly visible symptoms. At this time, the chloroplasts, mitochondria and microbodies appeared to be damaged, the types of damage sustained being similar to that resulting from treatment with diquat. However, in addition to these changes, variable amounts of damage to both the plasma membrane and the tonoplast were evident at this stage (Figs. 69 - 72). This is contrary to the finding with diquat-treated fronds in which both of these limiting membranes appeared to remain intact during the early stages of cellular damage. Similarly, Dodge & Lawes (1974) did not observe disruption of the plasma membrane or tonoplast of flax cotyledon cells until more than 12 hours after changes in the ultrastructure of chloroplasts had first become apparent. Stokes et al. (1970) make no mention of these membranes in their algal work and scrutiny of their published micrographs does not help to determine the fate of these structures in diquat-treated cells of C. vulgaris.

At present it is unclear which of these membranes sustained damage first

and whether damage to these structures followed or indeed preceded ultrastructural changes in other parts of the cell. Harris & Dodge (1972 a) reported that cellular disruption in the mesophyll cells of detached flax cotyledons treated in the light (2250 lux) with 10^{-4}M paraquat began with blistering and subsequent rupture of the tonoplast. This was first evident after approximately 6 hours and, at the same time, the plasma membrane was seen to have moved away from the cell wall although it did not appear to have lost its structural integrity. The mitochondria were stated to be intact at this time. The condition of the chloroplasts during these early changes was not specifically mentioned by these workers. However, one of their micrographs illustrating rupture of the tonoplast after 6 hours appears to show localized swelling of an adjacent chloroplast. The pattern of cellular disruption following rupture of the tonoplast was not described in detail. However, the plasma membrane was disrupted some time after its detachment from the cell wall and it was reported that the mitochondria appeared to have swollen or ruptured by 8 hours. By this time, the chloroplasts had sustained considerable damage and their condition steadily worsened, being almost totally disrupted by 30 hours.

In an earlier study, Baur *et al.* (1969) reported that the first ultrastructural change in mesquite mesophyll cells exposed to paraquat (10^{-2}M) in the light was the disintegration of the plasma membrane. This occurred after treatment for only 5 min. No mention was made of the tonoplast. Disruption of the chloroplast membranes was noted after 40 min. after which time the chloroplasts lost their turgidity. It was claimed that, while these changes were taking place, all other aspects of cell structure remained unaffected. Indeed, at the end of the sampling period (300 min.) only the plasma membrane and the chloroplasts had sustained ultrastructural

damage.

There is, therefore, no general agreement about the nature of the primary cellular damage in plant cells following treatment in the light with paraquat. However, the observations of the changes in cell structure following treatment of fronds of S. oligorrhiza with paraquat are not inconsistent with the claim that cellular disruption begins with rupture of the tonoplast (Harris & Dodge, 1972 a) and tend to support the suggestion (Dodge & Lawes, 1974) that paraquat and diquat may differ slightly in their action.

Harris & Dodge (1972 a) observed a massive build-up of osmiophilic plastoglobuli in the degenerating chloroplasts of paraquat-treated flax cotyledons. According to these workers, the plastoglobuli were probably formed from lipid material derived from the disintegrating thylakoids. However, this author observed no comparable accumulation during the disruption of chloroplasts in paraquat-treated fronds of S. oligorrhiza. No build-up of plastoglobuli was reported following the exposure of Chlorella (Stokes et al., 1970) or flax cotyledons (Dodge & Lawes, 1974) to diquat in the light. However, similar electron dense deposits were observed during the disruption of chloroplasts in diquat-treated fronds of S. oligorrhiza (Figs. 44 - 55), but this was not found to be a constant feature of chloroplast breakdown.

Early ultrastructural damage in fronds of S. oligorrhiza exposed to either diquat or paraquat was accompanied by changes in the appearance of the microbodies. These organelles, which normally have a spherical shape and occur singly in the cytoplasm of untreated cells, were commonly observed in clusters after the onset of ultrastructural damage. At this time the shape of these structures was also more varied (Figs. 46, 48, 50,

52, 66, 68 and 78). The significance of this finding is uncertain. The microbodies were eventually totally destroyed by herbicide treatment in the light but they did appear to be slightly more resistant to damage than nearby mitochondria (Figs. 46, 48, 56, 66, 68, 74 and 78). A similar resistance to ultrastructural change was shown by the microbodies in dark-treated fronds (Figs. 116 and 118). None of the published accounts of ultrastructural damage to plants following exposure to bipyridylium herbicides in the light contains an account of the fate of these organelles.

The demonstration that ultrastructural damage to diquat or paraquat-treated fronds of S. oligorrhiza was greatly retarded if the treatment was conducted in complete darkness is in agreement with the findings of Harris & Dodge (1972 a). After treatment of fronds of S. oligorrhiza with diquat or paraquat (10 ppm cation) in darkness for 140 hours, damage to chloroplasts, mitochondria, the plasma membrane and tonoplast was usually evident (Figs. 91 - 98). The extent of this ultrastructural damage was variable but never as severe as that observed in the light after only 24 hours. Harris & Dodge (1972 a) failed to detect any sort of cellular disruption in paraquat-treated flax cotyledons maintained in darkness for 18 hours. After 30 hours mitochondria still appeared unaffected but the chloroplasts appeared swollen and had sustained marked disintegration of their limiting membranes. However, in all respects, the cellular damage visible at this time was less advanced than in tissue treated in the light for the same duration.

In contrast to the above observations, Dodge & Lawes (1974) reported that the early changes in the appearance of the chloroplasts in cells of flax cotyledons exposed to 10^{-4}M diquat in the light also occurred in complete darkness at a similar, but slightly slower rate. Furthermore, Baur et al.,

(1969) had previously reported that ultrastructural damage to paraquat-treated mesquite leaf cells developed just as rapidly in darkness as in the light.

Thus, it appears that there is no general agreement about the extent to which the development of ultrastructural damage is retarded when bipyridylium-treated plants are maintained in complete darkness. However, it is possible that much of this lack of uniformity may stem from differences in the herbicide concentration and in the variety of treatment durations employed. In spite of gross swelling of the chloroplasts, no membrane damage was observed by Didge & Lawes (1974) in tissue treated with diquat (10^{-4}M in the dark. However, the treatment period was not extended beyond 18 hours. Harris & Dodge (1972 a), using paraquat (10^{-4}M), and the present author, using diquat and paraquat (10 ppm cation), have presented evidence of membrane damage in dark-treated tissue after 30 hours and 140 hours. respectively. It is therefore possible that damage to some of the cellular membranes would have occurred in flax cotyledons had they been exposed to diquat in darkness for a longer period.

The retarded damage in paraquat-treated tissue reported here and elsewhere (Harris & Dodge, 1972 a) was not observed by Baur *et al.*, (1969) who claimed that the plasma membrane of mesquite mesophyll cells was ruptured after treatment with 10^{-2}M paraquat for only 5 minutes in either light or darkness. These workers employed permanganate fixation which, as this author has found with a variety of duckweed species, can by itself cause extensive damage to cellular membranes. This was also pointed out by Dodge & Lawes (1974) who suggested that the rapid rupture of the plasma membrane observed by Baur and co-workers may in fact have been caused by the fixative rather than by the herbicide itself. Another possible cause of the disparity between the observations of Baur *et al.* (1969) and other

workers is the extremely high concentration of paraquat (10^{-2}M) which was used. However, ultrastructural investigation of fronds of S. oligorrhiza treated with a massive dose of diquat (500 ppm salt), both in the light and in darkness, still revealed a marked delay in the onset of cellular damage. Cell structure appeared to be undamaged after 1 hour in darkness (Figs. 107 - 109) despite obvious changes to the chloroplasts of light-treated tissue (Figs. 99 - 106). Even after 15 hour treatment, the extent to which damage had occurred in the dark was considerably less than in tissue treated for the same length of time in the light (Figs. 110 - 119).

Observations revealed that, at ultrastructural level, diquat was more toxic than paraquat to fronds of S. oligorrhiza in the light (Figs. 57 - 87). Cellular damage appeared first in diquat-treated fronds and the subsequent deterioration of cell structure proceeded more rapidly in these fronds. Investigation of the available literature has failed to reveal any report of a comparison of the effects of diquat and paraquat on the fine structure of plant cells. However, Hams & Dodge (1972 a), working with paraquat (10^{-4}M), and Dodge & Lawes (1974), working with diquat at the same concentration, used the same plant species and essentially similar experimental techniques and conditions in their ultrastructural investigations. The observations of these two sets of workers seem to indicate that the onset of ultrastructural damage in light-treated flax cotyledons occurs at the same time irrespective of which herbicide is employed. However, as indicated previously, the nature of the initial damage was not the same in the two instances. Paraquat appeared initially to cause rupture of the tonoplast after which time the remaining cell structure rapidly disintegrated (Harris & Dodge, 1972 a). On the other hand, diquat appeared to have its first effect on the shape of the chloroplasts, damage to the bounding membranes

of the organelles not taking place until after rupture of the tonoplast and plasma membrane which required a longer period in diquat-treated tissue (Dodge & Lawes, 1974). Thus, after the initial ultrastructural changes had occurred, cellular disruption in the light proceeded more rapidly in the presence of paraquat. This conclusion is contrary to the finding reported here for S. oligorrhiza.

No firm conclusion about the relative toxicity of diquat and paraquat to S. oligorrhiza in darkness can be reached from the results presented. Treatment for a shorter period in the dark would possibly have lead to less variation in the appearance of treated tissue and allowed a more exact conclusion to be drawn. However, the greater toxicity of diquat observed in the light was certainly less marked in darkness. On the contrary, comparison of the results of Harris & Dodge (1972 a) and Dodge & Lawes (1974) indicates that, at least in the case of flax catyledons, ultrastructural damage occurs more readily in darkness with diquat than with paraquat.

Growth Studies

Diquat was also found to have a more marked inhibitory effect than paraquat on the growth of S. oligorrhiza in the light (Pl. 2 - 12). The greater inhibition resulting from diquat-treatment was more pronounced at intermediate concentrations. At all concentrations within the range 5.0 - 0.002 ppm (cation) diquat treatment resulted in the greater reduction in frond production. After 168 hours in the presence of the lowest concentration employed (0.0004 ppm cation) frond numbers in diquat-treated cultures were lower than in controls but greater than in cultures treated with paraquat. However, at this concentration frond numbers in diquat-treated cultures were significantly lower than in paraquat-treated cultures after treatment for

96, 120 and 144 hours. In addition, despite the greater number of fronds in diquat-treated cultures after 168 hours, fresh and dry weight measurements at this time indicated a greater growth inhibition in the presence of diquat.

Except at the highest concentration used (5.0 ppm cation) diquat-treated cultures always exhibited the greater reduction in both fresh and dry weights when compared with controls. With both herbicides, at concentrations of 0.005 ppm (cation) and upwards, the reductions in both fresh and dry weights were greater than the reduction in frond number. This finding may reflect a loss of cellular material following extensive tissue disruption. The reductions in fresh and dry weights of treated cultures compared with controls were similar after any one treatment. However, except at higher concentrations, the fresh weight of treated cultures appeared to show a slightly greater reduction than dry weight. The "sweating" observed in many of the treated cultures may possibly account for this finding.

The sensitivity of duckweeds to bipyridylium herbicides has made them useful tools for the determination of low concentrations of paraquat and diquat (Funderburk & Lawrence, 1963; Blackburn & Weldon, 1965; Damanakis, 1970). Funderburk & Lawrence (1963), using L. minor, and Blackburn & Weldon (1965) using Azolla caroliniana and a variety of duckweed species investigated the effects of a wide range of concentrations of both paraquat and diquat. However, neither set of workers used growth as a measure of herbicidal activity. Damanakis (1970) used the reduction in dry weight of L. polyrhiza expressed as a percentage of controls as a bioassay for low concentrations of paraquat.

Zweig, Hitt & McMahon (1968) found that diquat (3×10^{-5}M) caused a slight decrease in cell number in light-grown cultures of Chlorella pyrenoidosa. Since no cell destruction was observed, the authors concluded that diquat was algistatic under these conditions. Diquat (2×10^{-4}M) has also been

demonstrated to be a bacteriostatic inhibitor of growth in Rhodospirillum rubrum under both photosynthetic (anaerobic) and non-photosynthetic (aerobic) conditions (Kaneshiro & Zweig, 1965). Inhibition of the growth rate of a number of nonphotosynthetic bacteria by paraquat and diquat has been shown by Breazeale & Camper (1972). Erwinia carotovora was more sensitive to diquat while Bacillus sp. and Pseudonomas fluorescens were more sensitive to paraquat. Bipyridylium herbicides have also been shown to affect root growth in higher plants. Merkle, Leinweber & Bovey (1965) found that paraquat (4×10^{-4}M - 4×10^{-6}M) caused an inhibition of the rate of radicle elongation in mesquite seedlings. In a more recent study, Damanakis, Drennan, Fryer & Holly (1970) demonstrated that paraquat (0.5 - 1.5 ppm), taken up through the roots, resulted in a marked inhibition of the growth of the roots of ryegrass seedlings after 21 days. In this study inhibition of the root growth exceeded that of the shoots.

The roots of mesquite and ryegrass seedlings bear little resemblance to those of duckweeds. Quite apart from the differences in dimension and the degree of tissue differentiation, the roots of the latter are green due to the presence in many of the root cells of chloroplasts which are believed to be photosynthetically active (Hillman, 1961). No special study was made of the effects of herbicide treatment on the roots of S. oligorrhiza. The exposure of fronds to sub-lethal doses of paraquat or diquat did not result in any obvious differences in root growth. However, it was observed that at higher concentrations the roots of treated fronds often broke away from fronds at their points of attachment.

The most obvious change in the appearance of fronds of S. oligorrhiza during treatment with paraquat or diquat was the development of chlorosis. Fronds became chlorotic much faster with diquat than with paraquat. This

is consistent with the observed effects on growth and with the findings of Funderburk & Lawrence (1963) and Blackburn & Weldon (1965). These authors used the development of chlorosis in duckweeds to demonstrate the presence of low concentrations of paraquat and diquat in water. Under the experimental conditions employed in the present work, the lowest concentrations of paraquat and diquat to cause visible chlorosis in S. oligorrhiza after 7 days were 0.005 ppm and 0.002 ppm of the cation respectively. Using L. minor, Funderburk & Lawrence (1963) found the limit of detection to be 0.00075 ppm (cation) for paraquat and 0.005 ppm (cation) for diquat. Blackburn & Weldon (1965) investigated the effects of a range of paraquat and diquat concentrations on Azolla and a variety of duckweeds and reported variable degrees of chlorosis after 7 days in all except Wolffia columbiana with the lowest concentration of paraquat and diquat used (0.005 ppm)

Exact comparison of the susceptibilities of S. oligorrhiza and those species used by Funderburk & Lawrence (1963) and Blackburn & Weldon (1965) is difficult since the experimental conditions employed differed in a number of important respects. Firstly, the intensities of illumination during treatment were unequal. Funderburk & Lawrence (1963) and Blackburn & Weldon (1965) used light intensities of 500 f.c. (5000 lux) while in the present investigation the light intensity was only 3500 lux. The importance of light intensity on the speed at which chlorosis occurred in S. oligorrhiza has already been shown (Pl. 24 - 26). Secondly, these two sets of workers floated their plants on solutions containing only the herbicide and distilled water while in the present study both paraquat and diquat were incorporated into ½ strength Hutner's nutrient solution. Nutrient solution was used in place of distilled water for two reasons, firstly in order to encourage the growth of control fronds and secondly since this more closely approximated

the conditions under which duckweeds would be exposed to herbicides in the field. However, Parker (1966) showed that the substitution of tap water for distilled water greatly reduced the toxicity of paraquat to L. minor and he concluded that this was due to the presence of calcium or other cations in the top water interfering with the uptake of herbicide by the plants. More recently, Damanakis (1970) found that the sensitivity of L. polyrhiza to paraquat was variable in different media, being greatest in distilled water whil Stokes et al. (1970) showed that bleaching of chlorophyll in Chlorella vulgaris was more rapid when diquat was supplied in distilled water rather than in Warburg or phosphate buffers. Thus the observed sensitivity of S. oligorrhiza to paraquat and diquat in nutrient solution is likely to be less than that which would have been obtained had the herbicides been supplied in distilled water. Thirdly, the test plants of Funderburk & Lawrence (1963) and Blackburn & Weldon (1965) were floated on 50 ml. volumes of herbicide solution while in the present study a smaller volume of 40 ml. was employed. The ability of aquatic plants to accumulate bipyridylium herbicides from water containing low concentrations is well documented (Coates, Funderburk, Lawrence & Davis, 1964; Newman & Way, 1966; Austin, Calderbank & Carter, 1968). Due to the greater volume of herbicide solution provided, the plants used by these workers therefore had the potential to accumulate greater concentrations of paraquat and diquat than those used in the present study. Lastly, Funderburk & Lawrence (1963) rated fronds of L. minor for chlorosis over a period of 11 days while Blackburn & Weldon (1965) and the present author terminated the observation period after only 7 days. The longer treatment period employed by Funderburk & Lawrence may account, at least in part, for the apparently greater sensitivity of L. minor to bipyridylium herbicides since it required more than 7 days for herbicide

concentrations of less than 0.01 ppm (cation) to produce visible evidence of chlorosis.

The differences in experimental conditions just described do not allow strict comparison of the relative sensitivities of the various species of duckweed to paraquat and diquat, although in all species chlorosis did develop more rapidly in the presence of diquat. However, there is no doubt that differential sensitivity to paraquat or diquat does exist among the duckweeds (Blackburn & Weldon, 1965). This may be due to unequal rates of metabolism under the set of conditions provided. However, these authors found that the same species differential was maintained in field trials. Perhaps differential uptake of herbicide is a more important factor in determining the relative sensitivities displayed by the duckweed species tested.

The present studies indicate that the growth of S. oligorrhiza, as measured by the increase in such parameters as frond number, fresh weight and dry weight was affected by concentrations of paraquat and diquat below those resulting in visible chlorosis. The lowest concentration of paraquat to result in any obvious degree of chlorosis after 7 days was 0.005 ppm (cation) while at the same time there were significant reductions in frond number, fresh weight and dry weight compared with controls in cultures treated with only 0.002 ppm paraquat (cation) and a significant reduction in frond number in the presence of paraquat at a concentration of 0.004 ppm (cation). No degree of chlorosis was visible after 7 days in fronds treated with diquat at concentrations of less than 0.002 ppm (cation) whereas significant reductions in frond number, fresh weight and dry weight would be measured after the same period in the presence of only 0.004 ppm diquat (cation). Thus, at least in the case of S. oligorrhiza, it would appear that measurement of

growth parameters provides a more sensitive indication of the action of paraquat and diquat than do observations on the onset and degree of chlorosis. These findings also suggest that the reduction in chlorophyll content of treated fronds is secondary to the effects of the herbicides on growth. This is supported by the observation that at all concentrations of both paraquat and diquat in the range 0.05 - 0.0004 ppm (cation) small but significant reductions in frond numbers compared with controls were evident after only 24 hours and these were found to be unaccompanied by any changes in the appearance of the fronds. In addition, at all concentrations resulting in complete inhibition of growth, it was noted that this occurred before chlorosis was complete.

Pigment Studies

Quantitative assessment of the chlorophyll content of fronds of S. oligorrhiza following treatment with a range of concentrations of paraquat and diquat once again showed that the destruction of chlorophyll was more rapid with diquat. Increasing the rate of applied herbicide (Pl. 18 and 19) the duration of treatment (Pl. 21 and 22) or the light intensity (Pl. 24 and 25) during the experimental period all resulted in increased chlorophyll loss induced by diquat. Investigation of the literature has failed to reveal any account of quantitative measurements of the chlorophyll content of duckweeds following exposure to bipyridylium herbicides. However, Barnes & Lynd (1967) demonstrated that the reduction of chlorophyll in Phaseolus leaf discs exposed to paraquat was related to the rate of applied paraquat, time, light intensity and temperature. Similar findings have also been obtained with Chlorella pyrenoidosa (Sue-Fei. Tsay, Jhy-Mei Lee & Lynd, 1970).

Investigation of the effect of the duration of exposure to diquat on the

chlorophyll content of fronds of S. oligorrhiza revealed that, after a short exposure time, the chlorophyll content of treated fronds remained almost unchanged whereas the chlorophyll content of control fronds had increased (Pl. 21 and 22). This suggests that an early effect of diquat may have been to interfere with chlorophyll synthesis. In this context it is interesting to note that Mees (1960) reported that, after being dipped in diquat solution and placed in the light, etiolated wheat seedlings failed to develop a green colour despite remaining alive for more than 4 days. In addition, Kaneshiro & Zweig (1965) showed that diquat had an inhibitory influence on the synthesis of bacteriochlorophyll by R. rubrum growing under photosynthetic conditions. Partial inhibition of bacteriochlorophyll synthesis by diquat has also been demonstrated with chromatophore syspensions fractionated from R. rubrum (Kaneshiro & Zweig, 1966).

The finding that chlorophyll destruction in diquat-treated cultures of S. oligorrhiza was directly related to the intensity of illumination is in agreement with the visible changes in cultures of giant duckweed (Spirodela polyrrhiza) reported by Blackburn & Weldon (1965). These authors also observed some chlorosis in dark-treated fronds after 72 hours. However, in the present study, measurement of the chlorophyll content of fronds of S. oligorrhiza exposed to 10 ppm diquat (salt) in complete darkness revealed no loss compared with controls after 24 hours (Pl. 24 and 25). Similarly, Davies & Seaman (1968) found that no destruction of chlorophyll occurred in shoots of Elodea canadensis after exposure to 10 ppm diquat (cation) in darkness for 24 hours. In fact shoots treated in the darkness showed a sizeable increase in chlorophyll content for which the authors offered no explanation. A slight, but in no way comparable, increase in the chlorophyll content of dark-treated fronds of S. oligorrhiza over dark controls was

also obtained in the present study (Pl. 25). The reason for this is unclear. Reducing the light intensity had the general effect of reducing the chlorophyll content of control fronds, the controls in complete darkness having the lowest chlorophyll content of all. It would appear that in complete darkness diquat may in some way retard the loss of chlorophyll caused by the absence of illumination. Kaneshiro & Zweig (1965) have shown that the normal tendancy of the bacteriochlorophyll content of cultures of R. rubrum to decrease upon switching from photosynthetic to non-photosynthetic growth was presented by diquat. The reduced loss of chlorophyll in dark-treated fronds of S. oligorrhiza may however be more apparent than real. For example, it has been reported here that diquat causes a marked stimulation of the rate of respiration in fronds of S. oligorrhiza in darkness (pl. 45 and 46). Thus, at the end of the treatment period, diquat-treated fronds can reasonably be expected to be somewhat lighter than control fronds. In this way, the higher chlorophyll content of dark-treated fronds compared with dark controls need not indicate the presence of more chlorophyll per se but may simply be a reflection of a diquat-induced reduction in frond weight.

The destruction of chlorophyll in fronds of S. oligorrhiza exposed to diquat in the light can be temporarily halted by a period of darkness (Pl. 27 - 29). The dependence on light for the continued breakdown of chlorophyll in diquat-treated cells of Chlorella Vulgaris has also been demonstrated by Stokes et al. (1970) and a similar dependency has been shown to occur in paraquat-treated flax catyledon leaves (Harris & Dodge, 1972 b).

In bipyridylium-treated fronds of S. oligorrhiza chlorophyll a was lost more rapidly than chlorophyll b causing a reduction in the chlorophyll a/chlorophyll b ratio. This ratio showed a general reduction with increasing time. Such an observation is in agreement with the findings of Baldwin,

Dodge & Harris (1968) and Harris & Dodge (1972 b) with paraquat-treated flax cotyledon leaves. These workers reported that loss of chlorophyll b became apparent only after destruction of chlorophyll a had been in progress for some hours. However in the present study the loss of chlorophyll a and b appeared to begin at about the same time. Since the chlorophyll a/Chlorophyll b ratio became smaller as chlorosis became more advanced, it is not surprising that the present investigations also revealed that the ratio decreased with increasing herbicide concentration and that the reduction was more marked with diquat at all concentrations tested.

Gas Exchange Studies

Treatment of fronds of S. oligorrhiza with paraquat or diquat in complete darkness caused an almost immediate increase in oxygen uptake which was maintained throughout the experimental period (330 min.). In contrast, Mees (1960) reported that diquat usually decreased oxygen uptake in broad bean leaf discs, although an initial stimulation was occasionally found. However, Mees' results are not directly comparable with those obtained in the present studies with S. oligorrhiza since the leaf discs were treated beforehand with diquat for 3 hours in the light and the manometric measurements were always made after removing the discs from the herbicide solution. On the other hand, in the present study treated fronds of S. oligorrhiza were at no time exposed to the light and remained in contact with the treatment solution throughout the duration of the oxygen uptake measurements.

However, the observed stimulation of oxygen consumption in bipyridylium-treated fronds of S. oligorrhiza is in agreement with the results obtained by other workers with a variety of photosynthetic material. Working with the

duckweed L. minor, Funderburk & Lawrence (1964) demonstrated a stimulation of oxygen uptake caused by paraquat and diquat. Davies & Seaman (1964) measured increased oxygen uptake in the leaves of the submerged plants Elodea canadensis, and Potamogeton pectinatus. A rapid increase in the rate of oxygen uptake was also observed in Chlorella culgaris treated in darkness with diquat (Stokes & Turner, 1971). Diquat has also been shown to stimulate oxygen uptake by broad bean root tips (Mees, 1960)

The finding that the increased oxygen uptake in diquat-treated fronds of S. oligorrhiza in darkness was accompanied by increased carbon dioxide evolution and that diquat did not significantly alter the R.Q. of treated fronds (Pl. 45 and 46) suggests that diquat stimulates respiration. Stimulation of carbon dioxide output by diquat with little change in the R.Q. has already been demonstrated by Mees (1960) with broad bean and mustard leaf discs and by Stokes & Turner (1971) with Chlorella vulgaris. Increased carbon dioxide evolution by flax cotyledon leaves after treatment with paraquat has also been demonstrated by Harris & Dodge (1972 b). This increase was shown to be independent of the light conditions during the treatment period. The effect of diquat on the oxygen consumption of fronds of S. oligorrhiza in the presence of different levels of respiratory substrate lends further support to the view that the herbicide-induced stimulation of oxygen uptake is related to carbohydrate metabolism. As one would expect, oxygen uptake in untreated fronds exposed to continuous illumination prior to treatment was greater than in fronds given a dark pre-treatment. It was also shown that the oxygen consumption of untreated fronds could be increased by the addition of glucose. Similarly, light pre-treatment and the addition of glucose to diquat-treated fronds both resulted in an increased oxygen consumption in the dark. In both cases the increase in oxygen uptake in treated fronds was greater than

that caused by light pre-treatment or by the added glucose alone. In other words, the diquat-induced stimulation of dark oxygen uptake was enhanced by conditions which tended to increase the level of available respiratory substrate. This is in agreement with manometric work carried out by Stokes & Turner (1971) with C. vulgaris. However, the increased stimulation of oxygen uptake by diquat observed by these workers after either light pretreatment or the addition of glucose to the treatment solution was more marked than that obtained with S. oligorrhiza. The concentration of of diquat used by Stokes & Turner was 10^{-3}M which was much higher than that employed in the present work (2.9×10^{-5}M). It is therefore possible that at this low concentration diquat was a limiting factor and that at a higher concentration, light pretreatment or the addition of glucose would have led to a greater herbicide-induced stimulation of oxygen uptake by treated fronds.

Further evidence in support of the stimulation of respiration indicated by manometric techniques has arisen from tracer studies performed by Stokes & Turner (1971). Working with C. vulgaris these workers have shown that treatment with diquat in darkness accelerates the breakdown of starch and the loss of labelled carbon from many of the intermediates of carbohydrate metabolism.

Oxygen exchange studies carried out in the light with fronds of S. oligorrhiza in the presence of paraquat or diquat indicated that both herbicides had an inhibitory effect on photosynthesis (Pl. 47 - 49). Both caused a rapid cessation of apparent photosynthesis, complete inhibition being achieved more rapidly in diquat-treated fronds. The observed inhibition of oxygen evolution by bipyridylium-treated fronds of S. oligorrhiza is in agreement with earlier findings. Funderburk & Lawrence (1964) reported decreased oxygen production in the duckweed L. minor treated with paraquat or

diquat. Complete inhibition of oxygen evolution by illuminated leaves of Elodea canadensis and Potamogeton pectinatus has been shown to occur rapidly in the presence of a low concentration of diquat (Davies & Seaman, 1964). Zweig et al. (1968) reported that diquat (3×10^{-5}M) had no inhibitory effect on oxygen evolution by Chlorella pyrenoidosa. However, Turner, Stokes & Gilmore (1970) have demonstrated a rapid and marked inhibition of oxygen production by Chlorella vulgaris with diquat at a slightly higher concentration (10^{-4}M).

The general pattern of oxygen exchange by fronds of S. oligorrhiza following the addition of paraquat or diquat closely resembles that obtained by Turner et al. (1970) with diquat-treated cells of C. vulgaris. The peak of net oxygen uptake by fronds following inhibition of apparent photosynthesis was also observed in C. vulgaris and in both studies the size of the peak was a positive function of herbicide concentration.

No attempt was made in the present studies to measure the uptake of carbon dioxide in light-treated tissue. However, such investigations by other workers have also provided evidence for the inhibition of photosynthesis by bipyridylium herbicides. Greatly reduced carbon dioxide uptake has been measured in diquat-treated leaves of Phaseolus vulgaris (Van Oorschot, 1964) and in paraquat-treated leaves of flax cotyledons (Harris & Dodge, 1972 b). In addition, tracer studies have shown that treatment with low concentrations of diquat resulted in reduced $C^{14}O_2$-fixation in corn, soybeans and cotton (Couch & Davis, 1966) and Chlorella pyrenoidosa (Zweig, Hitt & Cho., 1969).

No investigation was made of the ultrastructural condition of bipyridylium treated fronds of S. oligorrhiza throughout the duration of oxygen exchange measurements in the light. However, earlier studies revealed that ultrastructural damage did not become evident in fronds treated in the light with

diquat or paraquat at a concentration of 10 ppm (cation) for periods exceeding 4 and 6 hours. respectively. Complete inhibition of apparent photo synthesis was achieved in fronds exposed to the same concentrations of diquat and paraquat within approximately 90 and 160 min. respectively. Although the experimental conditions employed for the ultrastructural and manometric studies were not identical, the information obtained suggests quite strongly that diquat and paraquat have an inhibitory influence on photosynthesis which is not simply the result of cellular damage.

An early inhibition of photosynthesis before the onset of ultrastructural damage should result in an reduced amount of starch within the chloroplasts of treated tissue. Electron microscopical observations did reveal some evidence of this, however the quantity of starch in chloroplasts generally was too variable to allow firm conclusions to be made on this point. However, using the unicellular alga Chlorella vulgaris, Stokes, et al. (1970) were able to demonstrate electron microscopically that diquat-treated cells had not accumulated starch like control cells after a period of 1 hour in the light. This observation supports evidence from gas exchange studies with C. vulgaris (Turner et al., 1970) that diquat inhibits photosynthesis within 1 hour and was unaccompanied by any damage to the chloroplasts visible in the electron microscope. The apparent inhibition of photosynthesis in diquat and paraquat-treated fronds of S. oligorrhiza prior to the onset of ultrastructural damage is in agreement with the results obtained with paraquat-treated flax cotyledon leaves (Harris & Dodge, 1972 a, b). Complete inhibition of photosynthetic carbon dioxide uptake was achieved within 5 hours whereas ultrastructural damage first became evident after 6 hours.

In contrast to the situation indicated by the above results Baur et al. (1969) suggested from the results of their ultrastructural studies with

paraquat-treated mesquite leaves, that the blocking of anabolic processes in the chloroplasts was secondary to membrane damage. However, these workers made no gas exchange measurements. Their conclusions were based upon the failure of light pre-treated cells to accumulate starch following treatment with paraquat and upon the non-reappearance of starch grains in the chloroplasts of dark pre-treated plants following the same treatment. Electron microscopical observations indicated that ultrastructural damage to treated cells occurred within only 5 min., a finding which may have been due to the extremely high concentration of paraquat employed (10^{-2}M). In any event, under the conditions employed by Baur and coworkers the interval of time between the application of herbicide and the start of ultrastructural damage was too short to permit a realistic assessment of any effect of treatment on the photosynthetic rate by observations of the size or numbers of starch grains within the chloroplasts. This does not preclude the possibility that under the conditions employed, cellular damage does in fact precede the inhibition of photosynthesis. However, the bulk of existing evidence at present points to the opposite situation being the case, at least when more moderate doses of herbicide are applied.

DISCUSSION (continued)

MODE OF ACTION

According to the generally held conception of the mode of action of bipyridylium herbicides, the bipyridylium cations are converted within plants by acceptance of a single electron from sources of reducing power, to their free radical forms which undergo rapid reoxidation in the presence of molecular oxygen with the simultaneous production of hydrogen peroxide. Most of the available evidence suggests that photosynthesis is the most important source of reducing power for bipyridylium herbicides (see introduction). These herbicides are believed to interfere with photosystem I of photosynthesis by shunting electrons away from ferredoxin.

The findings of this author and other workers that the rate of development of chlorosis in bipyridylium-treated plants was a positive function of the light intensity during treatment are not inconsistent with the importance attached to photosynthesis as the main source of reducing power for these herbicides. However there is evidence that much of the chlorophyll loss occurring in light-treated plants is in fact due to photooxidation after damage to the photosynthetic apparatus rather than to a direct effect of the herbicide (Dodge, Harris & Baldwin, 1970; Stokes *et al.*, 1970; Dodge, 1971; Harris & Dodge, 1972 b). Thus different degrees of chlorosis in treated plants need not imply different levels of cellular damage.

However, ultrastructural observations of treated fronds overcome this difficulty. The results of ultrastructural studies with fronds of *S. oligorrhiza* undergoing treatment with bipyridylium herbicides in the light and in darkness provide further evidence for the implication of photosynthesis as the primary source of reducing power in the light. With both paraquat and diquat, the development of ultrastructural damage was greatly retarded in

dark-treated fronds. After treatment of fronds for 24 hours in the light with either paraquat or diquat (10 ppm cation) the disruption of cell structure was almost complete (Figs. 81 - 87) while treatment with the same concentration of herbicides in total darkness for 140 hours resulted in variable but significantly less damage (Figs. 91 - 98). Similarly, a slower rate of ultrastructural damage in darkness has been reported for flax cotyledon leaves treated with paraquat (Harris & Dodge, 1972 a) and diquat (Dodge & Lawes, 1974). In contrast to the above findings, Baur et al. (1969) failed to find ultrastructural evidence consistent with reduced paraquat toxicity in dark-treated mesquite leaves. Baur and coworkers used an extremely high concentration of paraquat (10^{-2}M). However, they discounted the possibility that the observed ultrastructural changes were caused by the direct action of paraquat on cellular membranes owing to their findings that damage appeared to be confined to the plasma-membrane and the membranes of the chloroplasts. The authors argued that photosynthesis was no more important than other sources of reducing power in converting paraquat to its free radical form. The extremely high concentration of paraquat used by these workers may however be an important factor in explaining the results which they obtained, Baur and coworkers suggested that reduced NADP was the main electron donor in the dark. At more modest herbicide concentrations it is possible that much of the existing supply of reduced NADP within the cells of dark-treated leaves would have been consumed by normal physiological processes before the build-up of paraquat within the cells had been achieved. However, at the high dosage employed, it could be that sufficient paraquat gained entry into the cells fast enough to divert a much greater amount of this reduced NADP towards free radical formation. Assuming the availability of sufficient oxygen within the cells it is perhaps possible that rapid reoxidation could have taken

place with the concomitant formation of enough hydrogen peroxide to cause the same degree of ultrastructural damage as was sustained in the light after the same length of time. Even although hydrogen peroxide may have been formed more rapidly in the light, a proportion of this toxic substance may in fact have been in excess of the amount required to cause the observed effects. However, this author has demonstrated a marked retardation of cellular disruption in dark-treated fronds of S. oligorrhiza in the presence of 500 ppm. diquat (Figs. 99 - 119). Admittedly this concentration although far higher than those normally used, is still lower than that employed by Baur and coworkers. However the results obtained failed to suggest that at high dosage levels the rates of cellular damage in the light and in darkness are in any way similar.

The results of manometric studies indicated that treatment with paraquat or diquat in the light caused the rapid inhibition of apparent photosynthesis whereas in darkness a stimulation of respiration was shown. These findings also point to the greater toxicity of paraquat and diquat in the light and to the possible importance of light-generated reducing power to the toxicity of these herbicides.

If bipyridylium herbicides interfere with photosynthesis by accepting electrons from the primary electron acceptor of photosystem I, they would be in a position to interfere with the reduction of ferredoxin which in turn would lead to a reduced production of reduced NADP. Indeed, in experiments with isolated chloroplasts Davenport (1963), Zweig; Shavit & Avron (1965) and Black (1966) have shown that bipyridylium herbicides competitively inhibited the photoreduction of NADP by ferredoxin. Such wastage of reducing power would have an inhibitory effect on carbon fixation. Thus both the electron microscopical evidence for an early reduction

in the starch content of chloroplasts of light-treated fronds of S. oligorrhiza and the demonstration by Stokes et al. (1970) that, unlike control cells, diquat-treated cells of C. vulgaris did not accumulate starch before the onset of ultrastructural damage are consistent with the proposed site of interference by bipyridylium herbicides with photosynthetic electron transport. The reduced growth rate of fronds of S. oligorrhiza in the presence of sub-lethal concentrations of paraquat and diquat (Pl. 6 and 7) also suggests a wastage of reducing power such as would result from a shunting of electrons away from ferredoxin.

Davenport (1963), Zweig et al. (1965) and Black (1966) have shown that bipyridylium salts can also be reduced by pulling electrons away from reduced NADP. In this way paraquat and diquat may be converted to their free radical forms in both light and darkness. This can therefore account for the observed toxicity of paraquat and diquat in the dark assuming that sufficient oxygen is available to the plants to allow reoxidation of the free radicals. The absence of photosysthesis in the dark will nto only prevent the photoreduction of bipyridylium cations but also lead to a lower oxygen tension within the plant tissues thus showing the reoxidation of the free radicals and the simultaneous formation of hydrogen peroxide. Both of these factors would help to explain the reduced toxicity of bipytidylium herbicides in darkness.

The slower loss of chlorophyll from fronds of S. oligorrhiza treated in the light with diquat and 2, 3, 5 - triphenyl tetrazolium chloride (TTC) together compared with the loss from those treated with diquat alone (Pl. 30 and 31) suggests that reduction of the herbicide is an important part of the toxic reaction. TTC is an electron acceptor with a redox potential of -80 mV and is therefore more readily reduced than diquat which has a redox potential of -349 mV. The reduced toxicity of diquat in the presence of TTC

strongly suggests a competition for electrons. If the reduction of diquat was not a necessary prerequisite for rapid herbicidal activity it is unlikely that this antagonistic effect would have been obtained.

Paraquat has a redox potential of -446 mV and so is less readily reduced than diquat. Thus, if reduction is an important step in their toxic action, one would expect diquat to be more toxic to plants than paraquat. This of course assumes that the uptake of both herbicides is similar and that the respective free radicals are veoxidized equally readily. Indeed diquat was always found to be the more toxic herbicide to S. oligorrhiza in the light whether the criterion was the development of ultrastructural damage, growth, the loss of chlorophyll or the rate of photosynthesis. In darkness diquat also had the greater stimulatory effect on respiration.

According to the proposed scheme to explain the mode of action of bipyridylium herbicides, the bipyridylium free radicals are readily reoxidized by molecular oxygen to the original cations which are then once again capable of undergoing reduction within the plant. Thus, in theory, the bipyridylium cations can participate in the toxic reaction over and over again. Such regeneration of bipyridylium cations is consistent with the very low concentrations at which both paraquat and diquat have been shown to interfere with the normal ultrastructure and physiology of green plants. The rapidity with which toxic symptoms develop in treated green tissue in the light is accounted for by the production of hydrogen peroxide, or free radicals derived from this, during the regeneration of the herbicide cations. Evidence for the formation of hydrogen peroxide within chloroplasts in the presence of bipyridylium herbicides is indirect (Davenport, 1963). The formation of hydrogen peroxide was indicated by detecting a metmyoglobin-peroxide complex on illuminating a crude chloroplast preparation

containing metmyoglobin in the presence of a catalytic amount of diquat.

While not providing further evidence for the generation of hydrogen peroxide or free radicals derived from this, the rapid and extensive ultrastructural damage to frond tissue in the presence of paraquat or diquat is not at variance with such an event. It has been suggested by Black & Myers (1966) that even if hydrogen peroxide is formed in plants as a result of treatment with bipyridylium herbicides it will be destroyed by catalase and various peroxidases within the tissues before any damage is done. However, Gregory (1968) found very little catalase activity associated with spinach chloroplasts and other workers have shown that the bulk of this enzyme is located outwith the chloroplasts in membrane-bound organelles termed microbodies or peroxisomes (Tolbert, Oeser, Kisaki, Hageman & Yamazaki, 1968; Tolbert, Oeser, Yamazaki, Hageman & Kisaki, 1969; Frederick & Newcomb, 1969; Vigil, 1969; Yamazaki & Tolbert, 1970; Frederick & Newcomb, 1971).

Attempts to determine the distribution of catalase within frond cells of S. oligorrhiza by a histochemical technique were not entirely successful (Figs. 120-125). Nonetheless, the results indicated that most of the catalase activity was located within the microbodies. Electron-dense deposits were also observed in the cell walls. Frederick & Newcomb (1969), using basically the same technique, also observed the slight deposition of electron-dense material within primary cell walls in some sections. On the basis of the results of inhibitor studies in conjunction with histochemical techniques, these workers argued that the deposits observed within the cell walls were due to the oxidation of the electron cytochemical reagent diaminobenzidine (DAB) by soluble and wall-localized peroxidases rather than by catalase. In view of this and the body of evidence that many plant parts contain both soluble and wall-localized peroxidases (Jansen, Jang &

Bonner, 1960; Lipetz & Garro, 1965; Gagnon, 1968; Tolbert *et al.* 1968) it seems likely that the electron-dense deposits observed in the walls of frond cells of *S. oligorrhiza* are indicative of peroxidase activity. If this is the case, destruction of hydrogen peroxide could occur within the cell walls provided that suitable electron donors were present.

In bipyridylium-treated fronds in the light, the bulk of the hydrogen peroxide production should occur within the chloroplasts following interference with photosynthetic electron transfer. Reduction of paraquat and diquat by reduced pyridine nucleotides and subsequent reoxidation may also give rise to some hydrogen peroxide production both within the chloroplasts and in the cytoplasm. In addition, some hydrogen peroxide may be formed inside the mitochondria as a result of interference with respiratory electron transport, although there is some evidence from work with rat liver (Gage, 1968) and potato mitochondria (Stokes & Turner, 1971) that bipyridylium herbicides are unable to penetrate mitochondrial membranes. In any event, owing to their apparent localization within the microbodies and cell walls, it would appear that the enzymes capable of destroying hydrogen peroxide are not suitably sited within the cells of *S. oligorrhiza* to fulfill this task. The histochemical evidence presented does not exclude the possibility of a little catalase or peroxidase activity in those parts of the cell in which hydrogen peroxide may be formed. However, it is considered that, given the existence of a ready supply of reducing power and sufficient oxygen for regeneration of the bipyridylium cations, hydrogen peroxide would be generated more rapidly than could be adequately dealt with by the small amounts of enzyme which may be present. It is perhaps significant that the catalase-rich microbodies were generally found to sustain ultrastructural damage at a slower rate than other organelles following

treatment in the light with paraquat or diquat. If this relative resistance to damage is related to the catalase content of these bodies, this finding would strongly suggest that hydrogen peroxide is involved in causing tissue damage following treatment with bipyridylium herbicides.

It is interesting to speculate that the presence of many microbodies often found crowded together in cells of S. oligorrhiza some time after treatment with paraquat or diquat may in fact represent a proliferation of these organelles in response to levels of hydrogen peroxide significantly in excess of those occurring under normal conditions. Certainly, in some of the micrographs presented microbodies do appear to have formed by the fission of pre-existing microbodies (Figs. 48, 50, 52, 66, 68, 94 and 119). De novo synthesis of microbodies has been observed in rat liver cells treated with ethyl - d - p - chlorophenoxyisobutyrate (CPIB) (Legg & Wood, 1970 a) and in regenerating liver (Rigatuso, Legg & Wood, 1970). Under the influence of the drug CPIB the increase in the number of microbodies is paralleled by an increase in total liver catalase activity (Svoboda, Grady & Azarnoff, 1967) Svoboda, Azarnoff & Reddy, 1969; Reddy, Chiga, Bunyaratvej & Svoboda, 1970) which suggests a close relationship between the synthesis of catalase and the proliferation of these organelles. However, in further work involving the use of CPIB combined with inhibitors of catalase activity it has been demonstrated that the increase in numbers of rat liver microbodies is independent of catalase synthesis (Legg & Wood, 1970 b). In the light of this finding the above suggestion of microbody proliferation in bipyridylium-treated fronds of S. oligorrhiza in response to an increased level of hydrogen peroxide within the cells must remain highly speculative.

A change in the permeability of cellular membranes appears to be one of the early symptoms of the treatment of plants with bipyridylium herbicides

(Merkle et al., 1965; Harris & Dodge, 1972 b). Harris & Dodge (1972 b) found that increased membrane permeability appeared to be related to a rise in the level of malondialdehyde in paraquat-treated flax cotyledon leaves. Malondialdehyde is a breakdown product of tri-unsaturated fatty acid hydroperoxides. It would therefore seem reasonable to suppose that changes in membrane permeability could be caused by the action of hydrogen peroxide or free radicals derived from this. The increased oermeability of cellular membranes could account for the swelling of the chloroplasts and the initial changes in the arrangement of their thylakoid systems. In the present work with diquat-treated fronds of S. oligorrhiza and in work with diquat-treated flax cotyledons (Dodge & Lawes, 1974), swelling of the chloroplasts occurred in the absence of visible damage to the cellular membranes. Thus it would appear that permeability may be altered without the actual rupture of the membranes themselves. This is supported by Harris & Dodge (1972 a, b) who measured potassium leakage from paraquat-treated flax cotyledon leaf tissue 3 hours before any visible ultrastructural damage. These workers also observed a rapid increase in membrane permeability which coincided with the rupture of the tonoplast. Dodge (1971) and Harris & Dodge (1972 b) proposed that free radicals derived from hydrogen peroxide attack unsaturated fatty acids in the cellular membranes and set off a chain reaction leading to their gradual destruction. The observed swelling of the chloroplasts prior to the rupture of the cellular membranes is consistent with this hypothesis.

How much of what follows the onset of membrane disintegration is due to the continued activity of the herbicide and how much is an inevitable consequence of this damage is uncertain. Dodge (1971) and Harris & Dodge (1972 a, b) argue that the release of the vacuolar contents after the rupture of the tonoplast is the most likely cause of much of the subsequent ultrastructural damage.

The rapid deterioration of cell structure which these workers observed after rupture of the tonoplast adds strong support to their argument.

As discussed earlier, there is a lack of agreement among workers about the sequence of events occurring in bipyridylium-treated plant tissue in the light. It is proposed here that one possible reason for this may lie in differences in the composition of the cell sap within the vacuoles of the plants used in the studies carried out so far. The vacuole is known to be, among other things, a dumping ground for toxic waste products (Dainty, 1968) and is also thought to have lysosomal activity (Graham, 1968). So the release of the vacuolar contents into the cytoplasm following the rupture of the tonoplast would be expected to cause considerable damage. However the magnitude of this damage should be related to the composition of the cell sap. In all probability, the nature of the vacuolar contents will change with the age of the cell and may also vary in different tissues and different species of the same age. Such differencies could therefore account, at least in part, for the apparent lack of uniformity of cellular destruction following treatment with bipyridylium herbicides.

Harris & Dodge (1972 a) and Dodge & Lawes (1974) found that in flax cotyledon leaf cells, treated with paraquat anddiquat respectively, the rupture of the tonoplast was rapidly followed by considerable ultrastructural damage. In the present work with S. oligorrhiza the destruction of the tonoplast had a less dramatic effect on the speed of deterioration of cell ultrastructure. According to the above argument, this difference can be explained by assuming that the contents of the vacuoles in the frond cells of S. oligorrhiza are less toxic than the vacuolar contents of flax cotyledon leaf cells. Support for the view that in the case of S. oligorrhiza the contents of the vacuoles are not sufficiently toxic to cause damage to cell structure

at the rates normally observed in treated cells may be obtained from the ultrastructural examination of cells in senescing frond tissue. Chloroplasts and mitochondria which had apparently sustained relatively little ultrastructural damage were commonly observed despite the total disruption of the tonoplast (Figs. 126 and 127). This suggests that, at least in the case of S. oligorrhiza the release of the vacuolar contents may not be a particularly important factor in the deteriorative chain of events following the rupture of the tonoplast. However, it may be that earlier changes in the permeability of the limiting membranes of the cellular organelles could increase the susceptibility of these bodies to damage by materials from the vacuole. Other reasons for the differences in the sequence of ultrastructural events following treatment with bipyridylium herbicides could include differences in the herbicide concentrations employed in the various studies. In the present study, treatment with large doses of diquat in the light resulted in a considerable change in the arrangement of the chloroplast thylakoids which preceded all other visible ultrastructural damage. This is in contrast to the findings with low diquat concentrations under otherwise identical conditions.

In conclusion, it is probably true to say that there is less agreement about the effects of bipyridylium herbicides on the ultrastructure of plant cells than on their biochemistry/physiology. Attempts have been made here to account for at least some of the disagreement. However there are difficulties involved in any attempt to follow, at an ultrastructural level, the course of events following treatment with a rapidly-acting herbicide such as paraquat or diquat. Not the least of these difficulties is the fact that cells in different parts of the same piece of tissue very often tended not to be affected by treatment in a uniform manner. This point has also been raised by Dodge & Lawes (1974). Nonetheless, even allowing for the observed

differences in the sequential pattern of cellular disorganization in bipyridylium-treated plants, the ultrastructural information at present available is not at variance with the currently accepted scheme accounting for the mode of action of these herbicides.

BLUE-GREEN ALGAL STUDIES

INTRODUCTION

The structure, physiology and ecology of blue-green algae (Cyanophyta) have been extensively reviewed in recent years (Holm-Hansen, 1968; Carr & Whitton, 1973; Fogg, Stewart, Fay & Walsby, 1973). Taxonomically, the blue-green algae occupy a position between the bacteria and the green algae. Electron microscopy has shown that the blue-green algae, like the bacteria, have a prokaryotic cellular organization (Lang, 1968; Fogg *et al*, 1973). A typical vegetative blue-green algal cell has a multilayered cell wall external to the plasma membrane. Within the cytoplasm there are no membrane-bound organelles although a system of membranes, the thylakoids, are to be found generally occupying peripheral positions. Also in the cytoplasm, a varied selection of granular inclusions are generally to be found. The nuclear complement of blue-green algal cells is confined to central parts of the cell.

Despite their simplicity and lack of structural compartmentalization, blue-green algae are capable of a wide range of metabolic processes. The mechanism of photosynthesis in blue-green algae is similar to that occurring in higher plants (Holm-Hansen, 1968; Fogg *et al*., 1973; Krogmann, 1973). Water is generally the ultimate electron donor and oxygen is evolved. The light reactions of photosynthesis take place on the thylakoids. There is evidence for the occurrence of both photosystem I and photosystem II in the vegetative cells of the blue-green algae. Chlorophyll a is the principal pigment in photosystem I while in photosystem II the main light-absorbing pigments are the phycobiliproteins. Both cyclic and non-cyclic photophosphorylation can occur and the fixation of carbon dioxide is mainly via the Calvin cycle.

The details of the respiratory pathways in blue-green algae are less well

understood (Holm-Hansen, 1968; Biggins, 1969; Carr, 1973; Fogg et al. 1973). Enzymes of the glycolytic and pentose phosphate pathways and the glyoxylate and tricarboxylic acid cycles have been demonstrated in cells of blue-green algae, although it appears that the latter cycle is not fully operative. Blue-green algae exhibit generally low rates of endogenous respiration and the rates of production of ATP by oxidative phosphorylation are correspondingly low. Bisalputra, Brown & Weir (1969) have presented histochemical evidence which strongly suggests that the thylakoids are the sites of respiration.

Many representatives of the Cyanophyta have the ability, unique among oxygen-evolving photosynthetic plants, to fix atmospheric nitrogen (Fogg et al. 1973; Stewart, 1973). Of the species which have so far been shown to carry out nitrogen fixation, the vast majority are heterocystous. However, in recent years the ability to fix atmospheric nitrogen has been demonstrated in a small number of non-heterocystous species or strains of blue-green algae. These organisms comprise both unicellular (Wyatt & Silvey, 1969; Rippka, Neilson, Kurisawa & Cohen-Bazire, 1971), and filamentous (Stewart & Lex, 1970; Kenyon, Rippka & Stanier, 1972) forms. Members of the latter group however, do not fix nitrogen under aerobic conditions.

Nitrogen fixation by blue-green algae contributes greatly towards fertility in a variety of terrestrial and aquatic environments throughout the world (Fogg et al., 1973; Stewart, 1973). However, blue-green algae can become troublesome in aquatic ecosystems, particularly when eutrophic conditions prevail. Under such conditions some species of blue-green algae can grow in great profusion resulting in the serious spoilage of water making it unfit for domestic or industrial use. The recreational and aesthetic value of bodies of fresh water may also be impaired by dense blooms of blue-green algae. Deoxygenation of the water caused either by their decay or by their

combined respiration at night may lead to the death of fish and other aquatic organisms. In addition, many species of bloom-producing blue-green algae release toxins which can result in the death of aquatic animals. Such toxic products may also render water poisonous to birds, domestic animals and even man (Fogg et al., 1973). Many methods have been employed in attempts to control the growth of bloom-forming blue-green algae. Some of these methods involve the use of chemicals such as copper sulphate and 2,3-DNQ (2,3-dichloro-1,4-napthoquinone). However, this author knows of no report of the control of blue-green algae using bipyridylium herbicides.

The blue-green alga used in this work is Anabaena cylindrica. This is a filamentous species with the ability to fix atmospheric nitrogen. During the life cycle of this alga three distinct cell types may be formed - the vegetative cells which carry out photosynthesis and, derived from these cells under suitable conditions, the heterocysts and the akinetes.

It has been shown that the frequency of heterocysts in A. cylindrica is inversely proportional to the nitrogen content of the alga (Fogg, 1944) and that the differentiation of vegetative cells into heterocysts is inhibited by the presence of combined nitrogen (Fogg, 1949). The transition from vegetative cell to heterocyst in A. cylindrica and other heterocystous blue-green algae involves a considerable degree of cellular reorganization (Wildon & Mercer, 1963; Lang, 1965; Lang & Fay, 1971; Kulasooria, Lang & Fay, 1972). There is considerable evidence that metabolic changes are also involved. Although, like vegetative cells the heterocysts appear to have a functional photosystem I (Fay, 1969 b; Donze, Haveman & Schiereck, 1972; Scott & Fay, 1972), many studies have pointed to the absence of an oxygen evolving photosystem II within these cells. This conclusion is based largely on the almost total absence within the heterocysts of the accessory

pigment phycocyanin (Fay, 1969 b; Stewart et al., 1969; Wolk & Simon, 1969; Fay, 1970; Thomas, 1970), the failure to demonstrate oxygen evolution by isolated heterocysts upon illumination (Bradley & Carr, 1971) and the apparent lack of Hill reaction activity in extracts of isolated heterocysts (Donze et al., 1972).

In spite of being unable to carry out photosynthesis, heterocysts do appear to have an active metabolism. A variety of investigations have provided evidence of high reducing activity within heterocysts compared with vegetative cells (Drawert & Tischer, 1956; Tischer, 1957; Talpasayi, 1967; Stewart et al., 1969; Fay & Kulasooriya, 1972). Also, significant rates of photophosphorylation have been measured by Scott & Fay (1972) in isolated heterocysts of A. variabilis. Fay, Stewart, Walsby & Fogg (1968) suggested that the heterocyst is the site of nitrogen fixation in blue-green algae. Since then, direct evidence of nitrogen fixation in heterocysts has been provided by Stewart et al. (1969) and Wolk & Wojciuch (1971). Some controversy exists as to whether or not nitrogen-fixation in heterocyst-forming blue-green algae is confined to the heterocysts. Stewart (1971) has suggested that under microaerophilic conditions vegetative cells have the ability to fix nitrogen but that under aerobic conditions all or most of this activity is centred in the heterocysts.

The akinetes are easily distinguished from vegetative cells and heterocysts by their considerably larger size. The formation of akinetes from vegetative cells involves not only an increase in size but also significant changes in structure, composition and function. The ultrastructure of akinetes has been illustrated in a number of electron microscopical studies (Wildon & Mercer, 1963 ; Miller & Lang, 1968; Lang & Fisher, 1969; Clark & Jensen, 1969). Akinetes are vegetatively produced spores which can

remain viable under unfavourable conditions for long periods. Germination of akinetes can occur under suitable conditions giving rise to new filaments.

Fay (1969a) reported that photosynthesis occurred more slowly in isolated akinetes of A. cylindrica than in whole filaments. This observation is consistent with a subsequent finding (Fay, 1969 b) that the formation of akinetes in A. cylindrica is associated with profound changes in pigment composition. Fay showed that phycocyanin is largely absent in mature akinetes and that the bulk of the chlorophyll a is converted into phaeophytin. Fay (1969 a) has also shown that respiration occurs at a more rapid rate in isolated akinetes of A. cylindrica than in whole filaments. Fay argued that the increased respiration was associated with events occurring within the akinetes prior to cell division and germination.

One of the aims of the work described here is to investigate the effects of treatment with bipyridylium herbicides on an oxygen-evolving photosynthetic plant exhibiting a prokaryotic cellular organization. The selection of A. cylindrica as the test plant not only satisfies this requirement but also allows the effects of treatment on structurally and metabolically different cell types to be compared.

II(A) MATERIALS AND METHODS

A. Plant Material

The plant chosen was the blue-green alga *Anabaena cylindrica* Lemm. Bacteria-free cultures were obtained from The Cambridge Culture Collection.

B. Chemicals

The formulation and source of the herbicides used in investigations involving *A. cylindrica* are described elsewhere (P.). Unless otherwise stated, all other chemicals were obtained from British Drug Houses, Poole, Dorset, England.

C. Culture Conditions

Stock cultures of *A. cylindrica* were maintained on slopes on a standard nitrogen-free medium (Allen & Arnon, 1955) solidified with 1.2% agar. The culture medium was sterilized by autoclaving at 15 p.s.i. for 15 minutes. To prevent precipitation, the phosphate and iron containing components of the medium were autoclaved separately and added to the other constituents after cooling.

Liquid cultures were grown in 250 ml. Erlenmeyer flasks containing 100 ml. sterile nitrogen-free nutrient solution. The flasks were inoculated with algae from the agar slope cultures and they were incubated at 25°C on a Gallenkamp refrigerated orbital incubator revolving at 90 oscilations per minute. Continuous illumination was provided by a built-in array of white fluorescent lamps giving a light intensity of 3500 lux at culture level.

Special procedures were devised to provide material suitable for electron microscopic studies. The alga was initially grown aseptically in Petri dishes on standard nitrogen-free medium solidified with 1.2%

agar under conditions identical to those provided for slope cultures. When a good even growth had been obtained over the agar surfaces, small discs (2 cm. diameter), complete with surface growth, were cut out by pressing downwards with an inverted glass vial. These discs were then picked up by means of sterile needles floated on fresh nitrogen-free culture solution in separate covered Petri dishes and re-incubated as before. Under these conditions, new growth occurred outwards from the edges of the agar discs onto the surface of the culture solution. This resulted in thin, floating "carpets" of young, actively growing algal filaments. Unless otherwise stated, this "carpet" material was used for all electron microscopic investigations as it comprised cells of uniform appearance and allowed closely similar availability of light, gases and nutrients.

D. Electron Microscopy

Algal "carpets" were treated with paraquat or diquat when approximately 2 days old. To this end, the agar discs complete with the encircling new growth were carefully transferred onto the surface of fresh culture solution containing the required concentration of herbicide. Control material was transferred onto pure culture solution. Some cultures were incubated at room temperature ($22\pm2^{o}C$) in the light (3500 lux) beneath an array of Crompton 80W white fluorescent lamps, and removed for sampling after the desired duration of treatment viz. 0, 15, 30, 45, 60, 80, 100, 120, 150, 180, 210, 240 and 300 minutes. Other cultures were immediately placed in complete darkness at room temperature and sampled after 300 minutes.

In other experiments, 2 day old "carpet" material was given an initial dark pre-treatment lasting 18 hours. This measure was taken to

produce cells free of polyglucoside granules and was achieved by enclosing the cultures in black, polythene bags. The culture solutions were subsequently replaced by fresh medium with or without added diquat (final concentration 10 ppm) and the cultures then returned to the light (3500 lux). Both control and treated cultures were harvested for examination at regular intervals after periods ranging from 15 to 300 minutes.

In some investigations, agar-grown colonies of algal material were used in place of the floating "carpets". When the surface of agar plate cultures contained large numbers of colonies, small discs (2 cm diameter) were removed as described earlier (p.161) and floated on fresh culture solution containing the desired concentration of herbicide which ultimately reached the colonies by diffusion through the agar. Control discs were transferred onto culture solution alone. Cultures were subsequently incubated at room temperature in the light (3500 lux) or in darkness (in black polythene bags) and sampled at desired intervals.

Immediately prior to fixation, sections (approximately 1 mm^2) of algal material were removed from the same relative regions of the floating "carpets" by means of a razor blade. In the case of experiments carried out with colonies, the latter were similarly sectioned into 1 mm cubes. Three different fixation methods were employed: -

(i) Permanganate Fixation

2% aqueous potassium permanganate (Mollenhauer, 1959) for 1 hour at room temperature.

(ii) Osmium Fixation

1% buffered osmium tetroxide (Kellenberger, Ryter & Sechaud, 1958) for 18 hours at room temperature.

(iii) Double Fixation (glutaraldehyde/osmium tetroxide)

3% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2 (Juniper et al., 1970) for 22 hours at room temperature. After washing in 3 changes of fresh buffer over a 2 hour period, post-fixation was carried out with 1% osmium tetroxide in the same buffer for 16 hours at room temperature.

After fixation was complete, the specimens were washed in distilled water (permanganate-fixed material only) or in fresh buffer for 1 hour with 3 changes. Subsequently, fixed material was dehydrated through a graded ethanol series, treated with propylene oxide and embedded in Ciba Araldite or in Taab embedding resin as described earlier (p. 17) for duckweed tissue.

Silver-grey sections (600-700 Å thickness) were cut on an LKB "Ultratome" III using glass knives and picked up on fine mesh (200 meshes/inch) copper grids either uncoated or coated with formvar (Pease, 1964). The sections on the grids were then stained in 2% aqueous uranyl acetate (osmium tetroxide and glutaraldehyde/osmium tetroxide fixed cells only) and lead citrate (Reynolds, 1963). Observations were carried out using an AEI EM6B electron microscope which was operated at an accelerating voltage of 60 kV.

RESULTS

Ultrastructural features of normal cells of A. cylindrica

(i) The Vegetative Cell

The normal cellular organization of a vegetative cell of A. cylindrica after fixation with 2% aqueous KMnO4 is shown in Figs. 135 and 138. Beneath a four-layered cell wall lies the plasma membrane which has a definitely crinkled appearance. In the cytoplasm the most notable structures are the thylakoids which are closed, flattened sacs similar to those of chloroplasts but they are never stacked together to form grana. Nor are they separated from the rest of the cytoplasm by enclosing membranes. In vegetative cells the thylakoids occupy a more or less peripheral position. The paired membranes of the thylakoids normally lie close together although small intrathylakoidal spaces are sometimes found. The width of the thylakoids depends upon the sizes of such intrathylakoidal spaces but was normally found to be about 14 - 18 nm. In the central region of the cell scattered areas of low electron density occur which constitute the nuclear material. Despite the lack of preservation of ribosomes by KMnO4, a varied assortment of granular inclusions can be seen in the cytoplasm. The largest of these are the cyanophycin granules and the polyhedral bodies. Also, scattered throughout the cell and especially numerous around the thylakoids are small, roughly spherical electron dense granules. These structures about 25 nm in shortest diameter, have been termed polyglucoside granules and they are thought to represent photosynthetic products in light-grown cells. The appearance of a normal vegetative cell after fixation with 1% osmium tetroxide is shown in Fig. 136. The plasma membrane can just be discerned to the inside of the darkly-staining cell wall Figs. 136 and 139. The cytoplasm appears densely granular and the thylakoids are so lacking in

contrast that their position can be detected only with difficulty.

Double fixation with 3% glutaraldehyde and 1% osmium tetroxide resulted in vegetative cells which displayed an electron density lower than that resulting from osmium fixation alone (Figs. 137 and 140). Sheath material was much more distinct following double fixation but the plasma membrane was again difficult to discern. As in cells fixed with osmium tetroxide alone, the cytoplasm appeared densely granular throughout while the thylakoids were yet again difficult to distinguish. Indeed, in many areas of some cells the thylakoids could not be distinguished (Fig. 137). However, the preservation of the cyanophycin granules, which appeared as large empty areas following fixation with $KMnO_4$, was much improved using this double fixation schedule. Due, however, to the greater degree of membrane detail revealed by $KMnO_4$ fixation this method was employed throughout most of the ultrastructural work with A. cylindrica.

(ii) The Heterocyst

The heterocysts of A. cylindrica differ in many ways from vegetative cells. The light microscope reveals that heterocysts are somewhat longer and wider than vegetative cells (Figs. 133 and 134). Around the outside of the cell wall heterocysts possess a thick envelope (Fig. 141). Three envelope layers have been described (Fay and Lang, 1971). These comprise an outer, fibrous layer, a middle homogeneous layer and an inner laminated layer. The heterocyst envelope is particularly thick in the polar regions. However, at the point of connection of heterocysts with adjacent vegetative cells the outer envelope is absent (Fig. 142) and a pore channel is formed.

The heterocyst cell wall resembles that of vegetative cells as does the plasma membrane. However, in heterocysts the thylakoids assume a reticulated

formation unlike the normal arrangement in vegetative cells (Figs. 141 and 143). Also, in the region beneath the pore channels the thylakoids are contorted into a honey-comb like pattern (Fig. 143).

The numbers and types of granules visible within $KMnO_4$-fixed heterocysts varied. Cyanophycin granules were never observed within heterocysts. On the other hand, polyglucoside granules and, to a lesser extent, polyhedral bodies were often present although in reduced numbers. In addition, scattered areas of low electron density were often observed in the central region of the heterocysts but were less noticeable than in vegetative cells.

(iii) The Akinete

Akinetes of A. cylindrica, which are asexually produced spores are considerably larger than either the vegetative cells or the heterocysts (Figs. 144 and 145). Theakinetes are protected by a thick coat external to the usual four-layered cell wall. The most characteristic internal feature of the akinete cell is the presence of large numbers of cyanophycin granules (Fig. 144). Polyglucoside granules, so numerous in the cytoplasm of light-grown vegetative cells, are plentiful in some akinetes (Fig. 144) but not others (Fig. 145). It is possible that the age of the akinete is a significant factor in this regard. In all other respects akinetes closely resemble vegetative cells in their ultrastructural characteristics.

Under favourable conditions, akinetes may germinate producing new filaments by repeated cell division. Germinating akinetes were occasionally observed during ultrastructural examination of ageing colonies (Fig. 146).

Ultrastructural changes in light-treated cells of A. cylindrica

The developmental sequence of changes in fine structure was investigated

in cells of A. cylindrica growing in the light in the presence of 10 ppm diquat (salt). Studies were confined mainly to the effects of treatment on vegetative cells and unless otherwise stated, all the micrographs presented are taken from KMnO4-fixed material.

(i) Vegetative Cells

The typical appearance of a vegetative cell of control material is shown in Fig. 147. In all cells the plasma membrane had a characteristically crinkled appearance. Within the cytoplasm, polyglucoside granules were present in large numbers, especially in the region of the thylakoids. The paired membranes of the thylakoids were always closely apposed and in the central region of the cells the areas of low electron density constituting the nuclear material were easily distinguished. The number of cyanophycin granules and polyhedral bodies varied and the latter structures were generally distinct in outline.

For the first 60 min. after the application of diquat, no ultrastructural changes were observed (Fig. 148). During the following 40 min. however, there was a steady reduction in the number of polyglucoside granules present in the cytoplasm although other aspects of cell structure appeared to remain unchanged (Figs. 149 and 150). A general loss of granular material from the cytoplasm was also evident in OsO4-fixed cells prior to membrane damage (Fig. 151). In such cells the thylakoids could then be distinguished with ease (compare with Fig. 139).

After 120 min. herbicide treatment and occasionally before the complete disappearance of the polyglucoside granules, two other important changes occurred usually at about the same time - the nuclear regions became less distinct and the plasma membrane was ruptured (Fig. 152). Observations also revealed that after the plasma membrane was ruptured it lost its characteristic

small undulations. This can be seen in Fig. 152 and in greater detail in Fig. 153.

Once the disintegration of the plasma membrane was initiated, disorganization of cell ultrastructure proceeded rapidly. No effects on the cyanophycin granules were evident but, from this point onwards, the polyhedral bodies appeared less discrete their outermost edges acquiring a fuzzy outline (Figs. 152, 156, 160 and 165).

Figs. 154 and 155 show a dividing cell exhibiting more advanced breakdown of the plasma membrane. Destruction of the plasma membrane was complete in many cells after only 150 min. (Figs. 156 and 157). Complete destruction of the plasma membrane in OsO4-fixed material is shown in Figs. 158 and 159.

During the time in which the above changes were taking place, the structural integrity of the cell wall and the thylakoids appeared to be maintained. However, as the duration of the treatment was increased the paired membranes of the thylakoids tended to become less tightly apposed. Fig. 160 illustrates the typical appearance of vegetative cells which had been treated with diquat for 180 min.

After this stage two contrasting patterns of ultrastructural deterioration became apparent. In many cells the intrathylakoidal swelling continued together with the gradual loss of cytoplasmic material (Figs. 161 and 162). In other cells, however, fragmentation of the thylakoids occurred before any appreciable swelling could be observed (Fig. 163). This fragmentation continued, many of the fragments then swelling further to form rounded vesicles (Figs. 164 and 165). After complete destruction of the thylakoid system (after approximately 200 min. diquat treatment) breaks in the cellwall became apparent (Fig. 165). These breaks allowed any remaining cytoplasm to escape leaving behind only a mass of swollen and completely disorganized

thylakoids (Fig. 166).

One characteristic of many treated vegetative cells just after the onset of damage to the plasma membrane was the appearance of unusual membranous structures in the cytoplasm. These inclusions differed widely in appearance but were generally confined to the peripheral regions of the cytoplasm. Vegetative cells containing such structures are seen in Figs. 152 154 and 156. A selection of these membrane inclusions is illustrated in greater detail in Fig. 167, a - h. Despite their presence in many treated cells, these structures were never observed within the cytoplasm of control cells.

Cells in individual samples removed for ultrastructural examination throughout the treatment period did not all develop symptoms of toxicity at exactly the same rate so that some of the various stages illustrated tended to coexist to some extent. For example, Fig. 168 shows three consecutive vegetative cells after exposure to diquat for 160 min. By this time all three cells had suffered destruction of the plasma membrane. However, damage to other parts of the cell varied quite markedly from cell to cell. Nonetheless, after the analysis of very many micrographs, it can be concluded that in most vegetative cells treated with 10 ppm. diquat in the light the plasma membrane had begun to break down after 120. min. while the complete disorganization of the thylakoids and loss of the cytoplasmic matrix generally required 240-300 minutes. However, the first recognizable ultrastructural change in the treated cell was in all instances the disappearance of the polyglucoside granules.

(ii) Heterocysts

Examination of heterocysts throughout the treatment period suggested

that these structures were less susceptible to ultrastructural damage than were the more numerous vegetative cells. Fig. 169 shows a heterocyst which is apparently undamaged after exposure to diquat for 120 min. On the other hand, the adjacent vegetative cells have suffered destruction of the plasma membrane and show additional signs of ultrastructural damage. This apparent difference in sensitivity to diquat is illustrated in greater detail in Fig. 170. Fig. 171 shows a heterocyst after exposure to diquat for 160 min. The adjacent vegetative cells are severely damaged at this stage while the heterocyst has remained intact. However, this micrograph does reveal indications that some damage to the heterocyst has occurred. The plasma membrane although it appears unbroken has a less crinkled appearance than in control heterocysts. In addition, membranous inclusions are visible in the peripheral regions of the cytoplasm. Such structures were not normally found in control heterocysts but were, as already mentioned, not an uncommon feature of diquat-treated vegetative cells after rupture of the plasma membrane (see p. 169).

During the treatment period observations did occasionally reveal heterocysts which had suffered actual destruction of the plasma membrane (Figs. 172, 173 and 174). However, ultrastructural damage was first noticed in vegetative cells and, after the onset of cellular damage, vegetative cells were always more seriously affected than heterocysts.

Ultra-thin sections prepared from 'carpets' of algal material contained at best only a very small number of heterocysts. A significantly greater number of cells, including heterocysts, were obtained per section by using colonies instead of the algal 'carpets'. Examination of sections of diquat-treated colonies revealed the same general pattern of ultrastructural damage in both vegetative cells and heterocysts. However, cells in colonies

sustained structural damage more slowly and at far less uniform rates than cells in the 'carpets'.

Examination of cells from colonies exposed to 10 ppm diquat in the light for 48 hours revealed many totally disrupted heterocysts (Fig. 175). In such cells both the plasma membrane and the cytoplasmic matrix had been lost leaving a mass of twisted thulakoids and membranous vesicles. These heterocysts appeared remarkably similar to senescent heterocysts which were occasionally found in control colonies (Fig. 176).

(iii) Akinetes

Ultra-thin sections of material sampled from the young algal 'carpets' used in this study seldom revealed akinetes. The effect of exposure to diquat on the fine structure of these differentiated cells was therefore investigated using colonies. Observations showed the akinetes to be far less susceptible to ultrastructural damage than vegetative cells. Figs. 177 and 178 show cells of A. cylindrica from a colony treated with 1 ppm diquat (salt) for 48 hours. At this stage the akinetes appeared to be undamaged while nearby vegetative cells had been reduced to membranous 'skeletons'. A small portion of one of the akinetes is shown in greater detail in Fig. 178 (inset). For purposes of comparison an untreated akinete from a control colony is illustrated in Fig. 179.

The effect of diquat treatment in darkness

During the time in which cultures of A. cylindrica were undergoing treatment with diquat in the light, other cultures along with controls were similarly treated in complete darkness.

After exposure to 10 ppm diquat for 300 min. in darkness vegetative cells of A. cylindrica showed no signs of ultrastructural damage. At the end of the treatment period, control cells (Fig. 180) and cells treated with diquat (Fig. 181) appeared closely similar. However, the darkness itself did appear to have an effect on cell ultrastructure. As the micrographs show, both control and treated cells differed slightly from control cells growing in the light. The polyglucoside granules found in great numbers in the cytoplasm of light-grown cells were comparatively rare in cells after 300 min. in the dark. In addition, the slight amounts of intrathylakoidal swelling visible in Figs. 180 and 181 were characteristic of vegetative cells after this length of time in darkness. However, there was no evidence that diquat had any structural effect on vegetative cells during the treatment period.

The effect of diquat treatment in the light on dark pre-treated vegetative cells.

Vegetative cells of A. cylindrica growing in the light always contained numerous polyglucoside granules in the cytoplasm around the thylakoids (Fig. 182). By placing cultures in complete darkness for 18 hours, vegetative cells completely free of polyglucoside granules were obtained (Fig. 183). All the membranes of such dark pre-treated vegetative cells remained intact but, as Fig. 183 shows, a certain degree of intrathylakoidal swelling was generally present.

On returning cultures of dark pre-treated cells to the light, polyglucoside granules gradually reappeared within the cytoplasm. The granules first became obvious in control cells 60 min. after commencement of illumination (Fig. 184). After this time there was a rapid build-up in their numbers. Fig. 185 shows the typical appearance of control cells after 120 min. in the light. By this time the frequency of polyglucoside granules in the cytoplasm was similar to that observed in cells grown under continuous illumination.

No such reappearance of polyglucoside granules was observed over the same period in dark pre-treated cells which had been simultaneously treated with 10 ppm diquat and returned to the light. Fig 186 shows the appearance of a treated vegetative cell after 60 min. illumination in the presence of diquat. The typical appearance of treated cells after 120 min. in the light is shown in Fig. 187. Although the polyglucoside granules did not reappear in the cytoplasm of the treated cells, observations failed to reveal any other kind of ultrastructural damage during this experimental period.

Comparison of treatment with paraquat and diquat in the light

Vegetative cells of A. cylindrica exposed in the light to paraquat or diquat at concentrations of 10 ppm (cation) sustained ultrastructural damage at similar rates.

(i) Paraquat treatment

Paraquat-treated vegetative cells appeared to be unaffected by the presence of the herbicide after 60 min. (Fig. 188). At this stage all the membranes of the cell were intact and polyglucoside granules were still present in great numbers.

After 90 min. most vegetative cells revealed a considerable reduction in the number of polyglucoside granules in the cytoplasm (Fig. 189). However, in all other respects the cells appeared unaltered.

In treated cells after 120 min. polyglucoside granules were generally absent and in many cells the plasma membrane had been destroyed (Fig. 190). After rupture of the plasma membrane its characteristic small undulations were no longer present (Fig. 191). Other changes were noted in those cells which had suffered damage to the plasma membrane. Firstly, the nuclear regions were less discrete. In addition, the polyhedral bodies were no longer distinct in outline. Despite the severe damage to the plasma membrane, the structural integrity of the thylakoid system remained intact at this stage.

Following breakdown of the plasma membrane, a rapid deterioration of general cell structure was observed. In the least badly affected cells viewed after 150 min. all that remained of the plasma membrane was a large number of small fragments close to the periphery of the cytoplasm. In addition the early stages of intrathylakoidal swelling were evident (Fig. 192) However, other cells observed after 150 min. revealed more advanced destruction.

Fig. 193 is representative of the most seriously affected cells at this time. In such cells, little of the cytoplasmic matrix remained and intra-thylakoidal swelling was more marked. As Fig. 193 also shows, disintegration of the thylakoids and the subsequent rounding-off of the fragments had occurred in many cells by this time.

After exposure to paraquat for 180 min. most vegetative cells were reduced to little more than a mass of swollen, often fragmented thylakoids contained within the remains of the cell wall (Figs. 194 and 195). In almost all of the treated cells viewed at this time, the remains of the thylakoid system were still confined within the cell wall (Fig. 194). Small breaks were frequently found in the cell walls and in occasional cells these breaks were sufficiently large to allow some of the remains of the thylakoids to spill out (Fig. 195).

(ii) Diquat treatment

Although paraquat-treated vegetative cells appeared unchanged after 60 min. cells treated for 60 min. with the same concentration of diquat showed a slight reduction in the number of polyglucoside granules in the cytoplasm (Fig. 196). In all other respects, however, cell structure remained unchanged.

After exposure to diquat for 90 min. the majority of cells possessed few polyglucoside granules but showed no signs of other untrastructural damage (Fig. 197). However at this stage cells showing more advanced symptoms were occasionally observed. One such cell is shown in Fig. 198. Although no actual breaks could be observed, the plasma membrane of this more seriously affected cell had lost its crinkled appearance and was almost completely straight. In addition the electron transparent areas corresponding

to the nuclear content of the cells were no longer visible.

After 120 min. diquat-treated cells appeared similar to those treated with paraquat for the same length of time (Fig. 199). In all cells the plasma membrane had become ruptured in many places and, from this time onwards the polyhedral bodies were no longer sharply outlined.

The appearance of diquat-treated cells after 150 min. was variable as it also was among cells treated with paraquat (Figs. 200 and 201). Even in the least seriously disrupted cells the plasma membrane had become reduced to small fragments. (Fig. 200). Other cells viewed at this time had sustained more severe damage. Varying degrees of intrathylakoidal swelling followed the destruction of the plasma membrane in most cells. Fig. 201 illustrates the appearance of those cells which had undergone the greatest degenerative changes after 150 min. Here the cell wall had become fragmented and most of the cytoplasmic matrix was lost leaving behind a mass of swollen thylakoids.

After 180 min. there was less variability in the appearance of the treated cells. Although occasional cells did reveal a greater degree of destruction, most cells examined at this time were similar to that illustrated in Fig. 202. Paraquat-treated cells presented a similar appearance after 180 min., being little more than the swollen remnants of the thylakoid system contained within what was left of the cell wall.

Ultrastructural investigation of senescing vegetative cells of A. cylindrica

Ultrastructural examination of vegetative cells of A. cylindrica from colonies of different ages revealed degenerative changes. The pattern of changes observed was unlike that resulting from treatment in the light with either paraquat or diquat. The earliest changes were found in cells sampled from large colonies which were still dark blue-green in colour. Although most of the cells in such colonies appeared normal, many exhibited varying degrees of intrathylakoidal swelling (Figs. 203, 204 and 205). Despite considerable intrathylakoidal swelling in some cells, polyglucoside granules were generally visible in the cytoplasm though often in reduced numbers (Figs. 203 and 205).

After about 2 months the colonies were yellowish-green in colour. In most vegetative cells observed at this time intrathylakoidal swelling was extensive and the normal arrangement of the thylakoids was totally disrupted (Figs. 206, 207 and 208). These micrographs also reveal the accumulation of lipid granules in the cytoplasm which was characteristic of cells at this time. Despite these changes to the thylakoids, the plasma membranes of senescing vegetative cells were intact and polyglucoside granules were present in the cytoplasm. Rupture of both the plasma membrane and the cell wall was however observed in a minority of vegetative cells. Such a cell is shown in Fig. 209. In spite of the more advanced state of disruption visible in this cell, intrathylakoidal swelling was relatively slight. At the most advanced stage of senescence observed in yellow-green colonies, the vegetative cells were reduced to the swollen remains of the thylakoid system bounded in parts by the remnants of the cell walls (Fig. 210).

II(B) METHODS

Manometric Studies

Measurements of oxygen exchange in the light in A. cylindrica were made in the presence and absence of bipyridylium herbicides using a Gilson respirometer at a temperature of 25±0.5°C. Illumination from beneath was provided by a light bank giving a light intensity of 3,000 lux at flask level.

Immediately prior to all investigations, algal material was collected by centrifugation from 3 approximately 2-week old liquid cultures and re-suspended in 50 ml. Warburg buffer No. 9 (Umbreit et al., 1964). In order to determine the concentration of algae in suspension (on a mg. dry weight per ml. basis), 20 ml. was removed and the algae separated by centrifugation. The supernatant was discarded and the algae re-suspended in distilled water. After stirring briefly with a glass rod, the algal material was re-separated by further centrifugation and finally dried to constant weight in an oven at 100°C.

Oxygen exchange measurements were carried out using manometric flasks with single side-arms. In all experiments, the main compartments of the flasks received exactly 2 ml. algae suspended in Warburg buffer No. 9. 0.5 ml. of paraquat or diquat solutions were added to the side-arms of test flasks such that, upon mixing, the desired final concentrations were achieved. The side-arms of control flasks received the same volume of distilled water. In addition, control flasks double-wrapped in aluminium foil and enclosed in black polythene bags were utilized for the determination of control dark respiration.

Following a 20 minute equilibration period, oxygen exchange measurements

were made at 10 minute intervals for 140–180 minutes depending upon the experiment concerned. The contents of the side-arms were tipped into the main compartments of the flasks 20–30 minutes after the end of the equilibration period.

RESULTS

Analysis of variance was carried out on the data collected in the following experiments according to Snedecor (1967). The differences between means were tested by Duncan's multiple range analysis ('D' test) (Duncan, 1955).

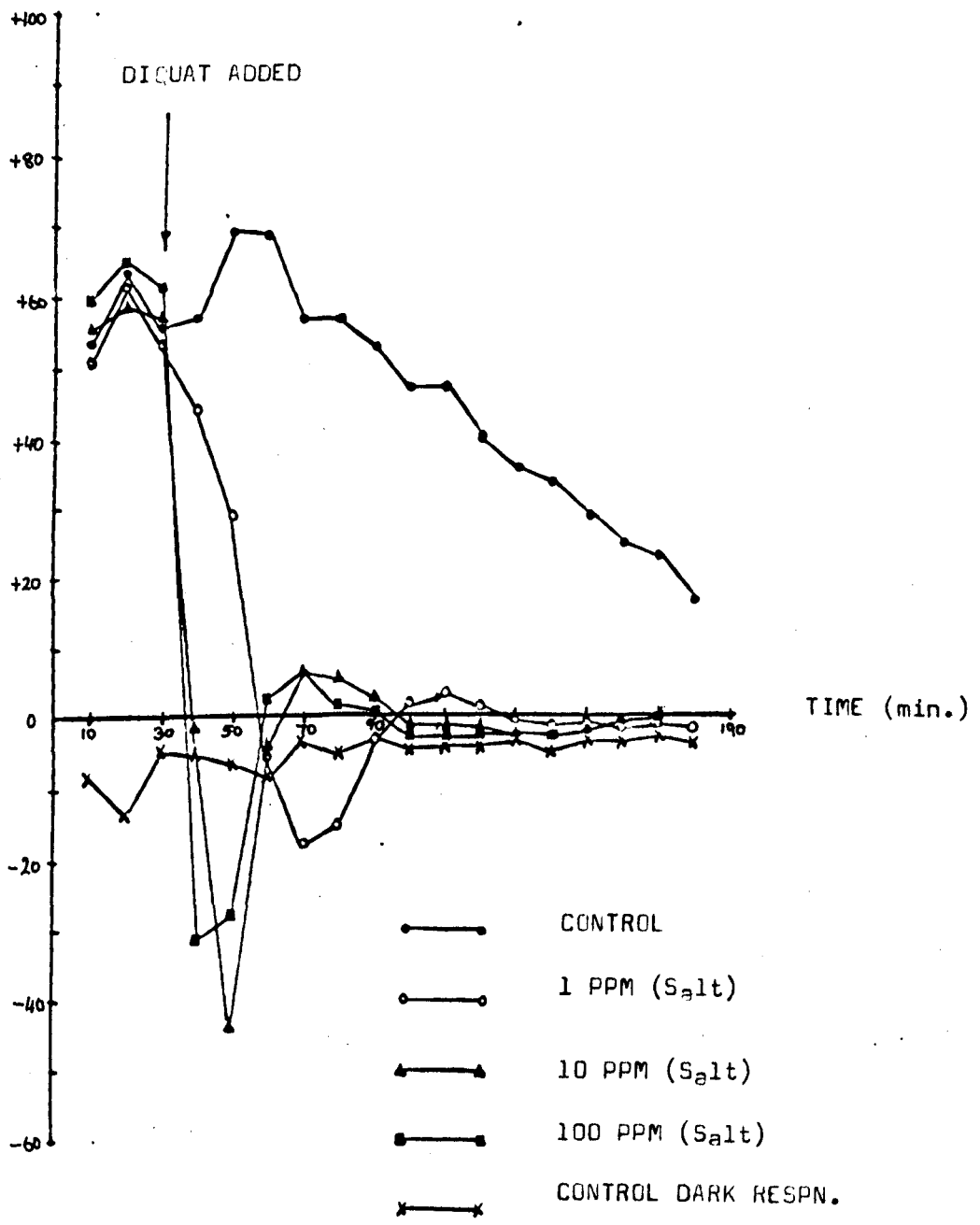
(i) The effect of diquat on oxygen exchange in the light

Treatment of A. cylindrica with diquat at concentrations of 1.0 - 100 ppm (Salt) had a rapid effect on oxygen exchange in the light (Pl. 50 and 51). The rate of oxygen output in treated samples dropped considerably soon after the addition of herbicide, the rate of fall being a positive function of diquat concentration. In typical trials, apparent photosynthesis was reduced to zero within 10 minutes of the addition of diquat at concentrations of 10.0 and 100.0 ppm. complete inhibition being reached a little sooner at the higher concentration. Complete inhibition of apparent photosynthesis in the presence of 1.0 ppm diquat required a longer period, usually ranging between 20 and 30 minutes.

For a brief period after the complete inhibition of apparent photosynthesis, treated cells absorbed oxygen at a high rate. In samples treated with 10.0 and 100.0 ppm. diquat, oxygen consumption reached a peak within 10 minutes of complete inhibition, 10 - 20 minutes being required in the presence of diquat at a concentration of 1.0 ppm. The peak of oxygen uptake was smallest in cells treated with the lowest concentration of diquat. Although the most rapid rate of oxygen uptake was measured in cells exposed to 10.0 ppm. diquat, the results suggest that an even greater rate was reached earlier by cells treated with 100.0 ppm. diquat. It would seem probable that such a peak occurred between measurements taken 10 and 20 minutes after the addition of herbicides (Pl. 50 and 51).

After oxygen uptake had reached its peak in treated cells, the rate

Plate 50 The effects of a range of concentrations of diquat on oxygen exchange in Anabaena cylindrica in the light



All values represent the mean of 2 replicates.

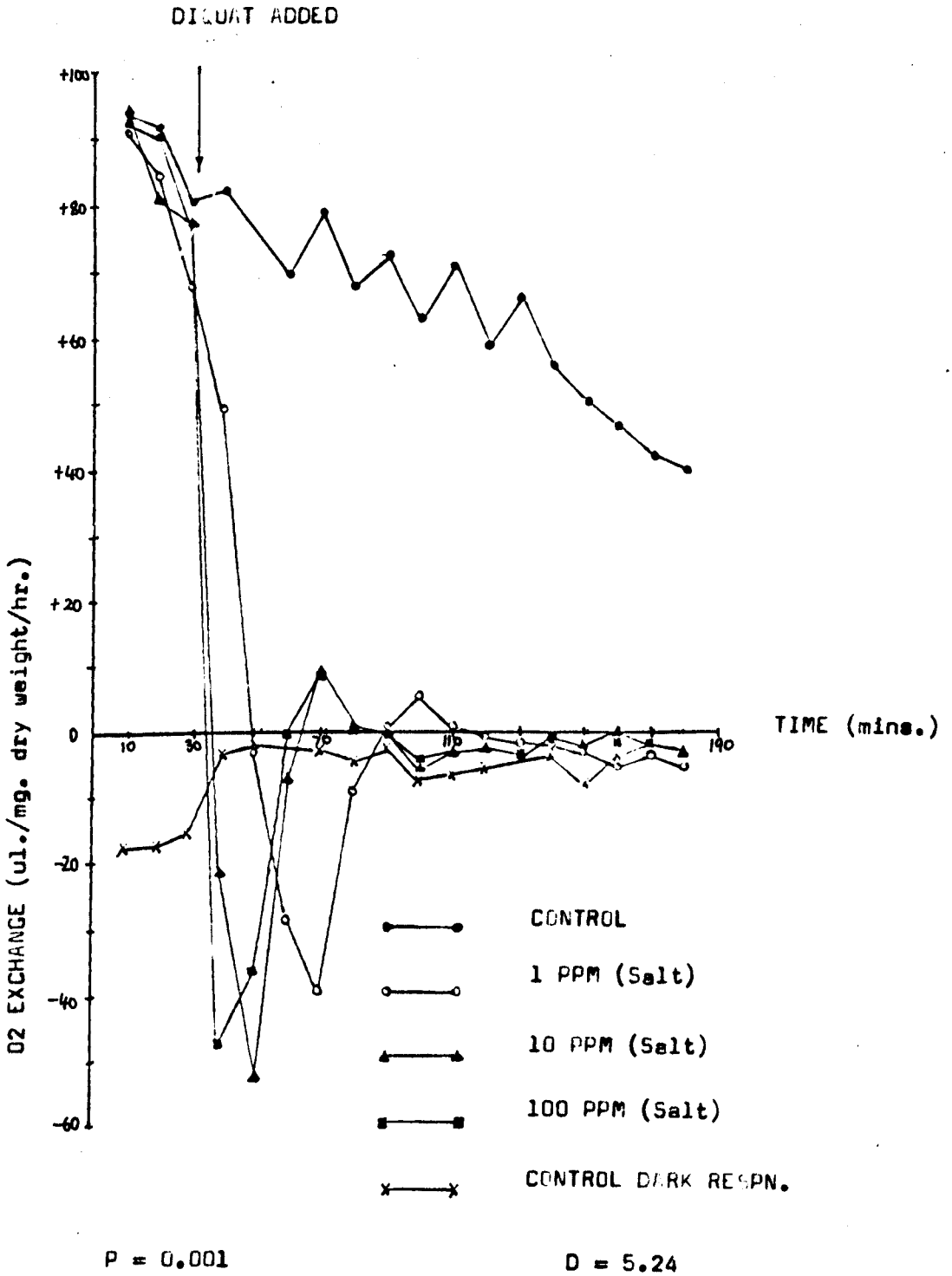
dropped quickly and was succeeded at all concentrations by a short period of oxygen output. During this period, which had a duration of 20 - 30 minutes depending upon the trial, oxygen output rose steadily reached a peak and then dropped steadily to zero. At all concentrations the magnitude of oxygen output at the peak was similar to the rate of control dark respiration.

Following the brief period of oxygen output, the cells of A. cylindrica absorbed oxygen at a slow rate until the end of the experimental period. At all concentrations, the rate oxygen consumption during this final period was similar and generally slightly below that of control dark respiration.

(ii) Comparison of the effects of paraquat and diquat on oxygen exchange in the light

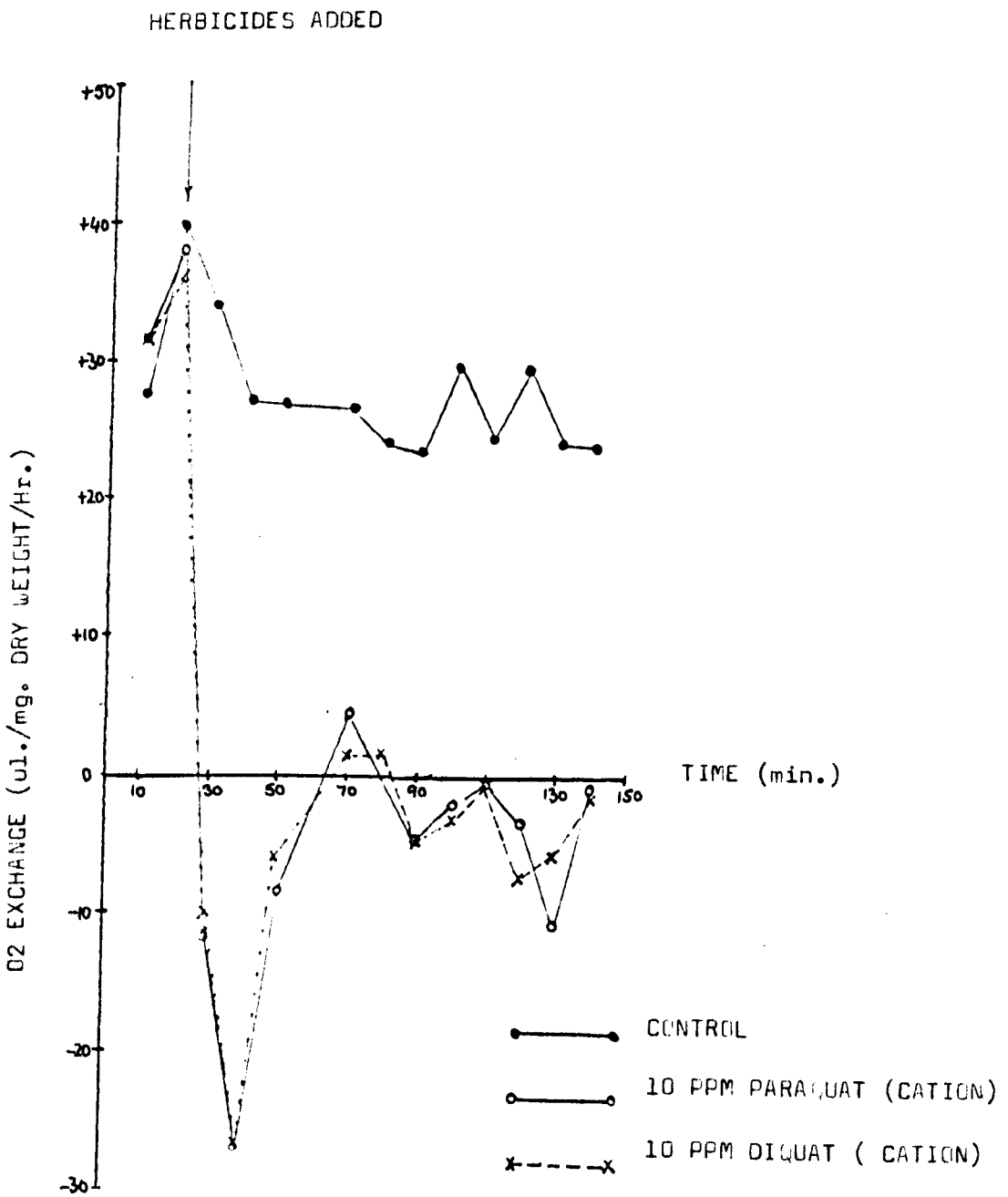
The effects of both herbicides on oxygen exchange in A. cylindrica in the light at 10.0 and 1.0 ppm. (cation) are illustrated in Pl. 52 and 53 respectively. At both concentrations, paraquat-treated cells showed the same pattern of osygen exchange exhibited by cells treated with diquat. Oxygen output dropped sharply and was succeeded by a period of increasing and then decreasing oxygen uptake leading to a transient period during which time there was a low oxygen output. The rate of oxygen exchange subsequently returned to below zero and there followed a period of low, variable oxygen uptake. Analysis of the data obtained indicated that at neither concentration was there any significant difference between the effects of paraquat and diquat on oxygen exchange in the light.

Plate 51 The effect of a range of concentrations of diquat on oxygen exchange in Anabaena cylindrica in the light



All values represent the mean of 2 replicates

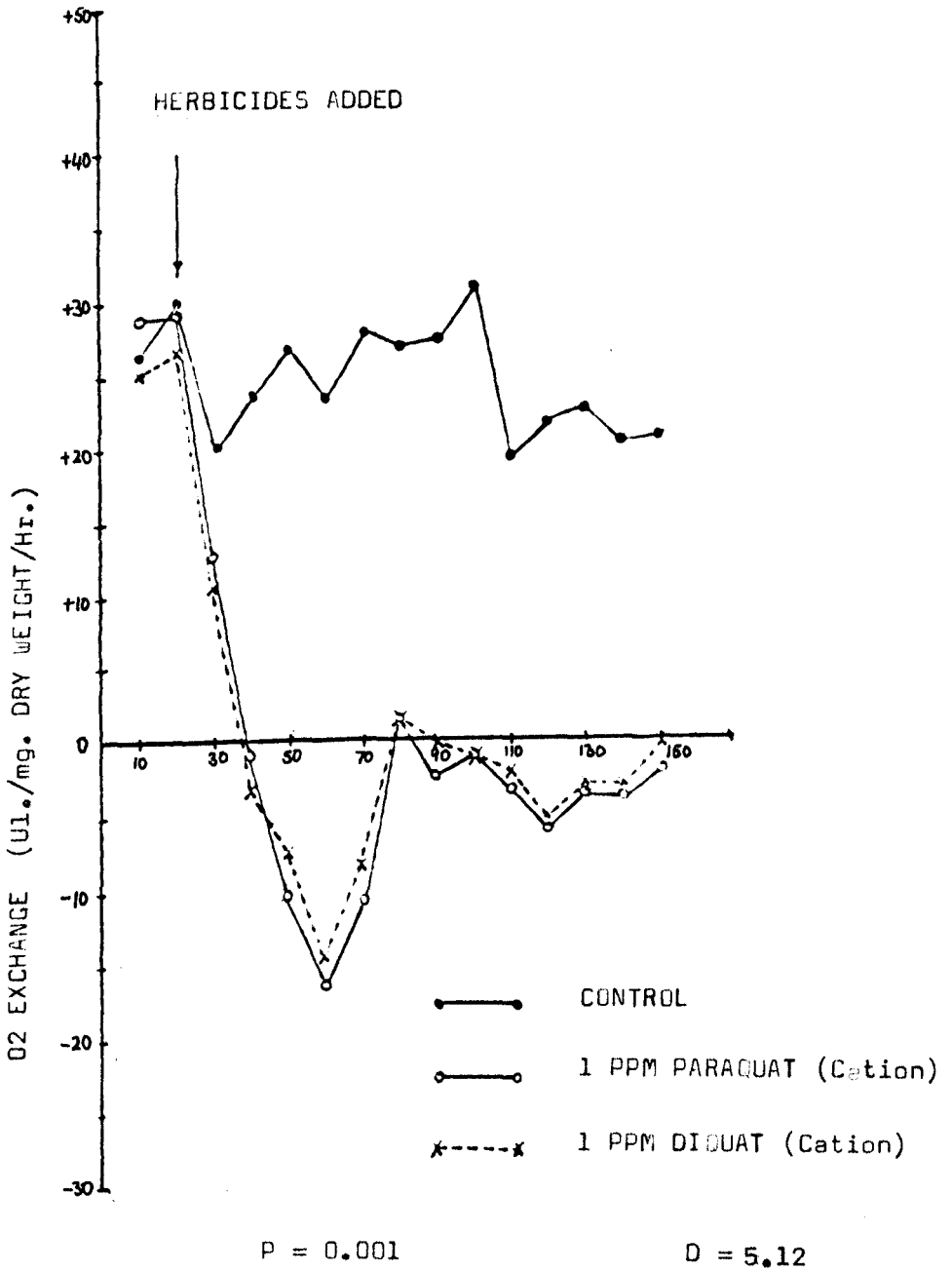
Plate 52 A comparison of the effects of paraquat and diquat on oxygen exchange in Anabaena cylindrica in the light



P = 0.001 D = 6.02

All values representthe mean of 3 replicates.

Plate 53. A comparison of the effects of paraquat and diquat on oxygen exchange in Anabaena cylindrica in the light



All values represent the average of 3 replicates.

DISCUSSION

Published accounts of ultrastructural changes in plants treated with bipyridylium herbicides are few (Baur *et al.*, 1969; Stokes *et al.*, 1970; Dodge, 1971; Harris & Dodge, 1972; Dodge & Lawes, 1974). As described earlier, all reports indicated rapid and profound changes in the cell structure of plants treated in the light. However, all of the above investigations involved eukaryotic plants. In the present study, treatment in the light with low concentrations of bipyridylium herbicides has been shown to cause rapid disruption of the prokaryotic cellular organization of vegetative cells of the blue-green alga *Anabaena cylindrica*.

The first observed ultrastructural damage to vegetative cells of *A. cylindrica* treated in the light with either paraquat or diquat was the rupture of the plasma membrane and the apparent disappearance of the nuclear regions. $KMnO_4$ fixation was employed throughout most of the ultrastructural work with this alga. Since this method of fixation destroys nucleic acids (Juniper *et al.*, 1970) the nuclear regions appear in the electron microscope merely as electron-transparent areas. Thus, it is unclear whether the nuclear material contained within the vegetative cells was actually destroyed or merely dispersed thinly throughout the cytoplasm. It is also uncertain whether the apparent disappearance of the nuclear regions was an indirect result of the damage to the plasma membrane or, alternatively, a direct result of herbicide treatment. In any event, observations indicated that the nuclear regions were always visible in vegetative cells prior to rupture of the plasma membrane.

Support for the view that the plasma membrane is the site of primary ultrastructural damage comes from the work of Baur *et al.* (1969). In their study of the ultrastructural changes in mesophyll cells of mesquite

(Prosopis juliflora) following treatment with paraquat, these workers observed extremely rapid rupture of the plasma membrane prior to damage to any other cellular components. Interestingly no damage to nuclei was observed even in severely affected cells. On the other hand, Stokes et al., 1970 found that the disappearance of the nucleus was one of the first signs of ultrastructural damage in cells of Chlorella vulgaris following treatment with diquat in the light. Unfortunately, these authors made no mention of the effect of herbicide treatment on the plasma membrane and examination of their micrographs fails to elucidate this point. Harris & Dodge (1972) presented evidence that in flax cotyedons leaf cells the tonoplast was the primary site of ultrastructural damage after treatment in the light with a low concentration of paraquat (10^{-4}M). Damage to the plasma membrane was observed but not until after rupture of the tonoplast. These observations are not necessarily in conflict with the possibility that the plasma membrane is the primary site of ultrastructural damage in the vegetative cells of A. cylindrica since blue-green algal cells do not possess a tonoplast. Dodge & Lawes (1974) working with flax cotyledon leaves and the present author working with fronds of S. oligorrhiza have shown that, following treatment with diquat in the light, the first sign of damage was a swelling of the chloroplasts.

The present investigation revealed that destruction of the plasma membrane of vegetative cells was followed by the general destruction of cell structure. Such results are not surprising since destruction of the plasma membrane inevitably leads to cell death. The odd membranous includions which were observed in many cells subsequent to rupture of the plasma membrane (Fig. 167 a - h) bear a resemblance to the mesosomes observed by Allen (1972) in the vegetative cells of two species of blue-green algae. However, since this author observed these inclusions only in treated cells and since they were

often found to lie close to breaks in the plasma membrane, it is considered that they do not have an active function but simply represent portions of the plasma membrane which have become contorted following herbicide-induced damage. The rapid onset of damage in A. cylindrica is consistant with the view that bipyridylium herbicides undergo alternate reduction and re-oxidation within plant cells with the simultaneous production of hydrogen peroxide. This is an extremely toxic substance which is capable of causing great damage to a biological system. It is worth noting at this point that blue-green algal cells are not noted for their catalase activity. Patterson & Myers (1968) sought catalase activity in 5 species of blue-green algae and found it only in Anacystis nidulans. (The other species investigated were not named). Honeycutt and Krogman (1970) also failed to find any detectable catalase activity in Anabaena variabilis.

If destruction of the plasma membrane was in fact caused by the action of hydrogen peroxide, the question remains whether the subsequently observed changes were also caused by continued production of this toxic substance or by some other factor such as osmotic shock. Perhaps indeed more than one cause is involved. This would help to explain why the breakdown of thylakoid structure appeared to follow different courses in different vegetative cells. Severe intrathylakoidal swelling without fragmentation (Fig. 162) may have been due largely to osmotic effects following the loss of integrity of the plasma membrane. On the other hand, fragmentation of the thylakoids (Figs. 164 & 165) may have been mainly the result of attack by hydrogen peroxide.

Most of the available evidence suggests that in plants photosynthesis is the most important source of reducing power for the bipyridylium herbicides (Calderbank, 1968). These herbicides are believed to interfere with photosystem I of photosynthesis by shunting electrons away from ferredoxin

and undergoing rapid reoxidation in the presence of molecular oxygen with the simultaneous production of hydrogen peroxide, or possibly free radicals derived from this. Support for the implication of photosynthesis as the primary source of reducing power in the light comes from the ultrastructural examination of vegetative cells of A. cylindrica after their exposure to diquat in complete darkness. While most vegetative cells had undergone complete destruction after exposure to diquat for 300 minutes in the light, no ultrastructural damage was visible after treatment for the same period in darkness. This finding is in keeping with the retarded deterioration of fine structure observed in both paraquat - and diquat - treated fronds of Spirodela oligorrhiza in darkness.

Further evidence suggesting the importance of photosynthesis in the mode of action of bipyridylium herbicides is obtained from observations on the development of ultrastructural damage in heterocysts after treatment with diquat in the light. There is evidence that heterocysts of blue-green algae have a functional photosystem I (Donze et al., 1972; Scott and Fay, 1972) and therefore a possible source of reductant for the bipyridylium herbicides. However many studies have pointed to the absence of an oxygen-evolving photosystem II within these cells. This conclusion is based largely on the almost total absence of the accessory pigment phycocyanin (Fay, 1969 b; Walk and Simon, 1969; Thomas, 1970). In addition, Thomas (1970) demonstrated the absence in vivo of all of the other major pigments of photosystem II in Anabaena sp. L-31. Furthermore, Bradley and Carr (1971) failed to demonstrate oxygen evolution by isolated heterocysts of A. cylindrica upon illumination and Donze et al., (1972) obtained no Hill reaction with extracts from isolated heterocysts of the same species. The absence of the oxygen-evolving photosystem II would result in conditions of low oxygen tension

within heterocysts. In addition, by the use of nitro-blue tetrazolium chloride, Fay and Kulasooriya (1972) have obtained evidence which suggests that adjacent vegetative cells play a part in promoting reducing conditions within the heterocysts of A. cylindrica.

The existence of a low oxygen tension within heterocysts would be unfavourable for hydrogen peroxide production. If the herbicidal properties of bipyridylium herbicides are associated with the generation of molecules of hydrogen peroxide concomitant with the reoxidation of the bipyridylium free radicals one would not expect heterocysts to sustain ultrastructural damage as quickly as vegetative cells which do possess an oxygen-evolving photosystem. Indeed, present observations (Figs. 169 - 171) do indicate retarded injury to the fine structure of heterocysts compared with the vegetative cells.

Nonetheless, damage to heterocysts was often noted within the 300 minutes in the light in the presence of 10 ppm diquat (salt) (Figs. 172 - 174). Such damage, although less marked than in vegetative cells, was still fairly rapid and severe. The finding that heterocysts sustained ultrastructural damage, albeit at a slower rate than did vegetative cells, is not necessarily incompatible with reduction of the herbicide followed by reoxidation and hydrogen peroxide production. Oxygen is unlikely to be completely absent within heterocysts. Bradley and Carr (1971) have demonstrated oxygen uptake by heterocysts of A. cylindrica isolated by lysozyme treatment. Thus, some oxygen may still be available for re-oxidation of bipyridylium free radicals. Considering the extrme toxicity of hydrogen peroxide and the general absence of catalase in blue-green algae (Patterson & Myers, 1968; Honeycut & Krogman, 1970), it is likely that even very low levels of hydrogen peroxide generation may be sufficient to cause considerable ultrastructural damage. It is therefore

not inconceivable that hydrogen peroxide may still be produced even under conditions of low oxygen tension.

There is evidence that reducing conditions within heterocysts are promoted by the activities of adjacent vegetative cells (Fay & Kulasooriya, 1972). The early destruction of these vegetative cells by herbicide treatment in the light would be expected to remove their contribution towards maintaining a low oxygen tension within the heterocysts and thus perhaps make the re-oxidation of the herbicide free radicals and hydrogen peroxide production more likely.

Damage to heterocysts need not necessarily imply direct interference with the metabolism of these cells. The plasma membranes of heterocysts are in direct contact with those of adjoining vegetative cells by means of structures termed microplasmodesmata (Wildon & Mercer, 1963 a; Lang & Fay, 1971). It is believed that metabolic products may pass into heterocysts from adjoining vegetative cells via these connections (Fogg, 1951; Wolk, 1968; Fay et al., 1968; Stewart et al., 1969). It is therefore possible that hydrogen peroxide, generated within vegetative cells, may also gain entry into heterocysts via the microplasmodesinata and subsequently cause ultrastructural damage to these cells. In addition, Fay & Lang (1971) have shown that damage to the plasma membranes of vegetative cells by physical means usually results in damage to the plasma membranes of adjoining heterocysts. Thus, herbicide-induced rupture of the plasma membranes of vegetative cells may be expected to result in damage to the plasma membranes of heterocysts with which they are continuous.

Fay (1969 a) has shown that isolated akinetes possess a much lower level of photosynthetic activity than vegetative cells. Thus, the failure to detect ultrastructural damage in akinetes at a time when vegetative cells had

been totally destroyed (Figs. 177 - 179) is also consistent with the involvement of photosynthesis in the mode of action of bipyridylium herbicides.

The possibility remains, however, that the relative resistance of heterocysts and akinetes to ultrastructural damage in the light may be due to slower penetration of the herbicides through their greatly thickened cell envelopes. This may be particularly important in the case of akinetes which are completely surrounded by envelope material. If the penetration of bipyridylium herbicides into heterocysts and akinetes is significantly restricted, the responses of these cell types to herbicide treatment can no longer be regarded as providing evidence in favour of the generally accepted mode of action theory. However, both paraquat and diquat are extremely soluble in water (Calderbank, 1968) and it is felt unlikely that their rates of penetration would be reduced sufficiently to cause the observed delay in the onset of cellular damage, particularly in the akinetes.

Observations of vegetative cells of A. cylindrica exposed to paraquat or diquat in the light revealed the disappearance of polyglucoside granules from the cytoplasm prior to any visible ultrastructural damage (Figs. 148 - 150). This finding can be explained by assuming that these granules, which have been shown to represent a glycogen-type storage product of photosynthesis (Chao & Bowen, 1971), are consumed by a respiratory process without being replaced by carbon fixation. The disappearance of the polyglucoside granules can therefore be regarded as analagous to the apparent reduction in the amount of starch in the chloroplasts of S. oligorrhiza prior to any visible ultrastructural damage. However, the amount of starch normally observed in the chloroplasts of S. oligorrhiza was somewhat variable making it difficult to draw firm conclusions about the effect of treatment on starch content from this work alone. The small size of the polyglucoside granules (23 - 30 nm

in diameter) and the speed with which they are synthesised (Figs. 183 - 185) makes them, however, more sensitive indicators of carbon fixation than the starch grains of eukaryotic green plants.

Stokes et al. (1970) showed that unlike control cells, diquat-treated cells of Chlorella vulgaris did not accumulate starch during the first hour of illumination. During this time no treated cells revealed ultrastructural damage. Subsequent to this there was a progressive loss of starch from the chloroplasts of treated cells. However, this loss was accompanied by changes in the fine structure of the cells.

Baur et al., (1969) reported that mesophyll cells of light pre-treated mesquite plants contained fewer starch deposits than control tissue following treatment with paraquat in the light. In addition they failed to detect the reappearance of starch grains in the chloroplasts of dark pre-treated plants following the same treatment. However, according to these workers, disintegration of the plasma membrane was visible in treated tissue after only 5 minutes and disruption of the chloroplast envelope was noted after 40 minutes. The possibility that the effects of paraquat on the starch content of chloroplasts was the indirect result of damage to important cellular membranes cannot be ruled out.

The failure of dark pre-treated vegetative cells of A. cylindrica to re-synthesize polyglucoside granules following illumination in the presence of diquat adds further support to a possible interference with carbon fixation by bipyridulium herbicides. The fact that the non-reappearance of these granules was not accompanied by any visible ultrastructural damage during the period in which their formation was occurring in control cells points to a direct interference with carbon fixation rather than an indirect result of damage to the photosynthetic apparatus or other cellular components.

Inhibition of carbon fixation is consistent with the proposed interference of bipyridylium herbicides with photosynthetic electron flow and the resulting inhibition of NADP reduction. Such wastage of reducing power (Davenport, 1963; Zweig et al., 1965; Black, 1966) is unlikely to result in the type of rapid damage which was observed in treated vegetative cells but is consistent with the ultrastructural evidence pointing to inhibition of carbon fixation. Manometric studies with A. cylindrica revealed a rapid inhibition of apparent photosynthesis in the light in the presence of both paraquat and diquat (Pl. 50 - 53). Complete inhibition of apparent photosynthesis was followed by a period of net oxygen uptake which reached an early peak and then dropped to a low level. A similar pattern of oxygen exchange was obtained by Turner et al., (1970) with Chlorella vulgaris exposed to diquat in the light. The effect of treatment on real photosynthesis is, of course, masked by the oxygen consuming processes which occur simultaneously. Several processes could contribute towards oxygen uptake in the light. The reoxidation of reduced bipyridylium salts could be involved as could the photooxidation of photosynthetic pigments. Also, a stimulation of respiration in the light may be involved. As described earlier, both paraquat and diquat caused a significant stimulation of oxygen uptake in the dark by fronds of S. oligorrhiza. Turner et al. (1970) showed manometrically that at least part of of the enhanced oxygen uptake in light-treated cells of Chlorella vulgaris was paralleled by an increase in carbon dioxide output.

The rapidly observed inhibition of apparent photosynthesis need not necessarily imply damage to cells. The effect may simply be caused by paraquat or diquat shunting electrons away from the normal photosynthetic electron chain. However, Turner et al. (1970) presented evidence that the inhibition was irreversible in diquat-treated cells of Chlorella vulgaris indicating that

more than a simple short-circuiting of the normal electron flow was at work. If hydrogen peroxide or an intermediate radical is in fact formed as a result of the interference by bipyridylium herbicides with photosynthetic electron flow and is the primary cause of the ultrastructural damage sustained by cells of A. cylindrica, one might perhaps expect that the thylakoids would have sustained the initial damage. In addition to being the sites of the light reactions of photosynthesis, the thylakoids also appear to be the sites of respiration (Bisalputra et al., 1969).

Thus, the thylakoids would seem to be the most likely sites of production of any hydrogen peroxide or toxic radicals resulting from the interference of these herbicides with respiratory electron flow. However, ultrastructural damage to the thylakoids of vegetative cells of A. cylindrica did not become apparent until after destruction of the plasma membrane. However, owing to the close proximity of the plasma membrane to the thylakoids and the absence of any bounding membrane separating the two, it is likely that hydrogen peroxide, if formed, will reach the plasma membrane very rapidly. Dodge (1971) and Harris & Dodge (1972)(b) argue that free radicals derived from hydrogen peroxide attack the lipids of the cellular membranes and initiate a chain reaction leading to their gradual disintegration. The membranes of the thylakoids may receive a degree of protection by virtue of being associated with the photosynthetic pigments and other complex molecules. It is conceivable, therefore, that the plasma membrane, having a less complicated structure, is more vulnerable to damage and consequently ruptures earlier.

During the work conducted with A. cylindrica, paraquat and diquat were shown to affect cell ultrastructure in the same way and at a similar rate. The apparently equal toxcity of these herbicides to A. cylindrica

was also evident in oxygen exchange studies carried out in the light (Pl. 52 and 53). These findings are in direct contrast to the results of ultrastructural and oxygen exchange studies conducted with the duckweed S. oligorrhiza in which diquat was always the more toxic herbicide (except after prolonged exposure in darkness when both herbicides caused similar amounts of damage). One would expect diquat to be more toxic than paraquat due to its lower redox potential (diquat, -349 mV and paraquat -446mV). In other respects, however, the observed effects of treatment of A. cylindrica with paraquat and diquat are in agreement with the results obtained with S. oligorrhiza and consistent with the existing theory accounting for their mode of action. In both test organisms treated with paraquat or diquat, the development of ultrastructural damage was retarded by darkness. Also, in both organisms there was evidence of interference with carbon fixation prior to visible cellular damage. The patterns of oxygen exchange in the light following treatment with paraquat or diquat were also similar.

The initiation of ultrastructural damage and the subsequent deterioration of fine structure occurred, however, in bipyridylium-treated vegetative cells of A. cylindrica at a much greater rate than in similarly treated fronds of S. oligorrhiza. This difference may be related to a more ready uptake of herbicide by the smaller blue-green algal cells. Certainly the herbicides must travel greater distances before gaining entry into the duckweed cells. However, the greater sensitivity of A. cylindrica may, in part, be a consequence of its prokaryotic cellular organization. The absence of structural compartmentalization within its cells should facilitate both the movement of the herbicides to their active sites and the movement of hydrogen peroxide

or subsequently derived toxic radicals from their sites of production to all parts of the cells.

BIBLIOGRAPHY

AKHAVEIN, A.A., LINSCOTT, D.L. (1968). The dipyridylium herbicides, paraquet and diquat. Residue Rev. 23, 97 - 145

ALLEN, M.B. & ARNON, D.I. (1955). Studies on nitrogen-fixing blue-green algae. I Growth and nitrogen fixation by Anabaena cylindrica Lemn. Pl. Physid., Lancaster, 30, 366 - 372

ALLEN, M.M. (1972). Mesosomes in blue-green algae. Arch. Mikrobid 84, 199 - 206

BALDWIN, B.C., DODGE, A.D., HARRIS, N. (1968). Recent advances in studies of the mode of action of the bipyridylium herbicides. Proc. 9th. Br. Weed Control Conf. 639 - 644

BARNES, D.L., and LYND, J.Q. (1967). Factors in paraquat-induced chlorosis with Phescolus foliar tissues. Agron. J. Sq, 364-366

BAUR, J.R., BOVEY, R.W., BAUR, P.S. and EL SEIFY, Z. (1969) Effects of paraquat on the ultrastructure of mesquite mesophyll cells. Weed Res., 9, 81 - 85

BIGGINS, J. (1969). Respiration in blue-green algae. J. Bact. 99, 570 - 575

BISALPUTRA, T., BROWN, D.L. and WEIER, T.E. (1969). Possible respiratory sites in a blue-green alga Nostoc sphaericum as demonstrated by potassium tellurite and tetranitro-blue tetrazolium reduction. J. ultastruct. Res. 27, 182 - 197

BLACK, C.C. (1966). Chloroplast reactions with dipridyl salts. Biochim. biophys. Acta, 120, 332 - 340

BLACK, C.C. and MYERS, L. (1966). Some biochemical aspects of the mechanisms of herbicidal activity. Weeds, 14, 331 - 338.

BLACKBURN, R.D. and WELDON, L.W. (1965). The sensitivity of duckweeds (Lemnacaea) and Azolla to diquat and paraquat. Weeds, 13, 147 - 149

BLACKMAN, G.E., & ROBERTSON-CUMMINGHAME, R.C. (1955). Interrelationship between light intensity, temperature, and the physiological effects of 2:4 - dichlorophenoxyacetic acid on the growth of Lemna minor. Jour. Exp. Bat. 6, 156 - 176

BRADLEY, S. and CARR, N.G. (1971). The absence of a functional photosystem II in heterocysts of Anabaena cylindrica. J. gen. Microbiol. 68, xiii - xiv.

BRIAN, R.C. (1967). The uptake and absorption of diquat and paraquat by tomato, sugar beet, and cocksfoot. Ann. appl. Biol. 59, 91 - 99.

BUON, W.R. (1964) The chemistry and mode of action of the bipyridylium herbicide diquat and paraquat Outlook Agr. 4, 163 - 70

BREAZEALE, F.W. & CAMPER, N.D. (1972) Effect of selected herbicides on bacterial growth rates. Appl. Microbid. 23, 431 - 432

BRIAN, R.C., HOMER, R.F., STUBBS, J. & JONES, R.L. (1968). A new herbicide. 1:1' - ethylene - 2:2' - dipyridylium dibromide. Nature (London), 181, 446 - 447

CALDERBANK, A. (1968) The bibyridylium herbicides. Advanc. Pest Control. Res. 8, 127 - 235.

CARR, N.G. & WHITTON, B.A. (1973). The Biology of Blue-Green Algae. Blackwells, Oxford.

CHAO, L. and BOWEN, C.C. (1971) Purification and properties of glycogen isolated from a blue-green alga, Nostoc muscorum. J. Bact. 105, 331 - 338.

CLARK, R.L. and JENSEN, T.E. (1969). Ultrastructure of akinete development in a blue-green alga, Clyndrospermum sp. Cytologia 34, 439 - 448.

COATS, G.E., FUNDERBURK, H.H. Jr., LAWRENCE, J.M. and DAVIS, D.E., (1964). Persistence of diquat and paraquat in pools and ponds. Proc. S. Weed Conf. 17, 308 - 320

COUCH, R.W., and DAVIS, D.E. (1966). Effect of atrazine, bromacil, and diquat on $C^{14}O_2$ - fixation in com. cotton and soybeans. Weeds 14, 251 - 255.

DAINTY, J. (1968). In: Plant cell organelles, ed. J.B. Pridham. London-New York: Academic Press.

DAMANAKIS, M. (1970). A bioassay for the determination of low concentrations of paraquat. Weed Res. 10, 77 - 80

DAMANAKIS, M., DRENNAN, D.S.H., FRYER, J.D. & HOLLY, K. (1970) The toxicity of paraquat to a range of species following uptake by the roots. Weed Res. 10, 278 - 283

DAVENPORT, H.E. (1963). The mechanism of cyclic phosphorylation by illuminated chloroplasts. Proc. Royal Soc. London, Ser. B. 157 332 - 345.

DAVIES, P.S., and SEAMAN, D.E. (1964). Physiological effects of diquat on submersed aquatic weeds. Abstr. Weed Soc. Amer., P 100.

DAVIES, P.J. and SEAMAN, D.E. (1968). The tetrazolium reaction as a measure of the action of diquat in Elodea. Weeds, 16 329 - 331

DE KOCK, P.C. & INNES, A.M. (1970). The effects of amitrode on duckweed. Can. J. Bot. 48: 1285 - 1288

DODGE, A.D., HARRIS, N., BALDWIN, B.C. (1970). The mode of action of paraquat and diquat. Biochem. J. 118, 43 - 44P

DODGE, A.D. (1971). The mode of action of the bipyridylium herbicides paraquat and diquat. Endeavour 30, 130 - 135.

DODGE, J.D. & LAWES, G.B. (1974). Some effects of the herbicides diquat and morfamquat on the fine structure of leaf cells.
Weed Res., 14, 45 - 49

DONZE, M. HAVEMAN, J. & SCHIERECK, P. (1972). Absence of photosystem II in heterocysts of the clue-green alga Anabaena. Biochim. biophys. Acta 256, 157 - 161

DRAWERT, H. and TISCHER, I. (1956) Über Redox-Vorgänge bei Cyanophyceen Unter besonderer Berücksichtigung der Heterocysten.
Naturwissenschaften 43, 132

DUNCAN, D.B. (1955) Multiple range and multiple F tests
Biometries, 11, 1 - 42

FAY, P. (1969a) Metabolic activities of isolated spores of Anabaena cylindrica J. exp. Bot. 20, 100 - 109

FAY, P. (1969b). Cell differentiation and pigment composition in Anabaena cylindrica. Arch. Mikrobiol. 67, 62 - 70

FAY, P. (1970). Photostimulation of nitrogen fixation in Anabaena Cylindrica Biochim. biophys. Acta. 216, 353 - 356

FAY, P., STEWART, W.D.P., WALSBY, A.E. and Fogg., G.E. (1968). Is the heterocyst the site of nitrogen fixation in blue-green algae?
Nature, (Lond.) 220, 810 - 812

FAY, P. & LANG, N.J. (1971). The heterocysts of blue-green algae. Ultrastructural integrity after isolation. Proc. R. Soc. B. 178, 185 - 192.

FAY, P. and KULASOORIYA, S.A. (1972) Tetrazolium reduction and nitro - genase activity in heterocystous blue-green algae. Arch. Mikrobid. 87, 341 - 352.

FOGG, G.E. (1944). Growth and heterocyst production in Anabaena cylindrica Lemn. New Phytol. 43, 164 - 175.

FOGG, G.E. (1949). Growth and heterocyst production in Anabaena cylindrica Lemm. II. In relation to carbon and nitrogen metabolism. Ann. Bot. 13, 241 - 259.

FOGG, G.E., STEWART, W.D.P., FAY, P. & WALSBY, A.E. (1973). The Blue- Green Algae. Academic Press, London, England.

FREDERICK, S.E., and NEWCOMB, E.H., (1969). Cytochemical localization of catalase in Leaf Microbodies (peroxisomes). J. Cell. Biol. 43, 343 - 353

FREDERICK, S.E. and NEWCOMB, E.H. (1971) Ultrastructure and distribution of microbodies in leav s of grasses with and without CO_2-photorespiration. Planta 96; 152 - 174

FUNDERBURK, H.H. and LAWRENCE, J.M. (1963). A sensitive method for determination of low concentrations of diquat and paraquat. Nature (Lond.) 199, 1011 - 1012

FUNDERBURK, H.H., Jr., and LAWRENCE, J.H. (1964). Mode of action and Metabolism of diquat and paraquat. Weeds 12, 259 - 264

GAGE, J.C. (1968). The action of paraquat and diquat on the respiration of liver cell fractions. Biochem. J. 109, 757 - 761

GAHAN, P.B. (1968). In: Plant cell organelles, ed. J.B. Pridham, London - New York: Academic Press

GAGNON, C. (1968). Peroxidase in healthy and diseased trees investigated by the benzidine histochemical technique. Can. J. Bot. 46, 1491 - 1494

GLAUERT, A. A. (1965). The fixation and embedding of biological specimens for electron microscopy. In: Techniques for Electron Microscopy. F.A. Davis Company, Philadelphia.

GORHAM, P.R. (1941) Measurement of the response of Lemna to growth promoting substances. Amer. J. Bot. 28, 98 - 101.

GREGORY, R.P.F. (1968). An improved preparative method for spinach catalase and evaluation of some of its properties. Biochim. Biophys. Acta. (Amst.) 159, 429 - 439

HARRIS, N., DODGE, A.D. (1972a). The effect of paraquat on flax cotledon leaves; Physiological and biochemical changes. Planta (Bart.) 104, 210 - 219

HARRIS, N., DODGE, A.D. (1972b). The effect of paraquat on flax cotyledon leaves: Changes in fine structure. Planta (Bert.) 104, 201 - 209.

HILLMAN, W.S. (1961). The Lemnaceae, or duckweeds. A review of the descriptive and experimental literature. Bot. Rev. 27, 221 - 287

HOLM-HANSEN, O. (1968). Ecology, physiology and biochemistry of blue-green agae. A. Rev. Microbiol. 22, 47 - 70

HOMER, R.F. & TOMLINSON, T.E. Nature (London) (1959) Redox Properties of some dipyridylium quaternary salts. Nature (Lond.) 184, 2012 - 2013

HOMER, R.F., MEES, G.C., & TOMLINSON, T.E. (1960) Mode of action of dipyridyl quaternary salts as herbicides. J. Sci. Food Agri. 11 309 - 315

HONEYCUTT, R.C. and KROGMANN, D.W. (1970) A light-dependent oxygen-reducing system from Anabaena variabilis. Biochim. Biophys. Acta 197, 267 - 275.

HUTNER, S.H. (1953). Comparative physiology of heterotrophic growth in plants. In: Loomis, W.E. (ed.) Growth and differentiation in plants. Iowa State College Press.

JANSEN, E.F., JANG, R. & BONNER, J. (1960). Binding of enzymes to Avena colcoptili cell walls. Plant Physid. 35, 567 - 574

JUNIPER, B.E., COX, G.C., GILCHRIST, A.J., & WILLIAMS, P.R., (1970) Techniques for Plant Electron Microscopy. Blackwells.

KANESHIRO, T. and ZWEIG, G. (1965). Effects of diquat (1,1' - ethylene - 2,2' - dipyridylium dibromide) on the photosynthetic growth of Rhodospirillum rubrum. Appl. Microbid., 13, 939 - 944

KANESHIRO, T & ZWEIG, G. (1966). Effect of diquat (1,1' - ethyline - 2,2' - dipyridylium dibromide) on the formation & photo-reaction of chromatophores from Rhodospirillum rubrum. Biochemica et Biophysia Acta 126, 225 - 233

KELLENBERGER, E., RYTER, A. & SECHAUD, J. (1958). Electron microscope study of DNA containing plasms. II Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4, 671 - 678

KENYON, C.N., RIPPKA, R. and STANIER, R.Y. (1972). Fatty acid composit-ion and physiological properties of some filamentous blue-green algae. Arch. Mikrobid. 83, 216 - 236

KROGMANN, D.W. (1973). In: The Biology of Blue-Green Algae. ed. Carr, N.G. & Whitton, B.A., Blackwells, London.

KULASOORIYA, S.A., LANG, N.J. and FAY, P. (1972) The heterocysts of blue-green algae. III Differentiation and nitrogenase activity. Proc. R. Soc. B 181, 199 - 209

LANG, N.J. (1965). Electron microscopic study of heterocyst develop-ment in Anabaena azollae Strasburger. J. Phycol. 1, 27 - 134

LANG, N.J. (1968). The fine structure of blue-green algae. A. Rev. Microbiol. 22, 15 - 46

LANG, N.J. and FISHER, K.A. (1969). Variation in the fixation image of "Structured granules" in Anabaena. Arch. Mikrobiol, 67, 173 - 181

LANG, N.J. and FAY, P. (1971). The heterocysts of blue-green algae. II. Details of ultrastructure. Proc. R. Soc. B. 178, 193 - 203

LEGG, P.G., WOOD, R.L. (1970a). New observations on microbodies. A cytochemical study on CPIB-treated rat liver. J. Cell. Biol. 45, 118 - 129

LEGG, P.G. and WOOD, R.L. (1970b). Effects of catalase inhibitors on the ultrastructure and peroxidase activity of proliferating microbodies. Histochemic 22, 262 - 276

LIPETZ, J. & GARRO, A (1965). Ionic effects on lignification and peroxidase in tessue cultures. J. Cell. Bid. 25, 109 - 116

MEES, G. C. (1960). Experiments on the herbicidal action of 1,1 - ethylene - 2,2 - dipyridylium dibromide. Ann. appl. Bid. 48, 601 - 612

MERKLE, M.G., LEINWEBER, C.L. and BOVEY, R.W. (1965). The influence of light, oxygen, and temperature on the herbicidal properties of paraquat. Plant Physiol. 40, 832 - 835

MILLER, M.M. and LANG, N.J. (1968). The fine structure of akinete formation and germination in Cylindrospermum. Arch. Mikrobid. 60, 303 - 313

MOLLENHAUER, H.H. (1959) Permanganate fixation of plant cells. J. Biophys. Biochen. Cytol. 6, 431 - 435.

MULLISON, W.R. (1970). Effects of herbicides on water and its inhabitants. Weed Sci. 18, 738 - 750

NEWMAN, J.F. & WAY, J.M. (1966). Some ecological observations in the use of paraquat and diquat as aquatic herbicide. Proc. Brit. Weed Contr. Conf. 8, 582 - 585

NOVIKOFF, A.B., & GOLDFISCHER, S. (1968). Visualization of mierobodies for light and electron microscopy. J. Histochem. Cytochem. 16, 507.

PARKER, C. (1966). Influence of water hardness on the phyto-toxicity of paraquat.
Nature (Lond.) 212, 1465 - 1466

PATTERSON, P. & MYERS, J. (1968). Production and destruction of hydrogen peroxide by a blue-green alga. Pl. Physiol. 43, 5 - 12

REDDY, J. CHIGA, M., BUNYARATVEJ, S. & SVOBODA, D. (1970). Microbodies in experimentally altered cells VII. CPIB - induted hepatic microbody proliferation in the absence of significant catalase synthesis.
J. Cell Biol. 17, 226 - 234.

REYNOLDS, E.S. (1963). The use of lead nitrate at high pH as an electron opaque stain in electron microscopy.
J. Cell. Biol. 17, 208 - 213

RIGATUSO, J.L., LEGG, P.G., and WOOD, R.L. (1970). Microbody formation in regenerating rat liver. J. Histochem. Cytochem. 18: 893 - 900.

RIPPKA, R., NEILSON, A., KUNISAWA, R. and COHEN-BAZIRE, G. (1971). Nitrogen fixation by unicellular blue-green algae. Arch. Mikrobiol. 76, 341 - 348

SCOTT, W.E. and FAY, P. (1972). Phosphorylotion and animation in heterocysts of Anabaena variabilis. Br. Phycol. J. 7, 283 - 284

SNEDECOR, G.W. (1967). Statistical methods, 6th edition
Iowa State. Univ. Press.

STEWART, W.D.P., HAYSTEAD, A. & PEARSON, H.W. (1969). Nitrogenase activity in heterocysts of blue-green algae.
Nature, (Lond.) 224, 226 - 228.

STEWART, W.D.P. and LEX, M. (1970). Nitrogenase activity in the blue-green alga, Plectonema boryanum strain 594. Arch. Mikrobiol. 73, 250 - 260.

STEWART, W.D.P. (1971). Physiological studies on nitrogen-fixing blue-green algae.
Plant and Soil Special Volume, 371 - 391

STEWART, W.D.P. (1973). In: The Biology of The Blue-Green Algae. ed. Carr, N. G. & Whitton, B.A., Blackwells, London.

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 - 274

STOKES, D.M. and TURNER, J.S. (1971). The effects of the dipyridyl diquat on the metabolism of Chlorella vulgaris. III Dark metabolism; effects on respiration rate and the path of carbon.
Aust. J. Biol. Sci. 24, 433 - 437

SUE-FEI TSAY, JHY-MEI LEE & LYND, J.Q. (1970). The interactions of Cu^{++} & CN^{-} with paraquat phytotoxicity to a Chlorella.
Weed Sci. 18, 596 - 598

SVOBODA, D., GRADY, N., & AZARNOFF, D. (1967) Microbodies in experimentally altered cells. J. Cell. Biol. 35, 127 - 152.

TALPASAYI, E.R.S. (1967). Localization of ascorbic acid in heterocysts of blue-green algae. Cur. Sci. 36, 190 - 191

THOMAS, J (1970). Absence of the pigments of photosystem II of photosynthesis in heterocysts of a blue-green alga. Nature, Lond. 228, 181 - 183.

TISCHER, I. (1957). Untersuchunger über die granulären Einschlüssi und das Reduktions-Oxydations-Vermögen der Cyanophycaen.
Arch. Mikrobiol. 27, 400 - 428

TOLBERT, N.E., OESER, A., KISAKI, T., HAGEMAN, R.H., and YAMAZAKI, R.K. (1968). Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism.
J. Biol. Chem. 243, 5179 - 5184

TOLBERT, N.E., OESER, A., YAMAZAKI, R.K., HAGEMAN, R.H., and KISAKI, T. (1969). A survey of plants for leaf peroxisomes.
Plant Physiol. 44: 135 - 148

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.

UMBREIT, W.W., BURRISS, R.H., & STAUFFER, J.F., (1964). "Manometric Techniques". 4th Ed.
(Burgess Publ. Co.: Minnesota).

VAN OORSCHOT, J.L.P. (1964). Some effects of diquat and simetone on CO_2-uptake and transpiration of Phaseolus vulgaris. Proc. Brit. Weed Control Conf. 7, 321 - 324

VERNON, L.P. (1960). Spectrophotometric determination of chlorophylls and phaesphytins in plant extracts.
Analyt. Chem. 32, 1144 - 1150

VIGIL, E.L. (1969). Intracellular localization of calalase (Peroxidative) activity in plant microbodies.
J. Histochem. Cytochem. 17: 425 - 428

WILDON, D.C. & MERCER, F.V. (1963b). The ultrastructure of the heterogist and akinete of the blue-green algae.
Arch. Mikrobiol. 47, 19 - 31.

WILLIS, J.C. (1948). A dictionary of the flowering plants and ferns.
University Press, Cambridge.

WOLK, C.P. (1968). Movement of carbon from vegetative cells to heterocysts in Anabaena cylindrica.
J. Bact. 96, 2138 - 2143

WOLK, C.P. and SIMON, R.D. (1969). Pigments and Lipids of heterocysts.
Planta 86, 92 - 97.

WOLK, C.P. & WOJCIUCH, E. (1971). Photoreduction of acetylene by heterocysts.
Planta, (Cert.) 97, 126 - 134.

WYATT, J.T. and SILVEY, J.K.G., (1969). Nitrogen fixation by Gloeocapsa. Science. N.Y. 165, 908 - 909

YAMAZAKI, R.K., TOLBERT, N.E. (1970). Enzymic characterization of leaf peroxisomes.
J. biol. Chem. 245 5137 - 5144

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 - 346

ZWEIG, G., HITT, J.E. and McMAHON, R. (1968). Effects of certain quinones, diquat and diuron on Chorella pyrenoidosa Chick. (Emerson Strain).
Weed Sci., 16, 69 - 73

ZWEIG, G., HITT, J.E. & CHA, D.H. (1969). Mode of action of dipyridyls and certain quinone herbicides.
Agricultural & Food Chem. 17, 176 - 181.

ACKNOWLEDGEMENTS

The author acknowledges a grant from the Science Research Council. I also wish to express my gratitude to Prof. W. W. Fletcher for the use of research facilities and to Dr. I. G. Gardiner for her help and encouragement throughout this work. Thanks are also due to my parents for their tolerance and to Mrs. Skilling for typing the Thesis at short notice.