UNIVERSITY OF SYDNEY

USE OF UNICELLULAR ALGAE IN STUDIES OF PHOTOTOXICITY OF DIURON AND SOME OTHER HERBICIDES

By

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SUMMARY

The use of algae as bioassay test organisms for phenylurea herbicides was investigated and algal techniques developed to study soil-herbicide relationships.

Amongst many algae tested, Chlorella NMI (a Chlorella sp. isolated from soil) was found to be most sensitive to phenylurea herbicides and was used in the algal techniques.

The liquid algal technique could measure differences in growth inhibition over a range of 0.02 to 1.0 ppm of phenylurea herbicides. The paper disc technique was less sensitive, but concentrations as low as 0.2 ppm of active diuron or neburon could be detected in soil. It was rapid and easy to handle.

Based on the concentration of herbicide causing 50% inhibition of chlorella NMI in liquid medium, the following toxicity series for herbicides inhibiting photosynthesis was established: diuron (0.050 ppm)> neburon (0.063 ppm)> monuron (0.18 ppm)> bromacil (0.32 ppm)> atrazine (0.73 ppm)> simazine (0.83 ppm)> atratone (1.50 ppm)> amitrole (7.10 ppm).

Results obtained from algal trials were evaluated using the Freundlich-isothermal relationship to study the adsorption of neburon and diuron by soil. It showed that neburon was more strongly adsorbed than diuron by soil. The effects of organic matter and clay on the activity of phenylurea herbicides were separately studied by the paper disc technique. More neburon than diuron was adsorbed by the same amount of organic matter or clay. Organic matter adsorbed 18 times more diuron than clay.

The algal method was used to study the effects of five soil factors on the phytotoxicity of diuron in twenty soils collected from New South Wales and South Australia. Correlation and regression analyses of the results showed that diuron toxicity was closely related to organic carbon content ($r = o$) and to percentage herbicide adsorption ($r = o$). Clay and silt did not seem to affect the phytotoxicity of diuron. From the regression studies, it was concluded that diuron recommendations based on soil organic carbon content could be made. However, it might be unsatisfactory with soil or low organic carbon content (0.8%). Recommendations based on an adsorptive index proved to be more reliable for all the soils studied.

Bioassays with plants (oats) and Chlorella NMI were carried out using fourteen soils. A high correlation was noted between the responses (ED_{50} values) obtained by the two methods. The high degree of correlation provided a sound basis for the use of the algal technique to replace the lenghty higher plant assays and soil-herbicide studies.

Adsorption values, obtained by the liquid and paper disc techniques, were utilised to predict application rates of diuron and simazine in wheat field trials for three consecutive years in Camden, Castle Hill and Narrabri. The dosages determined by the algal technique were reliable, consistently gave good weed control without damage to crops, and left no harmful residues after harvest.

The mode of action of some herbicides, viz., diuron and amitrole was investigated by studying their effects, at low concentrations, on growth, photosynthetic pigments and ultrastructure of Chlorella NMI.

Modified fixation procedures in electron microscopy were used to establish the ultrastructure of Chlorella NMI and Euglena sp. The presence, distribution and conformation of DNA molecules in

the chloroplast of Chlorella NMI were demonstrated by light and electron microscopy. In both Euglena and Chlorella NMI, the implication of a protein synthetic system in the chloroplast distinct from a cytoplasmic protein synthetic system, was discussed on the basis of attachment of DNA fibrils to the lamellae membranes, with closely associated ribosomes and polysomes.

A simple set up was devised to grow synchronous cultures of Chlorella NMI. Cells were collected at various stages of the life-cycle, and used to study the process of cell division at an ultrastructural level.

Cells collected from synchronous cultures were utilized in the study of effects of diuron and amitrole on growth, photosynthetic pigments and ultrastructure. Experimental conditions were designed to minimise the likelihood of detecting secondary effects. Low and high levels of herbicide were used. Algal cell division was more sensitive to diuron than photosynthetic pigment synthesis. Very low concentrations of the herbicide stimulated growth and chlorophyll production. Theoretical implications were discussed with regard to chlorophyll a:b ratio, and the two photosystems in chloroplast. It appeared that the primary effect of diuron is

localised on chlorophyll pigments. Structural changes in the chloroplast were secondary to effects on the pigments. The results from studies with Euglena, which is physiologically different from Chlorella NMI, supported the above observations. Amitrole, was found to act primarily on the carotenoids, resulting in a probable secondary effect on nucleic acids in the chloroplast.

1 GENERAL INTRODUCTION.

1.1 Introduction

Pot trials with higher plants and chemical analyses had been used to assay and to study phenylurea herbicides in soil. Because of the mode of action of these herbicides (inhibition of photosynthesis), plant assay is a lengthy technique. Chemical analysis, on the other hand, may involve the long and tedious process of extraction and purification of the herbicide from soil, and may not always be specific. As a result of these observations, it was decided to investigate the possibility of using unicellular algae to assay and study the phytotoxic characteristics of these herbicides.

This thesis, therefore, is mainly concerned with the development of algal techniques for the bioassay of phenylurea herbicides, their application in herbicide soil relationships and in the recommendation of field application rates based on laboratory investigations (Part I). The use of algae is also extended to investigate the effects of diuron and other herbicides on growth, photosynthetic pigments, ultrastructure, and may thus contribute to the understanding of the mechanism of action of these herbicides on photosynthetic cells.

1.2 REVIEW OF LITERATURE

1.2.1 The Phenylurea Herbicides

Chemicals have been used against weeds since 1850. The arsenicals were the first chemicals to be tested widely for weed control and to gain general acceptance as herbicides. Since then, the development of new herbicides has progressed steadily. An important turning point, which led to the extensive use of chemicals for weed control in agriculture, came with the discovery of the herbicidal properties of the phenoxyacetic acids in 1944. Not long after, Thompson *et al* (1946), Bucha and Todd (1951) reported that the phenylureas had herbicidal properties. Bucha and Todd (1951) found that the compound 3-(p-chloro-phenyl)-1-1-dimethylurea (Monuron) was very effective in the control of annual and perennial grasses. After extensive field trials, McCall (1952) reported that monuron had shown promise as a selective herbicide in crops that were relatively tolerant to this material. Soon after, it was found that the chemical was extremely persistent and could be used effectively as a soil sterilant. The urge for rapid advance, associated with the discovery that the synthetic chemical could be used as a weed killer, led to more active research in this field. As a result a wide range of phenylureas was synthesized and screened for herbicidal activity in the laboratory and greenhouse.

The first phenylureas were chlorine-substituted dimethyl derivatives. Recently synthesized compounds have more complex ring substituents and alkyl groups. Compounds showing good herbicidal activity in the initial screening trials were then tested in field trials to evaluate their activities on weeds and crops. In the United States, West Germany, and Britain, this experimental work established the phenylureas as potent and selective herbicides. Today they are used extensively as selective, soil-applied, pre-emergent and post-emergent herbicides in agriculture and as soil sterilants. Because of their persistence in soils for very long periods, the phenylureas have received much attention in relation to their removal and inactivation in the soil.

1.2.2 Factors Affecting Loss of Phenylurea Herbicides from Soil

The susceptibility of the herbicides to loss or degradation after application in soils depends on soil properties and environmental conditions (Upchurch, 1966).

Factors influencing the loss of herbicides in soils include: volatility, photo-decomposition, microbial degradation, leaching, adsorption, chemical degradation, biological uptake and metabolism by plants. These factors can be influenced by the application rate, uniformity of

application, soil incorporation and the time interval between application and incorporation.

Photo-decomposition and Volatilization

Birk (1955) found that photo-decomposition might be a factor in the disappearance of monuron from soils. This was confirmed by Hill et al (1955), who showed that an 83% loss of monuron occurred when a standard solution, containing 88.3 ppm in glass distilled water sealed in quartz tubes, was exposed to sunlight for forty eight days.

Weldon and Timmons (1961a) demonstrated photo-chemical changes and reductions in biological activity of diuron and monuron, when these herbicides were subjected to ultra-violet radiation for 28 hours. They suggested that similar photochemical changes might occur in natural sunlight when these herbicides remain on the soil surfaces for prolonged periods after application.

Jordan et al (1962) using spectrophotometric analysis, studied the effects of controlled irradiation with ultra-violet light and sunlight upon the phenylurea herbicides under simulated field conditions. They showed photo-chemical changes in the herbicides by both treatments, but suggested that once those herbicides were adsorbed or incorporated into the soil the loss due to photodecomposition was negligible.

Sheets (1962) showed that monuron and diuron disappeared more rapidly from soil exposed to sunlight than from shaded soil. They reported that at high soil temperatures it was impossible to separate losses caused by photodecomposition from losses caused by volatilization. Under temperatures, varying from 20° to 50° C, loss of monuron by volatilization was negligible because of its extremely low vapour pressure. It can be concluded that loss of phenylureas by volatilization may be important only when herbicides remain on the soil surface exposed to hot and dry conditions.

Leaching

Some herbicides are displaced from the upper layers of soil (where most of the weed seeds are located) by leaching under heavy rainfall or irrigation. However, leaching plays little part in the disappearance of most of the phenylurea herbicides. Hill et al (1955) showed that after application of 1 to 2 lbs/ac (1.1 to 2.2 Kg/ha) of monuron and diuron, on a porous soil in extremely high rainfall conditions, very little herbicides appeared below a depth of 4 inches (10 cm). Upchurch and Pierce (1957) studied the effects of amount, intensity and frequency of simulated rainfall upon the leaching of monuron from a Lakeland sandy soil. They concluded that the leaching process depended on the solubility of monuron and the amount adsorbed to the soil from the percolating solution.

They also found that soil organic matter had a very marked effect on the leaching of monuron (Upchurch and Pierce, 1958). Burnside et al (1963) reported that 4 months after application of 5 lb/acre (5.6 Kg/ha) of linuron, 1.1 to 2.0 ppm were found in the 0-3" (0-7.5 cm) layer only, in 3 different soil types. Jordan et al (1963) also showed that leaching of linuron was very slight and it was related to its solubility. Dubey and Freeman (1965) compared the leaching of linuron and diphenamid in soils. At an application rate of 25 lb/acre (28 Kg/ha), linuron did not leach to the 5-6" layer in a sandy loam soil, even with the largest amount of water. In a silt loam, at 25 lb/acre (28 Kg/ha), linuron moved to 2-3 inch (5-7.5cm) depth with 8 inches (20 cm) of water, but still remained in highly phytotoxic concentration at the surface. Ashton (1961) in his studies with simulated furrow irrigation, found that the lateral movement of fenuron, monuron, diuron and neburon, showed variation with soil types. It was greatest in Yolo sandy loam, intermediate in Sacramento clay, and least in Staten Island peaty muck. Fenuron, which was more soluble and less strongly adsorbed by soils than monuron moved laterally more quickly than the other three herbicides.

Geissbuhler et al (1965a) also indicated that leaching of the phenylurea herbicides was related to solubility and adsorption. They compared the leaching of 3-[p-(p-chloro-

phenoxy)-phenyl]-1-1-dimethylurea (Ciba 1983) with a reference compound 1-butyl-3-(3,4-dichlorophenyl)-1-methylurea (Neburon) in three different soils and at two different soil-moisture levels. They found that the bulk of the Ciba 1983 remained in the 1 cm. layer of the three soils when 200 mm. of simulated rainfall was applied during a 45-hour period. On the other hand, a great proportion of the neburon had moved below the top 5 cm level. Ciba 1983 had a very low solubility (4 ppm) and was very strongly adsorbed by soil particles.

Hilton and Yuen (1966) also concluded that leaching is of minor importance in reducing the activity of diuron. They found that leaching was negligible below a depth of 2 inches (5 cm) in a Hawaiian sugarcane top soil when diuron was applied at rates up to 20 lbs per acre (22.4 Kg/ha).

Chemical Decomposition

The urea herbicides are sufficiently stable under normal temperature and soil conditions to resist breakdown by hydrolysis or chemical oxidation, (Hill et al 1955). Although it has been suggested that monuron could be broken down to simpler compounds by both acid and alkali hydrolysis (Lowen and Baker, 1952), such processes do not occur under normal soil conditions. This was confirmed by Hartley (1960), who pointed out that extreme acidic or alkaline conditions, necessary for hydrolysis of the phenylurea herbicides, are beyond the normal pH ranges of soil.

Deactivation by soil microorganisms

Most herbicides, when applied to soils are decomposed or partially degraded by soil microorganisms. However, the number of species capable of degrading the various compounds and the rate of decomposition varies. Some of the herbicides, such as 2,4-D and dalapon, disappear completely within a few weeks while others such as the phenylurea herbicides may persist for months in soil.

(a) Microorganisms

Searle and Cupery (1954), in their studies with C^{14} methyl-labelled monuron, found that 10% of the compound was decomposed by soil microorganisms in 90 days.

Investigating the effect of autoclaved and non-autoclaved soils on the residual activity of the phenylureas, Hill *et al* (1955), Hill and McGahen (1955) confirmed that microorganisms increase the breakdown of these herbicides in soil. They showed that certain soil bacteria belonging to the *Pseudomonas*, *Xanthomonas*, *Sarcina* and *Bacillus* groups and the fungi, *Penicillium* sp. and *Aspergillus* sp., were able to use monuron as a sole source of carbon in agar media. However, it has not been possible to obtain from such active organisms, cell-free systems or enzyme preparations capable of oxidising or degrading urea herbicides.

(b) Effects of temperature, moisture and pH

Loustelot et al (1953) showed that loss of monuron toxicity in the soil depended on the properties of the soil and on environmental conditions. From their results they suggested that factors such as temperature, adequate moisture, organic matter were favourable for microbial decomposition of the herbicides in the soil. Ogle and Warren (1954) also indicated that temperatures between 30°C and 40°C favoured the loss of monuron from muck soil.

Rahn and Baynard (1958) found that monuron disappeared more rapidly from a cultivated than from a non-cultivated soil. They suggested that cultivation accelerated microbial decomposition of the herbicide by improving soil aeration.

Reid (1963) reported that pH, moisture, and temperature affect the rate of degradation of the phenylurea herbicides. Sheets et al (1964) found that diuron was slowly inactivated in soil and that in a cool climate detoxication might still be proceeding 12 months after the application of a dose of 2 lb/acre (2.2 Kg/ha).

(c) Effects of organic matter

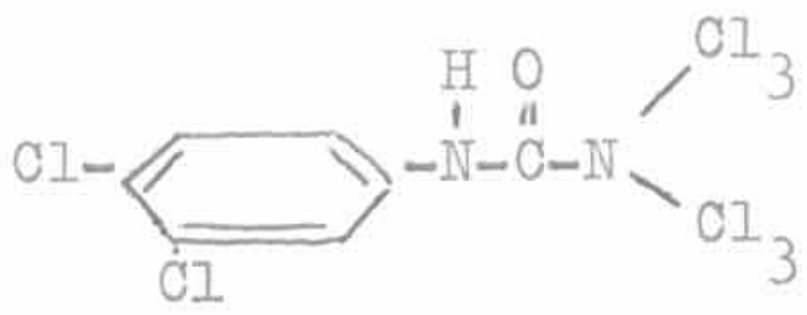
In general, microorganisms are more active in soils high in organic matter. The influence of soil organic matter on the persistence of phenylureas in soil, however, is not completely understood. Reid (1963) observed that monuron decomposition occurred more rapidly in soil containing a high percentage of organic matter than in soils containing

little or no organic matter. In contrast, Geissbuhler et al (1963) found that decomposition of chloroxuron occurred more rapidly in a sandy loam soil than in a humus soil. Corbin and Upchurch (1967) also reported a slower rate of detoxication of diuron in soils high in organic matter. They suggested that the herbicide molecules were so strongly adsorbed by the organic matter that they were not available for microbial attack. In addition, the high organic matter in soil provides microorganisms with a readily available food supply for a long period, thus delaying the decomposition of herbicide. More information is therefore required before the effect of soil organic matter on the microbial degradation of phenylurea herbicides can be adequately assessed.

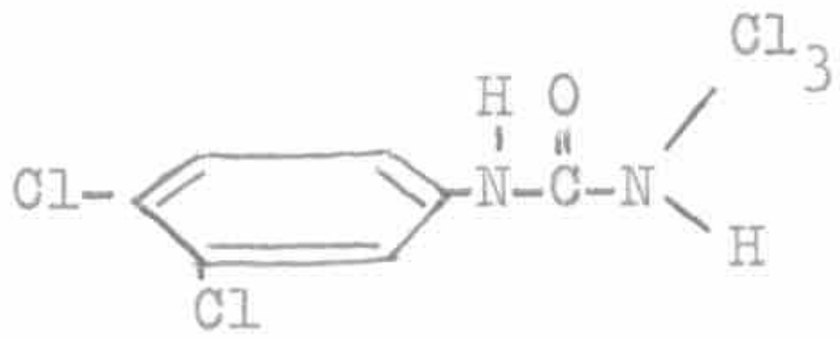
(d) Pathways of decomposition

The mechanism by which microorganisms degrade phenylurea herbicides has received much attention in the last few years. Geissbuhler et al (1963) showed that soil microorganisms degraded chloroxuron by successive demethylation, followed by hydrolysis of the phenylurea to aniline. Dalton et al (1966), using diuron, confirmed this mechanism of degradation in soil. Schroeder (1970) tested the ability of 81 fungi and 13 bacteria to decompose phenylurea herbicides in nutrient solution. He found that 78 out of the 94 organisms were able to break down the herbicides beyond

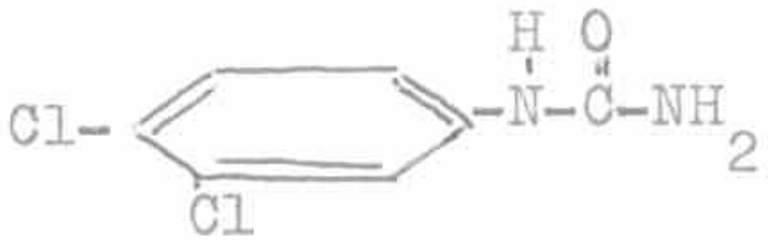
the chloranilines. Decomposition of the chemicals involved the following steps: demethylation, demethoxylation, hydrolysis and decarboxylation. The following is the hypothetical scheme for the partial degradation of diuron by soil microorganisms:



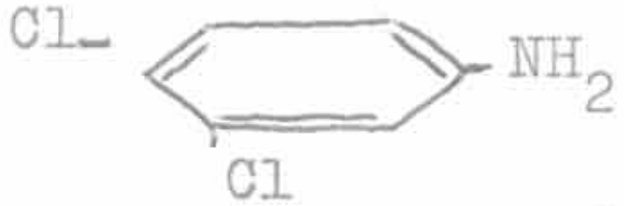
(Diuron)



3-(3,4-dichlorophenyl)-1-methylurea



3,4-dichlorophenylurea



3,4-dichloroaniline

$+ Co_2 + NH_3$

Adsorption by Soil

The greater amount of work on the adsorption of the phenylurea herbicides in soils has been carried out with monuron and diuron and, to a lesser degree with fenuron, ~~xxxxxx~~ and OMU. Various approaches have been used in the adsorption studies of these herbicides. In these investigations, the effect of soil type and organic matter on phytotoxicity was determined either chemically after shaking aqueous herbicide solutions with soil samples, or biologically by determining the reduction of toxicity against indicator plants (Sherbourne and Freed, 1954; Bingeman and Hill, 1955; Upchurch, 1958; Sheets, 1958; Coggins and Crafts, 1959; Yuen and Hilton, 1962; Hilton and Yuen, 1963, 1966; Harris and Sheets, 1965; Harris, 1966; Obien *et al*, 1966; Liu *et al*, 1970). Another approach used was to determine the adsorption isotherms or distribution coefficients using soil colloids and well characterised absorbents, (Frissel and Bolt, 1962; Geissbuhler *et al*, 1963; Hance, 1965a,b; Hance, 1967). Adsorption is considered to be the most important process which influences the effectiveness of the phenylurea herbicides (Audus, 1964; Upchurch, 1966). The extent to which they are adsorbed determines the herbicide concentration in the soil solution (Hilton and Yuen, 1963).

This affects the initial phytotoxicity of the herbicides in the soil.

Sherbourne and Freed (1954) found a very high degree of adsorption of monuron on organic matter and on clay particles. Further research has shown that organic matter content is apparently the most important factor influencing the adsorption of phenylureas in soil. (Ogle and Warren, 1954; Sheets, 1958; Upchurch, 1958; Upchurch and Mason, 1962; Dubey and Freeman, 1964; Hance, 1965a; Upchurch et al, 1966; Liu et al, 1970)

Coggins and Crafts (1959), on the basis of biological experiments, concluded that clay reduced the toxicity of phenylureas to barley. Neburon was most strongly affected followed by OMU, diuron, monuron and fenuron. Geissbuhler et al (1963) found that bentonite clay greatly reduced the toxicity of neburon and chloroxuron. It appears that the adsorptive effect of the clay is more important for phenylureas with low water solubility.

Diuron and neburon were found to be relatively more toxic under high moisture conditions than under low moisture levels, (Upchurch, 1957). The less polar herbicide molecules, under low moisture levels were found to compete more favourably with the polar water molecules for soil adsorption sites.

Yuen and Hilton (1962, 1963), in studies of adsorption of phenylurea herbicides in soils of Hawaiian sugar cane-fields, found that all soils tested showed a greater adsorptive capacity and bond strength for diuron than for monuron. Bonding energy, measured by the resistance to desorption, was found to be directly related to adsorptive capacity. They also observed that phytotoxicity increased in the following order: fenuron < linuron ≤ monuron < diuron, while water solubility was ranged as follows: diuron < linuron ≤ monuron < fenuron. However, Harris and Warren (1964), and Hance (1965a) found no close relationship between adsorption of phenylureas and their water solubilities.

Yuen and Hilton (1962) indicated that soil pH, presence or absence of soluble sulphates and phosphate ions, soluble monovalent cations (K+), and inorganic salts and bases had little or no effect on the phytotoxicity of the phenylureas in soil. Ries and Sweet also found no consistent effect of soil pH on the activity of monuron. In their studies with cotton and ryegrass, they found that variations of levels of N, K, and pH in White Store and Georgeville soils had little effect on the growth response of the plants treated with diuron. Coggins and Craft, (1959) reported that the solubility of phenylureas increased with increasing pH, resulting in the presence of more free herbicide molecules in solution and higher toxicity to plants.

Sheets (1958) and Upchurch (1958) found an inverse correlation between phytotoxicity and cation exchange capacity in the soil. In contrast, Hilton and Yuen (1963) reported no such relationship by direct adsorption measurements.

In general, it appears that adsorption of the phenylurea herbicides in the soil is related to organic matter, percentage of clay and cation exchange capacity. Soil pH does not seem to have a major importance, and there is a rough correlation between solubility of herbicide in water and the degree of its adsorption in soil.

The relationship between the structure of the herbicide molecule and its adsorption has received less attention. Coggins and Crafts (1959) suggested, from the electrophoretic behaviour of the phenylurea herbicides, that the shift in electron balance caused by the addition of a chlorine atom to the aryl substituent would affect adsorption relationships. They also found that the removal of one methyl group decreased toxicity in nutrient solution. Working with the phenoxyacetic acids, Shaik and Neal (1960) found that the chlorine substitution on the aryl group increased adsorption. From the work of Geissbuhler et al (1963), it was noted that the introduction of a second aromatic nucleus caused a more pronounced effect on adsorptive properties.

Hance (1965), in his studies of phenylureas in British soils, came to the same conclusions. He found that the phenylureas might be classified in the following order of increasing adsorption: fenuron < methylurea < phenylurea < monuron ≤ monolinuron < diuron ≤ linuron < neburon < chloroxuron.

1.2.3 Persistence in Soil

The persistence of phenylurea herbicides in various soils under different environmental conditions, has been studied (Loustalot et al, 1953; Hill, 1954; Ogle and Warren, 1954; Hill et al,1955; Rahn and Baynard, 1958; Bruns et al, 1962; Dawson et al, 1967). At recommended rates of annual applications, the phenylurea herbicides usually decompose fast enough that quantities toxic to most crop plants do not accumulate in the soil (Hill et al, 1955; Rahn and Baynard, 1958; Arle et al, 1965). However, several investigators have found that at the application rate of 2-4 lb/ac (2.2 to 4.5 Kg/Ha) or less, residual phytotoxicity of the phenylurea herbicides remained in soil from 6 to 12 months (Weldon and Timmons, 1961; Bruns et al, 1962; Sheets, T.J., 1964; Lee, W.O., 1966; Dawson et al, 1968; Dalton et al, 1966; Upchurch et al, 1969). Although persistence of phenylurea herbicides may be a distinct advantage in weed control, it may also constitute a disadvantage to sensitive crops during subsequent seasons (Upchurch et al, 1969).

Soil factors which increase the duration of residual phytotoxicity are primarily those that would tend to retard microbial activity and chemical reactions. Hill (1953) and Dubey and Freeman (1963) obtained conclusive evidence that microbes play a role in the disappearance of monuron and diuron in soil. Factors which affect microbial activity influence the breakdown of the phenylurea herbicides. Persistence of several phenylureas is reduced in moist soils compared with dry soils (Darding and Freeman, 1968). Increases in organic matter content of the soil are generally regarded as capable of decreasing residual phytotoxicity (Hill et al 1955; Ogle and Warren, 1954; Upchurch, 1958; Dubey et al, 1966). However, Geissbuhler et al, 1963, and Corbin and Upchurch, 1967, observed a slower rate of detoxication of chloroxuron and diuron in soils with higher organic matter content.

Soil pH may influence the reactivity of the herbicides and exert a great effect on the kinds of microorganisms active in the soil. In soils of neutral or alkaline pH, bacteria and actinomycetes predominate, while in acid soils, fungi are most numerous. Since fungi appear to be largely responsible for microbial decomposition of phenylurea herbicides (Schroeder, 1970), one might expect decomposition to occur more rapidly in acid soils. The effect of cation exchange capacity or residual phytotoxity has not been

adequately investigated. Reduced residual phytotoxity has been found to occur in surface soils when compared to sub-soils containing equal amounts of herbicide. This probably resulted from greater microbial or physico-chemical activity in the surface soils.

Monuron and diuron are widely used for the control of weeds in agricultural crops. Persistence problems are sometimes encountered when crops sensitive to monuron and diuron follow corn or cotton in crop rotations, (Upchurch et al, 1969). Wheat, soybeans, tobacco, peanuts, cucumber are all susceptible to low concentrations of diuron.

In Western Oregon, where diuron is used extensively for weed control in wheat, extensive herbicidal injury to the wheat itself has occurred on several occasions (Sedgeley and Boersma, 1969; Upchurch et al, (1969) reported a potential hazard from residue to the sensitive crops grown in rotation with diuron treated crops. In North Carolina, soybeans were injured 1 and 2 years after applications of 1.2 lb and 2.4 lb/ac (1.3 and 2.6 Kg/Ha) in both 2 year and 4 year rotations. In Nebraska oats were damaged 12 months after application of 2.5 lb/ac (2.75 Kg/Ha) of monuron (Burnside et al, 1963).

1.2.4 Uptake by Plants

Most experiments on the absorption of phenylurea herbicides by roots of plants have been conducted in nutrient solutions. These herbicides have generally been found to

be readily translocated in the xylem. Thus they follow the transpiration stream to the leaves, where they exert their effect by inhibiting photosynthesis (Bayer and Yamaguchi, 1965; Geissbuhler et al, 1963; Fang et al, 1955; Smith and Sheets, 1967; Minshall, 1954, 1957; Eshel, 1969).

The rate of toxic accumulation of the herbicide in the leaf tissue depends on the rate of uptake and translocation, binding of the herbicides at metabolically inactive sites, the ability of the species to detoxify the herbicide, and the rate at which such detoxication occurs.

When applied to leaf surfaces, the phenylureas remain localised and there is almost no translocation into stem or other leaves by transport through the phloem (Geissbuhler et al, 1963 b). Bayer and Yamaguchi (1965) have shown that there is practically no symplastic movement of diuron in soybean cotyledons.

The adsorption of linuron by roots appears to be passive and governed by the entrance of water (Hogue and Warren, 1968; Nashed and Ilnicki, 1970). Geissbuhler et al, (1963), however, reported resistance to the uptake of chloroxuron by roots. They compared the relative uptake from nutrient solution and soil medium containing the same amount of labelled chloroxuron. They found that both uptake by roots and translocation to stem and leaves were less efficient in soil medium.

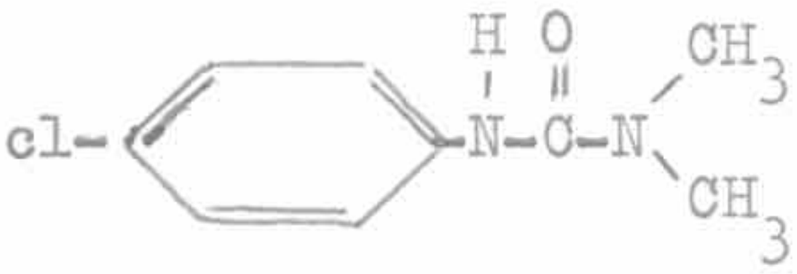
Hogue and Warren (1968) pointed out that selectivity of linuron on tomato and parsnip resided in the differential translocation of the herbicide within these plants. Tomato readily absorbed labelled C^{14} linuron from solution and translocated it to the leaves. The roots retained only a small percentage of the radioactivity. In contrast, parsnip roots retained the greatest part of radioactivity while only a small percentage was translocated to the leaves.

Recently, investigations have found that environmental and edaphic factors affect absorption of phenylurea herbicides from soil and their translocation to the leaves. Sedgeley and Boersma (1969) found that the soil water stress and soil temperature influenced the rate of diuron translocation to the leaves of wheat.

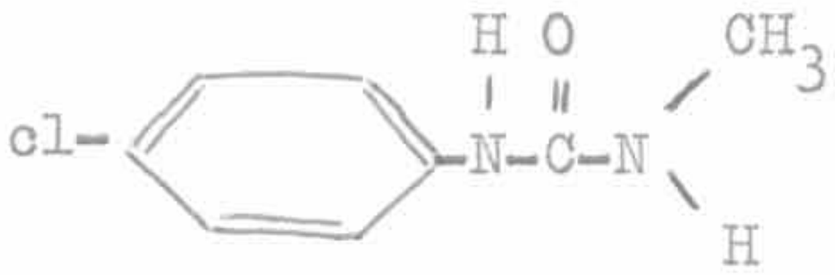
The metabolic fate of the phenylurea herbicides in plants has been studied by several investigators (Geissbuhler et al, 1963; Smith and Sheets, 1967; Swanson and Swanson, 1968; Rogers and Funderbunk, 1968).

Demethylation is the initial step of the metabolic pathway of degradation in plants. The process continues to the corresponding phenylurea and finally to the chloroanitine derivatives. Smith and Sheets (1967) showed that demethylation of diuron occurred in cotton, a tolerant species, and in soybeans, a sensitive species. In cotton, demethylation proceeded to the nonphytotoxic 1-(3,4-dichlorophenyl)

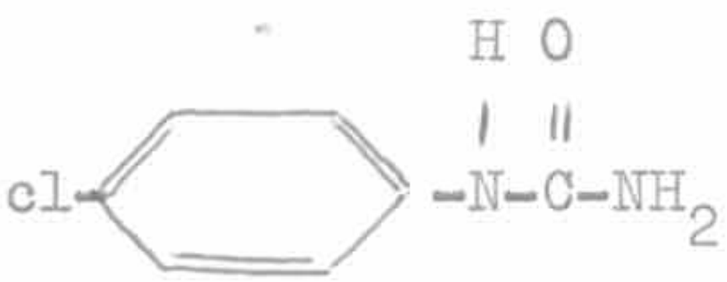
urea, whereas, in soybeans demethylation appeared to take place more slowly with the resulting accumulation of the phytotoxic 1-(3,4-dichlorophenyl) -3-methylurea.
Swanson and Swanson (1968) gave a scheme of degradation of monuron in plants:



monuron



mono-ethyl monuron



p-chlorophenyl urea



p-chloroaniline

1.2.5 Biological assay

The plant indicator test to study herbicides in soil was first developed by Crafts (1935). He used oats as test plants to examine the toxicity and persistence of sodium arsenite and sodium chlorate in four Californian soils. In the following years, a number of other small seeded plants were used by different workers in soil-herbicide studies.

Biological assays have been widely used for the intensive study of phenylurea herbicides in soil. Among the plants utilised in these assays, oat (Avena sativa L.) has most frequently served as the test plant.

Freed (1964) stated: "The validity of biological assay procedures rests on two basic assumptions. They are:

(a) that a given plant response to a chemical increases in an order related to the dose and

(b) that within the limits of sample variation, these responses are reproducible when the plant material and the environmental conditions are the same".

The accuracy of the plant bioassay in determining a herbicide concentration therefore depends to a great extent on the control of factors that can introduce variability.

Mitchell and Livingston (1968) pointed out that the seeds of the test plant to be used in the bioassay should be carefully selected and be as genetically homegeneous

as possible. Jacobsohn and Anderson (1968) reported significant differences in the response of different wild oat lines to several herbicides. In the bioassay of those herbicides, whose mode of action is the inhibition of photosynthesis, the seed size determines the amount of food available for the early growth of the test plant. Anderson (1968) found a significant correlation between soy-bean seed size and atrazine tolerance. The large seeded types were more tolerant than the small seeded types. The time of harvest of the test plants can affect the results of a bioassay of those herbicides. For example, Holly and Roberts (1963) reported that herbicide treated ryegrass harvested in the 3 to 3½ leaf stage of growth gave more reliable results when compared to plants harvested at the 2 - 2½ leaf stage. It was suggested that the early growth of the test plant depended on the food reserves in the seed, and was not influenced by the herbicide. However, when photosynthesis became the source of food supply, the interference with photosynthetic processes by the herbicide caused inhibition of growth of the test plant.

This inhibition was proportional to the herbicide concentration.

Upchurch and Mason (1962) showed that the moisture content of the soil could markedly change the toxicity of herbicides to plants. Thus, failure to maintain a uniform soil moisture throughout the bioassay period can introduce variability in the results. Biological assays of phenylurea herbicides require a considerable length of time. Three to four weeks are needed to obtain significant responses of the test plants to these herbicide. During this period, the plants may be exposed to a changing concentration of the herbicide.

Because of the persistence of the phenylureas in soils, bioassays have been mainly used to obtain information on the effect of soil and climatic factors on the phytotoxicity and dissipation of these herbicides in soil. Bioassays have established the major role played by organic matter on the herbicide toxicity in soil (Upchurch & Mason, 1962; Harris and Sheets, 1965; Upchurch et al, 1966). The influence of clay content, cation exchange capacity; individual cations and pH on toxicity has also been investigated by bioassays. (Upchurch & Mason 1962; Harris and Sheets, 1965; Upchurch et al, 1966). Leaching of these herbicides has been determined in a number of soils by bioassays. (Ashton, 1961; Burnside et al, 1963).

Furthermore, information on temperature, moisture content, microbial flora, chemical interactions of the soil in deativation of phenylurea herbicides has been obtained by bioassay techniques.

1.2.6 Chemical assay

The chemical methods of residual assay are sensitive to very small amounts of phenylurea herbicides. They are accurate and rapid, but are limited by cost of equipment, and problems usually encountered in extraction and purification of the herbicides from the soil. In the chemical methods, extraction procedures used may remove residues which are too firmly adsorbed in the soil to affect plants. They may not always be efficient to differentiate the herbicide from possible biologically inactive products of decomposition. Furthermore, chemical assays are of little value in investigations of effects of edaphic and climatic factors on the phytotoxicity of herbicides. However, they are of value for the determination of herbicide in extracts of biological material.

Bioassay techniques have an advantage over chemical methods in estimations of soil herbicide residues in that they give a direct measurement of the residues which are capable of affecting plant growth.

1.3 SCOPE OF THE INVESTIGATION

As low concentrations of phenylurea herbicides are very toxic to green algae, these organisms are expected to be suitable tools in the development of rapid and simple bioassay techniques for these herbicides in soil. Their use may reduce the difficulties encountered with higher plant bioassays (Section 1.2.5)

Thus, green algae have been tested as assay organisms and the unicellular alga, chlorella strain NMI, selected for further investigations. The algal bioassay has been extended to other herbicides which inhibit photosynthesis.

Against the background of the preceding review which indicates the various factors affecting phytotoxicity of herbicides, the algal techniques have been used to study phytotoxicity of phenylurea herbicides in artificial and natural soil conditions. Moreover, the algal techniques have been adapted to recommend rates of application of herbicides in the field.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 ALGAL CULTURES AND GROWTH CONDITIONS

2.1.1 Culture medium

The culture medium, referred to in the text as nutrient medium or nutrient solution, contained per litre:

$MgSO_4 \cdot 7H_2O$	2.5 g	Na_2HPO_4	0.89 g
KH_2PO_4	1.25 g	NH_4NO_3	0.57 g
$CaCl_2$	0.147 g	Fe.Na.EDTA	0.024 g

In addition, 1 ml of a 1000-fold solution of trace elements was supplied:

NH_4VO_3	0.023 g	$MnSO_4 \cdot 4H_2O$	2.03 g
$NiCl_2 \cdot 6H_2O$	0.0405 g	$CuSO_4 \cdot 5H_2O$	0.0785 g
Na_2MoO_4	0.252 g		
$ZnSO_4 \cdot 7H_2O$	2.20 g		

The pH of the complete solution was adjusted to 6.8

2.1.2 Growth Room

All experiments were carried out in a growth room, under controlled conditions. Cultures were illuminated continuously at a temperature of $26^{\circ} \pm 1^{\circ}C$, with a light intensity of 650 - 680 ft. candles, using 40 w "Warm white" fluorescent tubes. The cultures were not shaken.

2.1.3 Test Organisms

The test organisms used are uni-algae as listed in Table 2. The organisms were maintained on 2% agar slopes containing nutrient medium.

2.1.4 Preparation of Inoculum

The algal cells for the experiments were obtained as follows: an inoculum taken from an agar slope was suspended in 300 ml sterile nutrient medium in a 750 ml Erlenmeyer flask. The cells were grown either in a Gallenkamp shaker water-bath at 26°C, illuminated by incandescent lamps, or in a growth room, illuminated by a bank of fluorescent tubes. The algal suspension was flushed with a stream of air containing 5% CO_2. After three days, the cells were harvested by centrifugation, usually at 10,000 g for five minutes at 0°C. The cells were washed once in distilled water, and resuspended in 20 ml fresh medium. This cell suspension was then used to inoculate the nutrient medium to the desired density for the experiments.

2.2 Algal Growth Estimation

(a) Measurement of turbidity

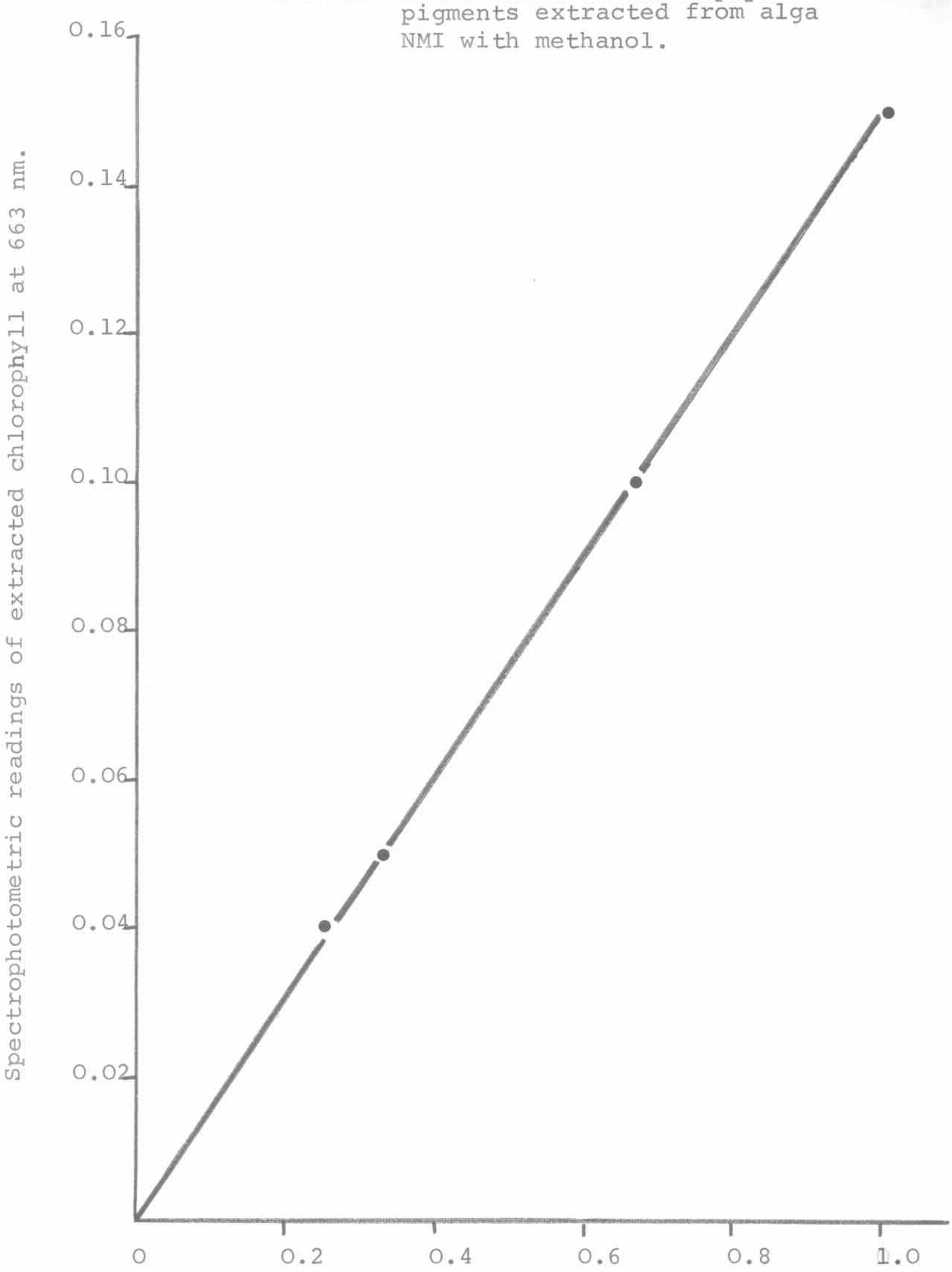
At the end of the growth period, the algal growth was measured by turbidimetry at 663 millimicrons, with a 1 cm path length cell in a Unicam SP600 spectrophotometer.

(b) Measurement of extracted pigments

At the end of the growth period, the contents of each experimental flask were transferred to a centrifuge tube and the supernatant discarded after centrifuging at 13,000 r.p.m. (20,200 g) for 10 minutes. About 0.3 gm of magnesium carbonate was added to the algal cells to minimise the degradation of pigments. 10 ml of methanol were added to the algal cell pellet in the centrifuge tube for pigment extraction. Another 10 ml methanol were poured into the experimental flask for pigment extraction of any algal cells remaining after transfer of the algal culture to the centrifuge tube. The closed centrifuge tubes and experimental flasks were left for 3 hours or overnight in the dark at $10^{o}C$. The contents of the flask were transferred to the centrifuge tube. A sample of the supernatant (methanolic pigment extract) after contrifuging at 12,000 g for 5 minutes, was used to read transmittance at 663 nm, with a 1 cm pathlength cell in a Unicam SP600 spectrophotometer.

The relation between spectrophotometer readings and the concentration of extracted pigments in arbitrary units is shown in Fig.1

FIG. 1. Measurement of chlorophyll pigments extracted from alga NMI with methanol.



2.3 HERBICIDES

Herbicides used are listed below together with information on the formulation of each chemical. For convenience, the common names recommended by the Terminology Committee of the Weed Society of America are used for the chemicals throughout this thesis. In the case of dalapon, the designation refers to the active agent although the sodium salt of the chemical was used. The formulation of 2,4-D contained the sodium salt. Stock preparations of herbicides in distilled water were stored in polythene containers at 5°C.

Common Name	Chemical Name	Formulation
Monuron	3-(p-chlorophenyl)-1, 1-dimethylurea	80% W.P.
Diuron	3-(3,4-dichlorophenyl)-1, 1-dimethylurea	80% W.P.
Neburon	1-butyl-3-(3,4-dichlorophenyl)-1 methylurea	80% W.P.
Dalapon	2,2-dichloropropionic acid	
2,4-D (Na)	(2,4-dichlorophenoxy) acetic acid (Na)	99% S.P.
Simazine	2-chloro-4, 6- bis(ethylamino)-s-triazine	50% W.P.
Atrazine	2-chloro-4-(ethylamino)-6-isopropylamino)-s-triazine	50% W.P.

Atratone	2-(ethylamino)-4-(isopropylamino)-6-methoxy-s-triazine	50% W.P.
Ametryne	2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-s-triazine	50% W.P.
Bromacil	5-bromo-3-sec-butyl-6-methyluracil	pure crystals
Triallate	S-(2,3,3-trichloroallyl) diisopropylthiocarbamate	Emulsifiable concentrate, (4lb/Imp. Gal)

W.P. = wettable powder

S.P. = soluble powder

2.4 SOILS

Soil samples were collected from Sydney University gardens (U_2), and different sites in New South Wales and South Australia. Clay from Chullora (N.S.W.) and peat from Mount Gambier (S. Australia) were also used in these experiments.

Composite soil samples were obtained by mixing 30 cores from the top 3 inches (7.6 cm) of each site. The soil samples were air-dried, crushed, and passed through a 2 mm mesh before use.

2.4.1 Analysis of soil properties

(a) pH

A Beckman glass electrode was used. The pH of each soil was measured in 2:5 soil - water mixture.

(b) clay and sand (Bouyoucos Method - 1936)

25 ml of 5% calgon (brought to pH 8.5 with $Na_2 CO_3$) and 200 ml distilled water were added to 30 g oven dry soil in a metal beaker. The mixture was shaken for 10 min., transferred to 500 cc graduated cylinder and made up to the mark with distilled water. Hydrometer readings were taken after 5 min., 60 min., and 120 min.,. Temperature correction was made by adding 0.3 g to the hydrometer reading for each $1^{o}C$ above $19.5^{o}C$, and subtracting 0.3 g for each $1^{o}C$ below. 3.0 g were subtracted for weight of calgon and $Na_2 CO_3$.

The supernatant suspension was discarded, and after repeated washings and decantations, the remaining sand was collected, dried and weighed.

(c) Organic carbon (Tinsley Method - 1950)

0.5 to 2.0 g of soil was used. 25 ml of a sulphuric acid - dichromate mixture (0.4N dichromate) were added to the soil in a 250 ml flask. The flask was fitted on to a cold thimble condenser on a hot plate and allowed to simmer for 45 min. After cooling, 100 ml water were added and the digest titrated with 0.4N ferrous sulphate. 2 drops of tri-orthophenanthroline ferrous sulphate were used as internal indicator.

From the equation

$$2\ Cr_2O_7^{--} + 3C + 16H^+ \rightarrow 4C_2^{+++} + 8H_2O + 3O_2$$

1 ml.N dichromate = 3 mg. carbon.

Percentage organic carbon content of each soil was calculated. Table 1 summarises the physical properties of soils analysed.

2.5 BIOASSAY METHODS

2.5.1 Liquid algal bioassay (Soil in medium)

The culture vessels were 100 ml or 150 ml Erlenmeyer flasks closed with beakers.

A small volume of herbicide solution (1 ml) was added to a small weighed sample of dry soil (2 gm) in the culture vessel to give the required final herbicide concentration. A range of 6 to 7 concentrations of herbicide in triplicate was used. After 2 hours, 19 ml of inoculated nutrient medium (10^5 algal cells/ml) were added, and the culture vessels incubated in the growth room. After 3 days, methanolic extracts of pigments were measured using a Unicam SP600 spectrophotometer at 663 millimicrons, with a 1 cm pathlength cell.

Table 1. Physical properties of soil

Soils	Organic carbon % (Tinsley)	Sand % (2.0-0.5mm)	Silt % (2.0-.002mm)	Clay % (<.002mm)	pH
Wolverton A (NSW)	2.64	37.10	30.80	33.0	5.0
Wolverton B (NSW)	1.35	31.70	24.30	41.7	5.0
Wolverton C (NSW)	0.98	43.30	24.80	38.4	5.0
Cobbity A (NSW)	0.69	42.15	12.63	47.7	5.2
Mt. Tomah (NSW)	4.80	48.65	28.08	30.5	4.8
Cobbity B (NSW)	2.95	54.93	13.25	34.7	6.0
Prospect (NSW)	3.39	33.40	17.80	61.3	5.5
Robe (SA)	2.55	84.00	5.00	7.2	7.7
Seddon (SA)	1.53	66.00	5.83	13.8	5.7
B.Springs (SA)	1.73	93.00	0.83	3.8	5.8
Cobbity C (NSW)	0.43	46.30	20.20	56.5	5.4
Cobbity D (NSW)	1.53	62.07	17.73	21.2	5.8
Cobbity E (NSW)	0.57	48.85	11.00	52.3	6.6
Wolverton D (NSW)	4.16	26.80	33.80	43.5	5.7
Wolverton E (NSW)	2.29	36.70	20.40	45.6	5.6
Wolverton F (NSW)	1.82	49.00	23.60	30.6	5.8
Waite (SA)	1.63	41.00	25.00	26.3	5.7
Turretfield (SA)	1.30	65.00	8.30	19.7	7.1
Ashbourne (SA)	2.86	24.33	22.50	42.2	7.5
Monarto (SA)	0.71	87.33	5.00	9.2	6.2
Parafield (SA)	1.68	59.00	14.20	18.8	5.9
Mount Burr (SA)	0.58	97.67	3.30	1.33	8.0
Moon Hills (SA)	1.15	91.30	1.67	1.33	5.5
Mt. Compass (SA)	1.12	95.33	2.5	0.50	5.3

2.5.2 Algal assay - Aqueous extracts

2 gm sample of air dry soil was mixed with 12 ml of nutrient medium containing 12 - 240 µg of herbicide. Five concentrations of herbicide and a control without herbicide were used. The mixture was shaken on a mechanical wrist shaker for 45 minutes, at room temperature (22°C). After shaking, 0.5 g Potassium sulphate was added as a flocculent (Hance, 1965). The liquid was then separated from soil particles by centrifuging at 12,000 rpm (17,300 g) for 10 minutes in a Sorvall RC-2 centrifuge with SS - 34 rotor. The amount of unadsorbed herbicide in the supernatant was then estimated by the algal technique. (Section 2.2)

2.5.3 Paper Disc Technique

50 ml of a 3-day alga NMI culture were centrifuged at 12,000 g for 10 minutes. The supernatant liquid was discarded, and the packed cells suspended in 1 ml of nutrient algal medium. This heavy suspension of alga NMI was used as the inoculum.

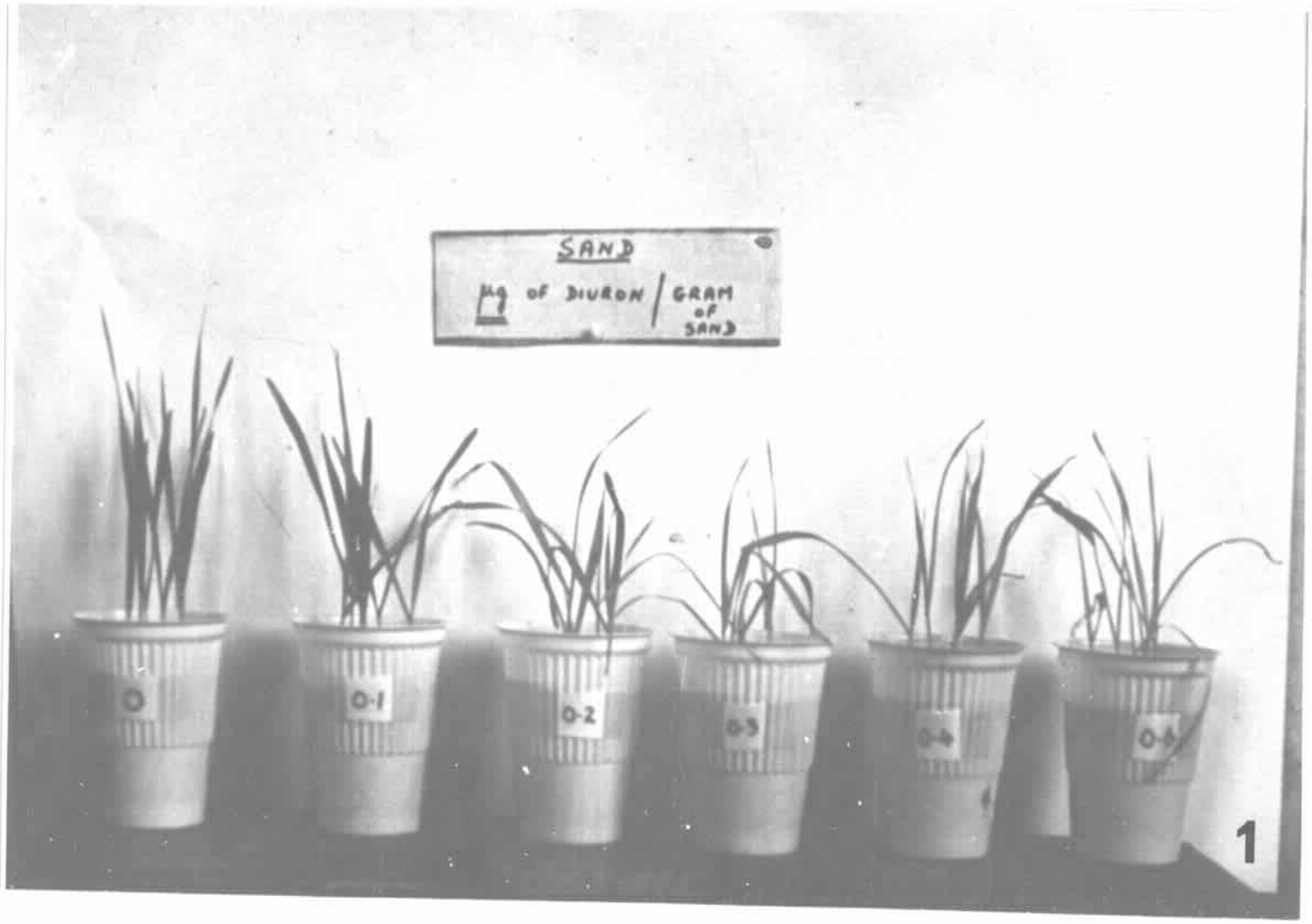
A weighed sample of soil was placed in a Petri dish and levelled to present an even surface. The soil was saturated with herbicide solution (in nutrient medium) to give the required concentration and allowed to reach equilibrium for 2 hours. An inoculating needle was used to deposit a point source inoculum on a disc (12.7 mm diameter) of highly absorbent filter paper (Penicillin assay type, No.740-E manufactured by Carl Schleicher & Schuell Co., Keene, N.Y., U.S.A.) The dry inoculated

disc was placed on a dry clean one and both placed on the herbicide treated soil surface. Three replicates were used in each Petri dish.

The dishes were sealed with white petroleum jelly, and incubated in the growth room for 24-30 hours. Visual estimates of inhibition of algal growth were recorded.

2.5.4 Oats - Pot trials

Algerian oats were used as indicator plants and stock solution of diuron was prepared from the 80% wettable powder formulation. Each assay was performed in duplicate. Samples (180 g) of the air-dried soils were dispensed in plastic cups. The stock solution of herbicide was diluted with nutrient medium, and applied directly to the soil - culture surface to give the required herbicide concentrations at field capacity. There were no holes in the bottom of the cups and thus no drainage was allowed. This system of watering was found to give excellent plant growth (Plate 2.1). Three hours after addition of the herbicide, 10 oat seeds, pre-germinated (30 hrs) on moist filter paper in covered glass petri dishes, at room temperature in darkness and high humidity, were transferred to the cups. The plants were grown under fluorescent light which provided an illumination of 365 ft. candles. The photoperiod was 16 hours at 28°C in the light and 8 hours at 16°C in the dark.


SAND
µg OF DIURON / GRAM OF SAND
0
0·1
0·2
0·3
0·4
0·6
1

The test containers were randomized on the growth room bench. A set of standards with acid washed sand was grown under the same conditions to provide a control for the data obtained with the herbicides in the soil series. The plants were watered daily with a diluted (1/20) nutrient solution. After germination the plants were thinned to 6 plants per cup. After three weeks, the plants were cut at the soil surface and their fresh or green weights were recorded.

To compare the effect of different soil types and diuron on shoot growth of oats, ED_{50} (equivalent dose for 50% response) values were calculated for each concentration series. This was done by plotting the fresh or green shoot weight as a percentage of the control against the logarithm of the herbicide concentration and a curve of best fit was drawn through the points. The logarithm of the herbicide concentration, which corresponded to 50% shoot growth was the logarithm of ED_{50}. The antilogarithm of this value gives the ED_{50} value in μg of diuron per gramme of soil.

In the above procedure, where percentages were graphed and the curve fitted by eye, the error of estimation for the estimates could not be determined. Observations from a preliminary trial seemed to indicate that the curve could be of the form $y = a\ b^{x}$ or $\log y = \log a + x \log b$

where y = estimated % response of the test organism

and x = concentration of herbicide used.

A transormation of the observed data to logarithms was done before fitting by least squares. The adequacy of the transormation was checked by an analysis of variance. The transformed data gave a straight line relationship. (Appendix A)

The transformation procedure was adopted in the determination of diuron ED_{50} values for both oats and algae trials in the soil - herbicide studies.

CHAPTER 3

SELECTION OF TEST ALGAE FOR HERBICIDES

3.1 INTRODUCTION

Many small seeded test plants such as oats, barley, mustard, wheat, soybeans, have been used by investigators for the biological assay of herbicide residues in soil, but the phenylurea and s-triazine herbicides, which act mainly as photosynthetic inhibitors, can only exert their full toxic effects on seedling plants at a late stage of seedling growth. Consequently bioassay methods for such herbicides may take 2-4 weeks and the result may sometimes be complicated due to dissipation of the herbicide during the assay period. In recent years, attempts have been made to develop rapid bioassay methods for these herbicides. Parker (1965) made use of the interaction between monuron and diquat (Mees, 1960) in the bioassay of phenylurea herbicides. With Lemna minor as a test plant, he reported that within the short experimental time of 4 days, he was able to detect monuron and diuron within the range of 4 oz. to 8 oz./ac (0.28 to 0.50 Kg/Ha) mixed in 2 in. (5.1 cm)of soil. However, the technique is complicated by the use of two herbicides, the choice of the correct amount of diquat to be used, and control of the factors governing the effects of diquat on the test organism.

In view of the lack of a suitable, rapid and simple bioassay, it was thought that photosynthetic microorganisms (algae) could be used to develop an assay technique for the phenylurea herbicides.

Algae have already been tried as test organisms for herbicide bioassay. Fitzgerald (1962) suggested that algae might be used as bioassay organisms for toxic chemicals in aqueous media. Atkins (1963) used an algal method, adapted from Tchan's method (1956) to detect residues of simazine and amitrole in nutrient solution and soil. Pillay (1964) reported that small amounts of phenylurea and s-triazine herbicides could be assayed in nutrient media by using unialgal cultures isolated from soil. Price and Estrada (1964), used a pure culture of Euglena gracilis to assay amitrole, while Gramlich and Frans (1964) investigated the phytotoxicity of a s-triazine in nutrient solution with a pure culture of Chlorella pyrenoidosa. Atkins (1965); Atkins and Tchan, (1967); Pillay and Tchan (1969) reported that the algal technique with small green algae NM1 and 1446/4 as test organisms, could be used for the bioassay of s-triazine and phenylurea herbicides.

As most of these trials have been carried out in aqueous media, the potential of algae as test organisms in rapid bioassay of herbicides in soil and in studies of soil-herbicide relationship is not known. In the present study,

several small algae have been tested against herbicides to select the most sensitive species to be used in the development of simple assay techniques for herbicides in soil.

3.2 Preliminary Selection of Test Organism

Small McCartney bottles containing 10 ml of nutrient solution inoculated with each of the uni-algal cultures (as listed in Table 2) were incubated in the growth room. 0, 1 and 5 p.p.m. of each of the following herbicides were used: dalapon, 2,4-D, monuron, diuron and neburon.

After 4-10 days, visual estimates of algal growth were made.

The results (Table 2) showed that some algae grew faster and were more sensitive to the herbicides than others. No appreciable differences in algal growth could be detected between the control (no herbicide) and the different levels of dalapon and 2,4-D in this experiment. Complete or partial inhibition occurred at 5 p.p.m. level of the phenylurea herbicides.

3.3 Sensitivity of the Selected Algae to phenylurea Herbicides

The sensitivity of the herbicides was tested quantitatively by the measurement of extracted pigments. Chlorella sp., Chlamydomonas sp. and alga NM1 had fast growth rates and were more sensitive to the phenylurea herbicides than the other algae (Table 2). They were therefore selected for this experimentation.

TABLE 2

Sensitivity of some uni-algal cultures to different herbicides

Organism	Source	Period of Incubation (days)	Sensitivity to herbicide in ppm*									
			Dalapon		2,4-D		Monuron		Diuron		Neburon	
			1	5	1	5	1	5	1	5	1	5
Alga NMI	Isolated from soil	4	-	-	-	-	++	++++	++++	++++	++++	++++
Chlorella sp.	Departmental algal collection	4	-	-	-	-	++	++++	++	++++	++	++++
Scenedesmus sp.	"	7	-	-	-	-	-	++	-	++	-	++
Chlorococcum sp.	"	7	-	-	-	-	++	++	++	++	++	++
Chlamydomonas sp.	Isolated from soil	4	-	-	-	-	++	++	++	++	++	++
Euglena sp.	Departmental algal collection	7	-	-	-	-	-	++++	++	++++	-	++++
Botrydiopsis sp.	"	4	-	-	-	-	++	++	++	++	++	++
Nostoc sp.	"	7	-	-	-	-	++	++	++	++	++	++
Anabaena sp.	"	7	-	-	-	-	++	++	++	++	++	++

* Visual estimation of algal inhibition by herbicides.

No inhibition -

Partial inhibition ++

Complete inhibition ++++

TABLE 3

Effects of urea herbicides on Chlamydomonas sp., Chlorella sp. and alga NMI

Herbicide conc.(ppm)	Algal Growth (expressed as % growth of control)								
	Monuron			Diuron			Neburon		
	Chlam.	Chlor.	NMI	Chlam.	Chlor.	NMI	Chlam.	Chlor.	NMI
0	100	100	100	100	100	100	100	100	100
0.001	-	-	-	105*	-	-	-	-	-
0.010	109*	-	-	101	-	-	104*	109*	-
0.050	-	-	-	-	-	60	-	-	60.5
0.063	-	-	-	-	-	50	-	-	-
0.075	-	-	-	-	-	-	-	-	50
0.080	109*	-	-	-	-	-	-	-	-
0.100	109*	104*	88	60	60	31	65	56	40
0.126	-	-	-	-	50	-	-	50	-
0.133	-	-	-	50	-	-	-	-	-
0.168	-	-	-	-	-	-	50	-	-
0.200	87	89	56	37	33	22	45	32	21
0.224	-	-	50	-	-	-	-	-	-
0.500	50	49	16	16	11	4	30	11	4
0.800	32	22	-	8	4	0	10	4	0
1.000	-	-	-	0	0	0	0	0	0

* Significant stimulation of algal growth

The results are summarised in Table 3. Alga NM1 proved to be superior to Chlorella sp. and Chlamydomonas sp. and was preferred for further experiments. At very low concentrations (0.001-0.01 p.p.m.) the phenylurea herbicides had stimulatory effects on the three test organisms. At higher concentrations (0.1-1 p.p.m.) they had a strong inhibitory effect on the algae. The algae showed measurable differences in growth over a range of concentrations of the herbicides (0.05-1 p.p.m.)

3.4 Discussion

For a comparison of the influence of the herbicides on the test organisms, sensitivity had been expressed as percentage of growth of control at a given concentration. Table 3 indicates the difference in sensitivity.

To compare the relative toxicities of the herbicides on the test organisms, the concentration of 50% inhibition was used (Table 3).

From the table it is apparent that alga NM1 was very sensitive to the phenylurea herbicides, as compared with the two other test organisms. There was no marked difference in sensitivity to monuron between Chlorella sp. and Chlamydomonas sp. When diuron and neburon were tested Chlorella sp. was more sensitive than Chlamydomonas sp.

Sheets and Crafts (1957) investigated the inherent toxicities of phenylurea herbicides, using oats in culture

solutions containing varying concentrations of the herbicides. Their results showed that diuron was nearly twice as toxic as monuron. Coggins and Crafts (1959), using barley in nutrient solution, also found diuron to be twice as toxic as monuron to the test plant. The results obtained with the algal technique compared favourably with the plant responses.

A few herbicides have been found to show stimulatory effects on plants at very low concentrations. Ashman (1964) in laboratory studies of the effects of chloramben (3-amino-2,5-dichlorobenzoic acid) on soybeans, reported that shoot growth of soybeans was stimulated by low concentrations of the herbicide. Atrazine and simazine stimulated algal and plant growth at low concentrations (Atkins, 1965; Swietochowski et al, 1964. Similar stimulatory effects by very low concentrations of phenylurea herbicides on alga were shown in the present study (Table 3). This stimulatory response could be attributed to

(i) the phenylurea herbicides might stimulate certain microorganisms and thus provide greater quantities of nutrients for algal growth, or

(ii) in very low concentrations the herbicides act as plant growth stimulators.

Sheets (1964) pointed out that in the presence of phenylureas, growth of soil microorganisms was not stimulated. He suggested

that certain microorganisms may utilise the phenylureas but not preferentially or selectively to other sources. Our results support hypothesis (ii). Since stimulatory responses were obtained with pure algal cultures (free of bacteria) the effect of the herbicide at very low concentrations is more likely to be due to the direct influence of the molecule on algal growth.

The work of Yuen and Hilton (1962) on Hawaiian sugar-cane soils, had shown that the intrinsic phytotoxicities of monuron and diuron were both such that less than 2 p.p.m. was needed in the soil solution for most weeds to be killed. As all three algal test organisms were completely inhibited at a herbicide concentration of less than 2 p.p.m. (monuron, diuron and neburon) and were sensitive to 0.1 p.p.m., these algae would be suitable as test organisms to assess the herbicides in soil.

3.5 Summary

(1) The three algae, Chlamydomonas sp., Chlorella sp. and alga NM1 are suitable as test organisms for the bioassay of phenylurea herbicides. They give measurable differences in growth over a wide range of low concentrations of the herbicides.

(2) Alga NM1 is the most sensitive test organism. Fifty percent inhibition of growth is produced by 0.063, 0.075 and 0.224 p.p.m. of diuron, neburon and monuron

respectively.

(3) Very low concentrations (0.001 to 0.01 p.p.m) of the phenylurea herbicides have stimulatory effects on the alga. It is suggested that the stimulatory response is due to a direct effect of the chemicals.

CHAPTER 4

EFFECTS OF CULTURAL CONDITIONS ON THE RESPONSE OF TEST ALGAE TO UREA HERBICIDES

4.1 Introduction

As reviewed earlier (Section 1.2.5) the precision of a bioassay to determine small concentrations of a herbicide depends to a large extent on the control of the factors that can introduce variability into the results. In the present study, the effects of inoculum size, period of incubation, and pH on the sensitivity of the test algae to phenylurea herbicides were investigated.

4.2 Inoculum size and period of incubation

The experiment was set up in liquid medium containing 10^6 and 5×10^7 cells/ml of alga NMI, and concentrations of 0 and 0.5 ppm diuron, were used. 10 ml samples were taken every day and growth of algae was followed by optical density measurements at 663 millimicrons.

The results (Table 4) clearly showed that inhibition of alga NMI was lower at the higher inoculum density. They also indicated that algal inhibition by 0.5 ppm diuron was highest after 3 days incubation, and decreased with increasing incubation time.

Table 4

Effect of inoculum size and period of incubation in inhibition of algal culture NMI by diuron

Time in days	Inhibition of alga (expressed as % of control) by diuron (0.5 p.p.m.)	
	10^6 cells/ml	5×10^7 cells/ml
2	78.4	66.8
$2\frac{1}{2}$	84	76
4	77	69
5	67.8	55
$6\frac{1}{2}$	59.4	45.3
7	43.3	35.7
8	36.1	29.3
9	27.7	25
10	24.5	21.3

4.3 pH

Algal nutrient media at the following pH values were used: 5.4, 6.0, 6.5, 7.0, 7.5 and 8.0. The pH of the solution was adjusted with IN NaOH or IN HCl. The levels of herbicides used were for monuron (0.5 ppm), for diuron (0.1 ppm) and for neburon (0.1 ppm). 20 ml samples of treated and untreated media (inoculated with 10^6 NMI cells/ml) for each pH were incubated in the growth room for 3 days. Growth was estimated by measurement of chlorophyll. (Section 2.2)

It was found that variation of inhibition for the three herbicides did not exceed 10% between pH values of 6.5-7.5 and only 15% from pH 5.4 to 8.0. The alga NMI showed optimum growth at pH 7.0-7.5.

4.4 Discussion:

From the results it was clear that the period of incubation and inoculum size influenced the inhibition of the algae by the s-phenylurea herbicides. Atkins and Tchan (1967) had shown that the sensitivity of algal bioassay of s-triazine herbicides was increased by reducing the size of the inoculum. The present results showed that the inhibition of alga NMI by diuron decreased with increasing inoculum density. Maximum inhibition occurred after 3 days incubation, and percentage inhibition decreased as incubation continued. These results showed that the maximum sensitivity for herbicide bioassay with alga NMI might be obtained with a light inoculum, and by measuring algal inhibition after a 3 day incubation period.

Selman and Upchurch (1963), using cotton plants grown in culture solutions at pH 4, 5, 6 and 7, found that diuron tended to be more toxic at higher pH values. The present results showed only a slight increase in toxicity of the herbicides as the pH increased from 6.0 to 7.5. At pH 5.4 and 8.0 a decrease in toxicity was noticed. However, as the variation of inhibition produced by pH from 5.4 to 8.0 was only 15%, it could be considered that alga NMI was suitable to study phytotoxicity of these herbicides in agricultural soils, where the pH is rarely as low as 5.0 and as high as 8.0. Upchurch et al (1971) had also indicated that with diuron, soil pH levels between 4.3 and 7.5 had no effect on the phytotoxicity of the herbicides.

4.5 Summary

(1) The sensitivity of the algal bioassay is affected by the period of incubation, and size of inoculum. Best results are obtained by using a small inoculum (aaprox. 10^5 cells/ml) and by measuring algal growth after a 3 day incubation period.

(2) The pH of the medium (6 and 7.5) does not markedly affect the toxicity of the herbicides. Alga NMI is considered to be a suitable organism for the bioassay of phenylurea herbicides in agricultural soils.

CHAPTER 5

APPLICATION OF ALGAL TECHNIQUES IN THE STUDY OF PHYTOTOXICITIES OF DIFFERENT HERBICIDES (PHOTOSYNTHESIS INHIBITORS)

5.1 Introduction

Upchurch (1966) pointed out that to a large extent the study of herbicide behaviour in soil is a study of the influence of soil properties and other related phenomena on the inherent phytotoxicity of herbicides. Unfortunately, very little work has been done to determine the inherent phytotoxicities of herbicides in the absence of soil factors. Therefore, in this investigation of a rapid bioassay tool to study soil - herbicide relationships, it is important to establish the inherent toxicities of those herbicides which can be assayed by algae. Such a study will help in a better understanding of the effects of soil properties on the activity of these herbicides in soil.

5.2 Inherent toxicities of herbicides (photosynthesis inhibitors) to test algae

The herbicides used were: monuron, diuron, neburon, atrazine, bromacil, simazine, atratone and amitrole. All of these herbicides inhibit photosynthesis.

Samples (20 ml) of the inoculated medium (alga NMI - 10^5 cells/ml) containing the required concentration of herbicide, were incubated in the growth room for 3 days. The algal growth was then estimated by optical density measurements. (Sect. 2.2)

5.3 Results

The results are summarized in Table 5. The differences in sensitivity towards different herbicides by the alga suggested that the technique would not be suitable for the detection of low concentrations of some herbicides, but excellent for others, e.g. phenylurea herbicides.

Table 5

Inherent toxicities of herbicides

Herbicides	Concentration causing 50% inhibition of growth of alga NMI (in ppm)
Diuron	0.050
Neburon	0.063
Monuron	0.180
Bromacil	0.32
Atrazine	0.73
Simazine	0.83
Atratone	1.50
Amitrole	7.10

Table 6

Inhibition of Chlamydomonas and alga NMI by phenylurea herbicides.

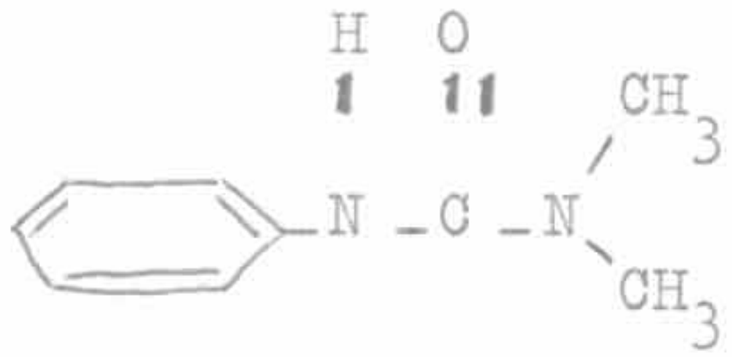
Herbicide	Concentration * ($x10^{-6}$M) causing 50% inhibition of alga NMI	
	Chlamydomonas	Alga NMI
Monuron	2.5	1.13
Diuron	0.57	0.27
Neburon	0.61	0.28

* Data taken from table 2

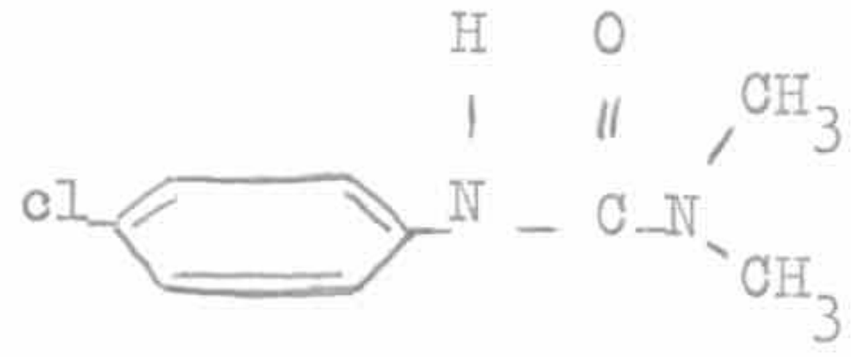
5.4 Discussion:

A knowledge of the inherent toxicity of a herbicide to a test plant can be useful as a guide to field experiments. Laboratory methods have been devised to evaluate and compare the inherent phytotoxicities of herbicides, by using sensitive test plants.

The present experiment was carried out to determine the inherent phytotoxicities of a number of soil-applied herbicides, which inhibit growth by interfering with the photosynthetic processes in plants. The minimum concentration which caused 50% inhibition of alga NMI by these herbicides in nutrient solution was determined.



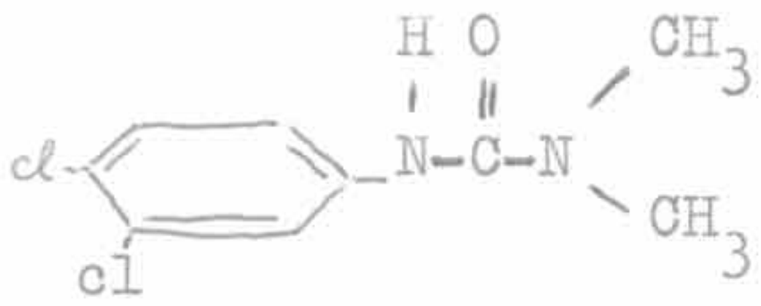
Fenuron



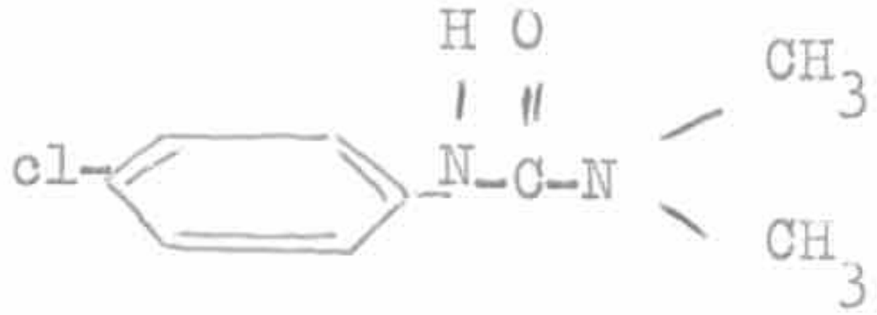
Monuron

When N-alkyl substitutions were the same, the dichoro-compound was more phytotoxic than the monochloro-compound, as in the case of diuron being more toxic than monuron.

In the present study, nearly four times as much monuron as diuron were needed to bring about 50% inhibition of alga NMI and chlamydomonas.



Diuron

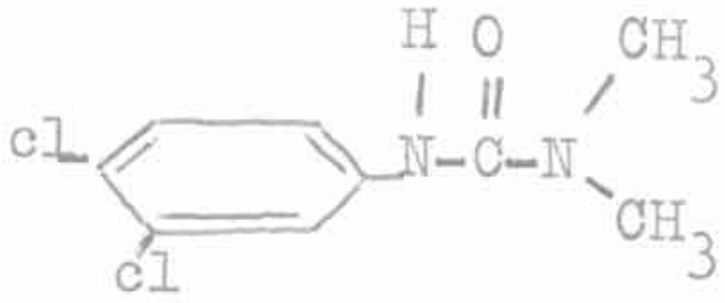


Monuron

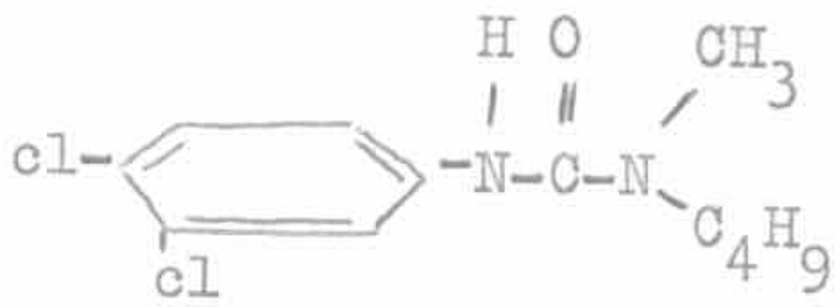
Coggins and Crafts (1959) found that diuron was more toxic than neburon in nutrient solution, and suggested that 2 methyl groups were necessary for maximum phytotoxicity to be expressed, and that the replacement of one of the methyl groups in a dichloro-compound lowered phytotoxicity. But, the results obtained with algae as test organisms were not in accordance

with Coggins and Crafts (1959) results. Neburon was found to be nearly as toxic as diuron to alga NMI and chlamydomonas in nutrient solution (Table 6).

When applied to higher plants, the phenylurea herbicides are absorbed by the roots and transported to the leaves through the xylem vessels. The herbicides must pass through the epidermis, cortex and endodermis of the root to the xylem and upward to the leaves where they exhibit their phytotoxic effects. Ennis (1964) pointed out that membranes of different plant species appear to be penetrated more readily by some compounds than by others. This difference in the translocation of materials to the leaves may be one of the factors affecting the sensitivities of different test organisms. Coggins and Crafts (1959) studied the behaviour of neburon in model systems and demonstrated that neburon was more strongly adsorbed to cellulose than other phenylurea herbicides. This greater adsorption of neburon to cellulose may partly determine to what extent the herbicide will penetrate to the site of action, and thus explain its lower toxicity to barley than diuron (Coggins & Crafts, 1959). On the other hand, with the small unicellular algae, problems of translocation, immobilisation at nodes are non-existent compared with higher plants. Consequently it was found that neburon was nearly as toxic to alga NMI and chlamydomonas. The present results suggested that substitution on the N-alkyl group did not affect inherent phytotoxicity but increased adsorption. The data



Diuron



Neburon

also suggested that the interpretation of the relationship between molecular structure and inherent toxicity at cellular level could not be satisfactorily derived if whole plants were used. Algal cells would provide a better experimental tool to this problem.

5.5 Summary

(1) The alga NMI is suitable to evaluate the inherent phytotoxicities of herbicides which are known to inhibit plant growth by interfering with photosynthesis. The following toxicity series is obtained: Diuron > neburon > monuron > bromacil > atrazine > simazine > atratone > amitrole.

(2) The algal technique can be useful for the rapid screening of soils containing high or low active amounts of the less sensitive herbicides (e.g. simazine, atratone, amitrole).

(3) Substitution on the N-alkyl group does not affect the inherent toxicity of the phenylurea herbicides, but substitution on the aryl group increases phytotoxicity.

CHAPTER 6

INFLUENCE OF SOIL ON THE ACTIVITY OF THE PHENYLUREA HERBICIDES

6.1 Introduction

The effectiveness of residual soil - applied herbicides depends to a great extent on their inherent phytotoxicities, their persistence and their leaching characteristics. Adsorption on to soil colloids is the main factor which determines these characteristics. Previous studies have indicated that several soil properties may be involved in adsorption. With regard to the phenylurea herbicides, most workers agree that soil organic matter is the major factor in adsorption. Opinions differ however, as to the degree of importance of clay colloids in adsorption of these herbicides (Sheets, 1958; Upchurch, 1958; Hilton and Yuen, 1963; Upchurch and Mason, 1962; Upchurch et al, 1966).

In general, herbicides adsorbed to soil colloids are not freely available to plants (Holly, 1962). Hannah (1954), however, claimed that CDAA (N, N-diallyl-2-chloroacetamide) and CDEA (2-chloro-N, N-diethylacetamide) were adsorbed by soil constituents but still remained available to plants. It was therefore thought advisable to check the availability of soil - adsorbed phenylurea herbicides to the test algae.

In this study, the algal bioassay method has been adapted to demonstrate: (1) that adsorption of phenylurea herbicides must be taken into account in calculating the amounts of these herbicides added to soil to give the desired amount

of the free and effective compound in the soil, (2) the effect of soil organic matter on the phytotoxicity of diuron, (3) the independent adsorptive capacities of peat (organic matter) and clay for diuron and neburon.

6.2 Effect of adsorbed herbicide on the test algae

2 g samples of University garden soil (U_2) were put in cellophane dialysis tubes tied at one end. One millilitre of monuron solution was added to give the following concentrations: 0, 6, 10 and 20 µg of herbicide per g of soil. The tubes were closed, and after 2 hours were placed in culture vessels containing 19 ml of inoculated nutrient medium (NMI - 10^5 cells/ml). Another series of flasks was set up, without cellophane tubes. Inoculated nutrient medium was added 2 hours after addition of monuron to the 2 g soil samples. The flasks were incubated for 3 days in the growth room. Table 7 summarises the results, obtained by measurement of pigment extracts.

Table 7

Effect of adsorption on toxicity of monuron in soil

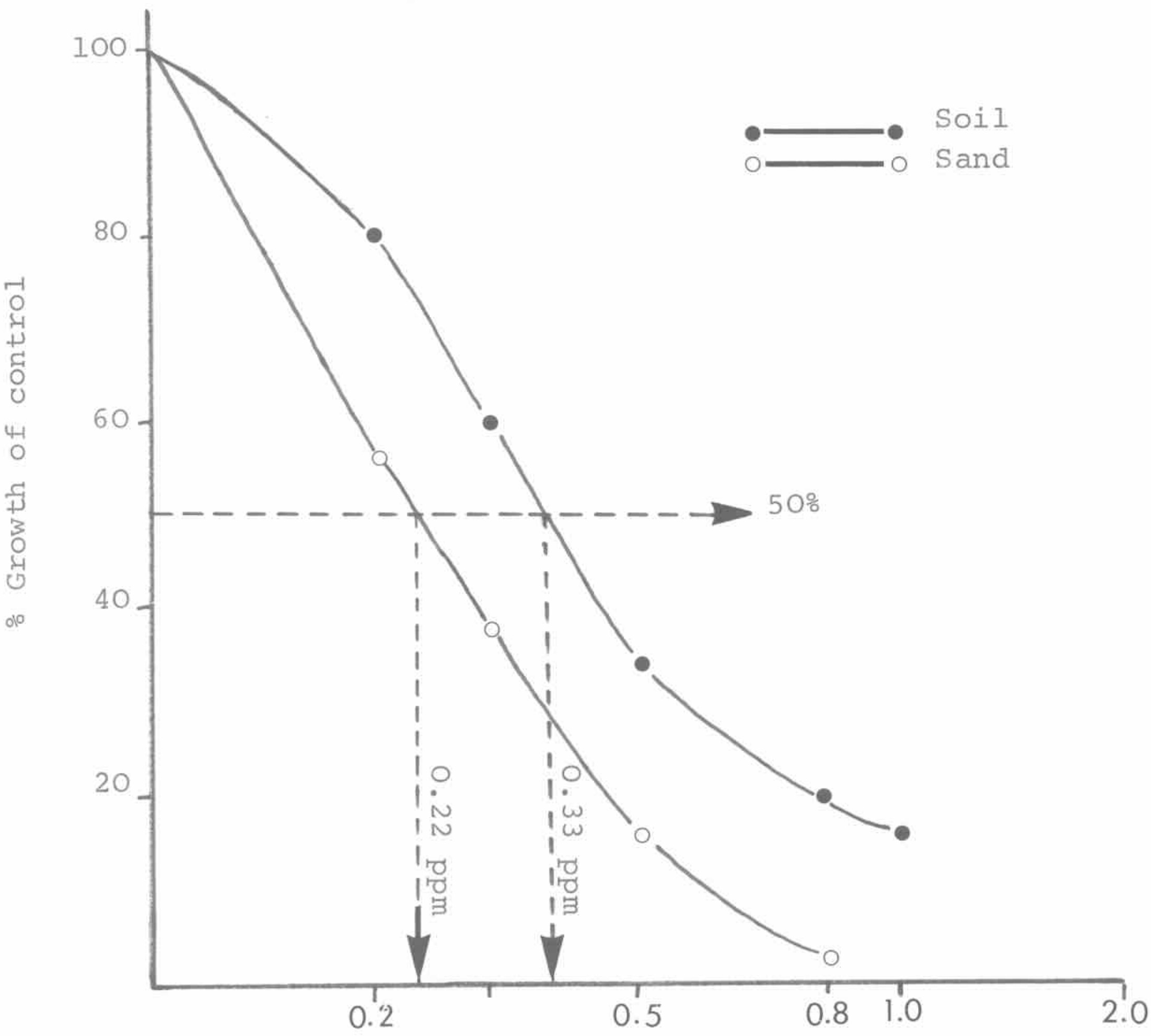
Amount of monuron in soil (μg/g)	Soil in cellophane bag % inhibition	Soil without cellephane bag % inhibition
0	0	0
6	24.2	17
10	90.8	87.6
20	100	100

6.3 Algal bioassay of monuron in soil

The experiment was set up with University garden soil (U_2) and washed sand containing monuron at different concentrations. Alga NMI was used for the bioassay. Figure 3 shows the % inhibition on alga NMI by various concentrations of monuron after 3 day incubation.

The figures shows that, to a slight degree, the soil had inactivated the herbicide. While 50% algal inhibition by monuron was obtained at 0.22 ppm in sand, it required 0.33 ppm in soil for the same inhibition. Furthermore, the minimum concentration of active monuron in soil solution (causing 20% inhibition of algae) which could be detected was 0.13 ppm.

FIG.3. Algal bioassay of monuron in soil



6.4 Aqueous extract method

The experiment was set up, as described in Methods and Materials (Section 2.5.2). The culture vessels were removed from the growth room after 3 days, and pigment extraction and measurement carried out. Growth was measured as the absorbancy at 663 nm. The results are expressed as a percentage of growth obtained without herbicide. The herbicide concentration which caused inhibition in the supernatant and diluted supernatant was derived by interpolation on a control growth - dose curve. Table 7a summarises the results and calculations. The results were evaluated using the Freundlich isothermal relationship -

$$\log \frac{x}{m} = \log K + n \log c$$

where $\frac{x}{m}$ is the amount of herbicide (in μg) absorbed by 1 gram of soil at equilibrium solution concentration c (in ppm). The constant K represents the concentration adsorbed on soil ($\mu g/g$) when in equilibrium with a herbicide solution of unit concentration, and can be used as an index of adsorption.

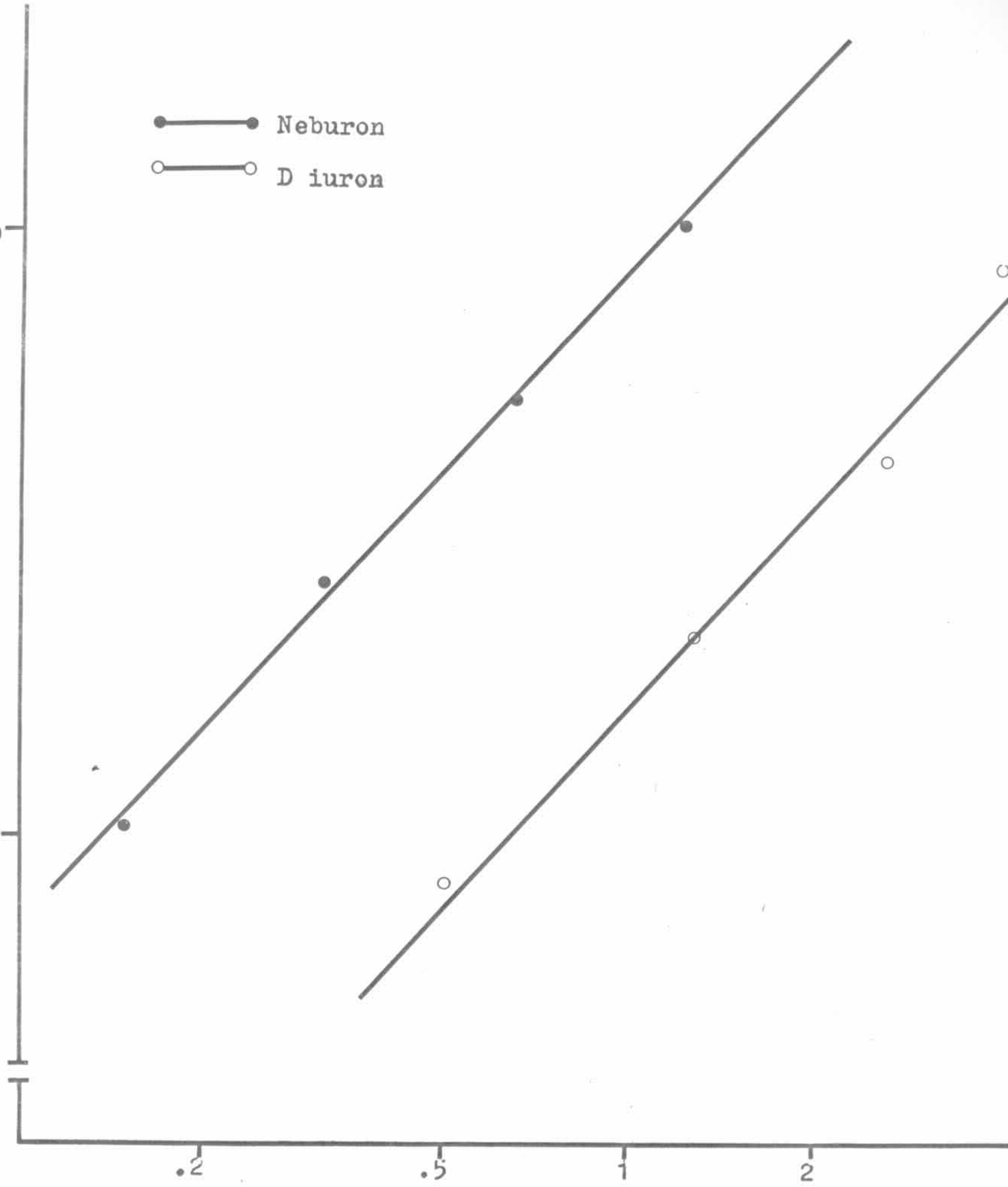
The value n obtained from the slope of the plot of $\log \frac{x}{m}$ against log c expresses the degree of linearity of the isotherm. When n = 1.0, the relationship between adsorption and equilibrium concentration is linear. Then K is independent of concentration and can be measured at any equilibrium concentration.

TABLE 7a. Adsorption of Diuron and Neburon by soil (U_2)

Supernatants from herbicide treated 2g. soil samples, were incubated with inoculated medium (alga NMI 10^4 cells/ml)

	Conc. of herbicide in supernatant (ppm) derived by interpolation on a control growth curve.	Amount of herbicide in µg used	Amount of herbicide in solution in µg	Amount of herbicide absorbed	X/m in µg	"C" in in p.p.m.
Diuron	-	-	-	-	-	-
	-	12	0	-	-	0
	0.45	24	6.0	18.0	9.0	0.5
	0.54	60	14.4	45.6	22.8	1.2
	0.56	120	30	90	45	2.5
	0.30	240	48	192	96	4.0
Neburon	-	-	-	-	-	-
	-	12	0	-	-	0
	0.14	24	1.86	22.14	11.07	0.155
	0.14	60	3.73	56.27	28.14	0.310
	0.14	120	7.50	113.5	56.75	0.625
	0.09	240	14.4	225.6	112.8	1.2
	0.07	240	14.0	220	113	1.17

FIG. 4. Adsorption Isotherms of diuron and neburon.



Adsorption isotherms were plotted in Figure 4 Straight lines based on 4 points were obtained for the herbicides.

Since the n values in both cases were close to unity, for practical purposes, K values for diuron and neburon were 18 and 98 µg per gram of soil.

These results were obtained under the experimental conditions of a fixed soil solution ratio and varying herbicide concentrations. They showed that members of the same group of herbicides were differently adsorbed by soil particles. Neburon was much more strongly adsorbed than diuron.

6.5 Effects of soil depth on the phytotoxicity of diuron

In this experiment alga NMI and oats were used as test organisms. Soil samples from different depths in one area were used. The differences between samples resided mainly in organic matter content.

The trials with oats and algae were set up as described in Materials and Methods (Sections 2.5.1 and 2.5.4) During the oats trial, it was observed that the very young oat seedlings grew equally well regardless of the concentration of the herbicide. Little difference existed between treatments until 5 - 7 days after emergence, when chlorosis of the leaf tips and leaf margins occurred in plant grown in herbicide treated soils.

Table 8

Effect of top and subsoils on toxicity of diuron on oat seedlings and alga NMI

Soil (depth)	WOLVERTON I. (0-7")*	WOLVERTON II. (8-15")	WOLVERTON III. (16-26")
pH	5.0	5.0	5.0
Organic Carbon %	3.59	2.64	0.98
Clay & silt %	65.8	63.8	63.2
Sand	39.5	36.5	43.3
% Inhibition of oat seedlings by diuron (µg/g soil) 0	0	0	0
0.8	0	0	12.5
1.33	0	19.5	40.5
2.66	30	54	87.2
3.33	46	74.5	92
3.99	100	100	100
Diuron (µg/g of soil) producing 50% inhibition of oat seedlings.	3.43	2.62	1.55
Diuron (ug/g of soil) producing 50% inhibition of alga NMI	7.2	4.8	3.37

* 1 inch = 2.54 cm.

Plants grown in soil containing higher concentrations of herbicide exhibited a very rapid spread of chlorosis until the entire leaf surface was affected and the plants died. A similar chlorosis occurred at lower concentrations, but its spread was not as rapid and many plants recovered and later grew normally. The green weights of harvested oat plants and the methanolic extracts of pigments of the alga were used to measure the inhibition caused by the different treatments. The herbicide concentrations causing 50% inhibition of oats and alga NMI were obtained by interpolation on the growth inhibition curves. Table 8 summarises the results of these experiments.

The amounts of diuron required to produce 50% inhibition of each test material decreased with increasing soil depth. A consideration of the organic carbon content of the three soils showed that the top soil had the highest carbon content and required more diuron than the other two soils to cause 50% inhibition of both algae and oat - seedlings.

6.6 Paper disc technique

6.6.1 Effect of soil on diuron phytotoxicity

This experiment was set up in Petri dishes, 8 cm diameter, 1.3 cm deep (Section 2.5.3) A standard series was set up using 10 grams of washed sand per dish and 5 ml of the herbicide (diuron) solution at the following concentrations 0, 0.2, 0.5, 1 and 2 ppm. (Plate 6.1.1)

The experimental series was set up with 10 grams of soil (U_2) containing 5 ml of the herbicide at the following concentrations: 0, 0.25, 0.5, 1, 2.5, 5 and 10 µg diuron/g of soil. (Plate 6.1.2)

At the different doses, a progressive increase in inhibition was observed. In the washed sand preparation a visual difference in algal growth could be detected between 0 and0.2 ppm of herbicide. Complete inhibition and killing of algae occurred at 1 ppm level of herbicide. (Plate 6.1.1) In the soil preparation, visual detectable inhibition of algal growth in the discs occurred at 2.5 µg diuron/g soil. There was almost complete inhibition of growth at 10 µg Plate (6.1.2)

6.6.2 Paper disc technique in the study of adsorption of the herbicides by clay and organic matter

(a) To demonstrate adsorption of herbicides by clay, samples of clay-sand mixture with 0, 10, 20, 60, 80 and 100% clay were used. 5 ml of diuron solution (1.5 ppm) was added to give a concentration of 0.75 µg of diuron per gram of clay-sand mixture. Plate 6.2.1 showed a progressive but very slight increase in algal growth in the treated dishes with increasing quantities of clay. The results suggested that the adsorption of the herbicides by clay is real but of little significance.

(b) To obtain semi-quantitative data, the results of (a) were compared with a standard series of discs on sand at the following diuron concentrations: 0, 0.05, 0.1, 0.2, 0.5 and 1 ppm. (Plate 6.2.2)

It was found that the dish containing 60% clay gave an algal growth corresponding to 0.2 ppm in the standard series. Hence, 1 gram of Chullora clay adsorbed approximately 1 µg of diuron under the experimental conditions.

(c) To test the differential adsorption of herbicides by clay, a series of clay-sand mixture at 0, 20 and 40% clay was set up with 5 ml of herbicide solution (2.6 ppm) (diuron and neburon) to give 1.3 µg of herbicide per gram of clay-sand mixture. A series of untreated clay-sand mixtures was included as a control.

No quantitative assessment of adsorption was made, but the results obtained showed clearly that there was comparatively more algal growth on the neburon-treated clay-sand mixtures. (Plate 6.3.1) One may regard the results as an indication of stronger adsorption of neburon by clay, since the difference in toxicity is not enough to explain the difference in growth.

(d) To demonstrate adsorption of herbicides by organic matter (peat), two series (A,B) of mixture (5 gms) of sand-peat (pH 6.5) were set up at 0, 1, 5 and 10% peat.

3 ml of diuron solution (1.5 and 3 ppm) was added to each series to give 0.9 µg and 1.8 µg per gram of sand-peat mixture respectively.

The results showed a progressive and rapid increase in algal growth on the treated discs with increasing amounts of peat. The two series of Petri dishes, A (5%) and B (10%) were compared to a control consisting of 10% peat without herbicide. From these observations of algal growth it was calculated that 1 gram of peat adsorbed approximately 18 µg of diuron.

(e) To test the differential adsorption of herbicides by organic matter, triplicate sets of sand containing 0, 1, 2, 4 and 6% of peat were set up. No herbicide was added to the first set, 1 ppm of diuron was added to the second set and 1 ppm of neburon for the third set. (Plate 6.3.2) Another triplicate set was made using 4 ppm of these two herbicides, with the following peat concentrations: 0, 1, 5, 10, 20 and 30%. (Plate 6.3.3)

In both series (1 ppm and 4 ppm herbicide) more algal growth was observed on discs treated with neburon than with diuron. Adsorption by peat was assessed. In the series with 1 ppm of herbicide 1 gram of peat adsorbed approximately 21 µg of diuron and 70 µg of neburon. In the series with 4 ppm, 1 gram of peat adsorbed 20 µg of diuron and 80 µg

of neburon. The results indicated a differential adsorption of these herbicides. Neburon was much more strongly adsorbed than diuron.

6.7 Discussion

Effect of adsorbed herbicide on the test algae

The algal assay method for herbicide in soil is based on the differences in algal growth in herbicide treated and untreated soils. By using cellophane containers to separate the treated soil from the inoculated liquid medium, it was found that the portion of herbicide, which was adsorbed to the soil particles, had no effect on algal growth.

Bioassay of monuron in soil

From a comparison of toxicity of monuron to alga NMI in sand and soil cultures, it was evident that more herbicide was required in soil to bring about the same degree of inhibition as in the sand cultures. This suggested that there were factors in the soil capable of rendering a portion of the applied herbicide unavailable to the algae.

The algal method indicated the amount of active monuron in the soil. By comparing the toxicity of monuron in soil U_2 and sand, it was found that, under the experimental conditions, the addition of a solution of 0.33 ppm of monuron to soil only caused an inhibition of NMI equivalent to that of 0.25 ppm monuron in sand.

If it is assumed there is no adsorption of monuron in sand, then it can be calculated that, from the amount of herbicide applied to the soil causing 50% inhibition, (0.40 - 0.25) x 20 ug = 3 µg of monuron were adsorbed by 2 gm of soil U_2 .

In addition, the results showed that the algal technique could detect very small amounts of active herbicide concentrations in soil, and could be useful for the study of adsorption of herbicide by soil.

Aqueous extract method

Yuen and Hilton (1962) reported that on addition of water to a herbicide treated soil, an equilibrium between herbicide in soil and herbicide in soil solution would be established. This equilibrium would depend on the concentration of herbicide in soil, on the water - soil ratio and on soil adsorptivity. Thus effective weed control on such a soil would occur as long as the amount of herbicide solution remained or was maintained at a level toxic to the weed population.

Adsorption studies such as those described here may, provide data on soil solution concentrations that could be expected from a given herbicide dose.

The application of the Freundlich isothermal relationship to study the adsorption of herbicides by soils had been used by many investigators (Bingeman and Hill, 1955; Hill, 1960; Yuen and Hilton, 1962; Geiss Buhler et al 1963)

The straight line isothermal equilibrium plot (Fig.4) showed that neburon and diuron were strongly adsorbed by the soil used. When in equilibrium with a herbicide soil solution of 1 ppm, the amount of neburon adsorbed (98 μg) by 1 gram of soil was approximately five times that of diuron (18 μg). It should be noted that these results were obtained under the experimental conditions of a fixed soil - solution ratio and varying herbicide concentrations.

These results suggested that substitution on the N-alkyl group, more particulary, substitution of a methyl group by a longer group (butyl) increases adsorption. A similar conclusion was reached by Crafts (1961), who pointed out that lengthening the N-alkyl chains led to higher adsorption of phenylureas by soil.

Effect of soil depth on herbicide toxicity

The soil analysis data clearly showed that there was less organic carbon in the subsoil and the horizon below the sub-soil than in the top soil. (Table 8). The ED_{50} values for both oats and algae showed that the herbicide exhibited a greater phytotoxicity in subsoils than in the surface layer. This is probably due to increased adsorption of the herbicide resulting from the higher organic carbon content in the surface layer. Thus, for the same amount of herbicide, the effective toxicity in such a soil would

be more pronounced in the subsoil layers than in surface layers. A knowledge of inherent toxicity of herbicide and soil characteristics is therefore extremely important in the application of herbicides in the field. Application of relatively low concentrations of a herbicide for weed control may produce disastrous results for deep rooted crop plants, if the herbicide molecules are translocated from a high organic surface soil to a mineral sub-soil of low organic content.

Paper disc technique

The Paper disc technique for the bioassay of s-phenylurea herbicides is very simple and rapid. It was however, less accurate and less sensitive than the liquid algal technique and plants pot trial. However, the rapidity of the assay and the advantage of visual assessments of growth favoured the technique for the rapid screening of soils containing high or low levels of active herbicide. As results were obtained within 30 hours, the difficulties mentioned by Cullimore (1966) in his studies of available N, K and P in the soil were not encountered.

In sand cultures the range of doses of diuron from 0.2 to 2 ppm gave differential responses under experimental conditions. It was also apparent that doses slightly less than 0.2 ppm could be visually detected by the technique. Similar results were obtained with neburon (Pillay and Tchan, 1969).

By comparing the algal growth on discs from the soil with those from the standard (sand) it was possible to assess the sensitivity of the technique as a method of assay of s-phenylurea herbicide in the soil. A concentration of 0.2 ppm of active diuron in soil could be detected under the experimental conditions. The difference between the quantity of diuron in soil and the quantity in sand required to produce the same algal inhibition, indicates the quantity adsorbed by the soil.

Since inhibitions produced by 5 ppm and 20 ppm herbicide solution in soil were identical to 0.2 ppm and 0.5 ppm in sand it can be assumed that the active concentrations in soil solution were 0.2 and 0.5 ppm. From these data, a semi-quantitative assessment of adsorption was made. A treatment of 5 ml diuron solution (20 ppm) to the soil gave an algal inhibition equal to 0.5 ppm diuron solution in sand. Therefore (20-0.5) x 5 = 97.5 μg = amount of diuron adsorbed by 10 grams of soil, or 1 gram of soil (U_2) adsorbed 9.75 μg of diuron.

The results of the Paper disc technique, with sand-clay and sand-peat mixtures, showed that organic matter (peat) adsorbed nearly 18 times more diuron than Chullora clay, under the same environmental conditions.

As both diuron and neburon were nearly equally toxic to the alga NMI in aqueous nutrient cultures (Table 6) the results (Plates 6.3.1, 6.3.2 and 6.3.3) showed that more neburon than diuron was adsorbed by the same amount of clay or peat.

The Paper disc technique shows great promise as a rapid and direct method of assay of active herbicide in the soil. The technique may also be adapted to study the effects of other soil properties on the effectiveness of s-phenylurea herbicides.

6.8 Summary

(1) Cellophane containers do not affect the toxicity of monuron, and the alga NMI is not affected by herbicide adsorbed by soil particles.

(2) More herbicide is required in the presence of soil than in aqueous medium to bring about 50% inhibition of alga NMI. The algal technique can detect small concentrations of active monuron in soil. (0.2 ppm), and can be used to calculate amount of herbicide adsorbed by soil under the conditions of the experiment.

(3) The algal - aqueous extract) technique shows that neburon is more strongly adsorbed than diuron by soil. Under the experimental conditions, a 1 ppm soil solution concentration is in equilibrium with 18 µg of neburon or 98 µg of diuron per gram of soil.

(4) The amounts of herbicide required to produce 50% inhibition of growth of oats and algae decreased with increasing soil depth. Same amounts of herbicide are more toxic in subsoil layers than in the top soil.

(5) The paper disc technique is very simple to set up and requires very little equipment. Errors involved in long bioassays due to microbial or chemical decomposition of the herbicide are reduced to a minimum with this technique, as results are obtained in 24 hours.

(6) This technique may be used quantitatively to assay herbicide residues in soil.

Although the paper disc method has a limited sensitivity (0.2 ppm of diuron or neburon), its advantages are obvious because of its rapidity, simplicity of operation, and potentialities as a quantitative analytical assay method.

From a practical point of view, it can be very useful in the rapid screening of soils, into those containing high (0.2 ppm) or low (0.2 ppm) levels of residual

soluble active phenylurea herbicides.

(7) The paper disc technique provides a rapid method for assessing the adsorptive capacities of different soils. It can be useful to study the effects of different soil properties (e.g. clay and organic matter) on the effective toxicity of the phenylurea herbicides. Although phenylurea herbicides are adsorbed by clay, the amounts adsorbed are almost negligible.

(8) Neburon is more strongly adsorbed than diuron by both organic matter and clay. 1 gm of organic matter adsorbs approximately 19 μg of diuron and 0.75 μg of neburon respectively.

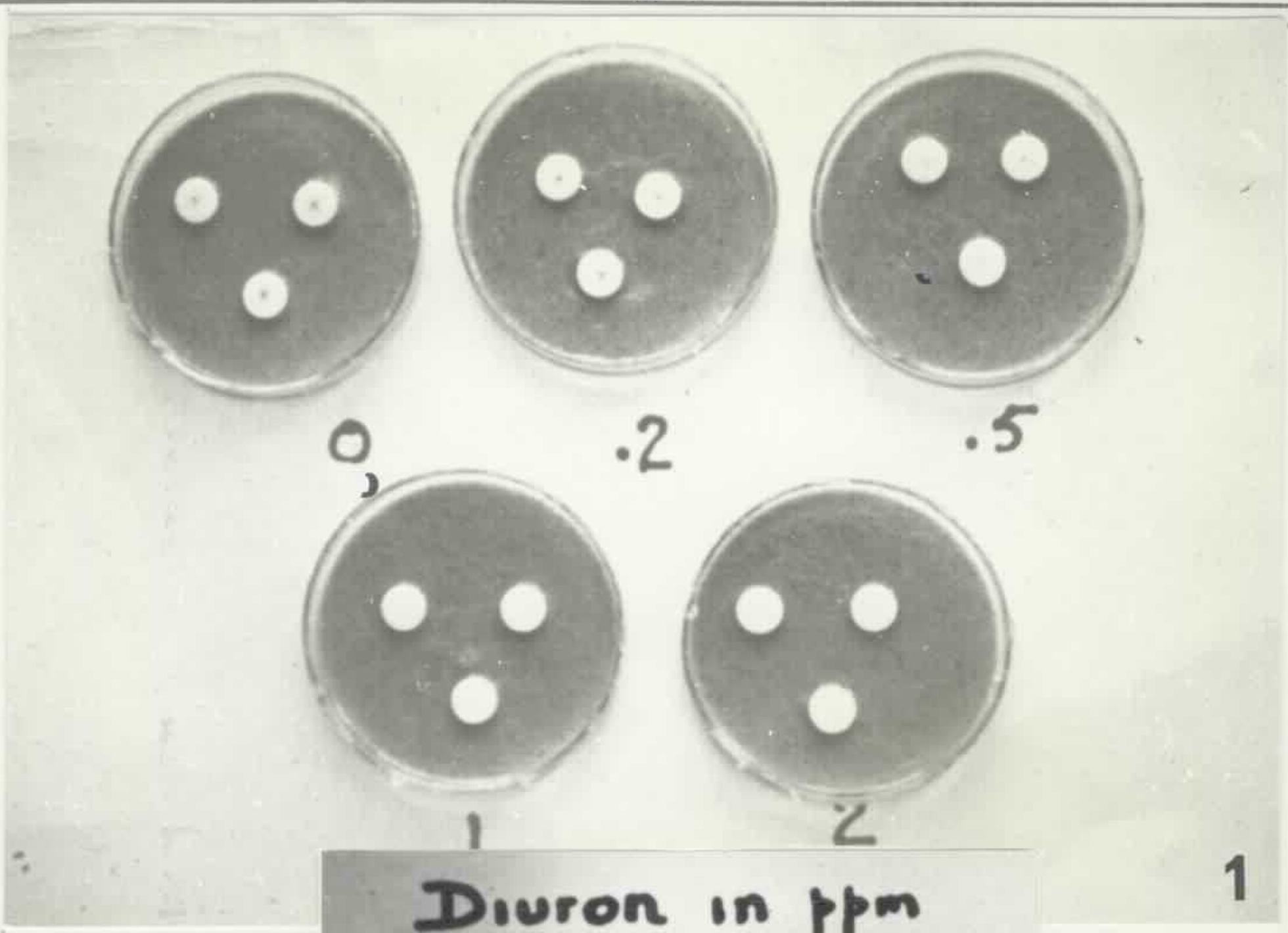
(9) Substitution on the alkyl group increases adsorption of the phenylurea herbicides.

Plate 6.1

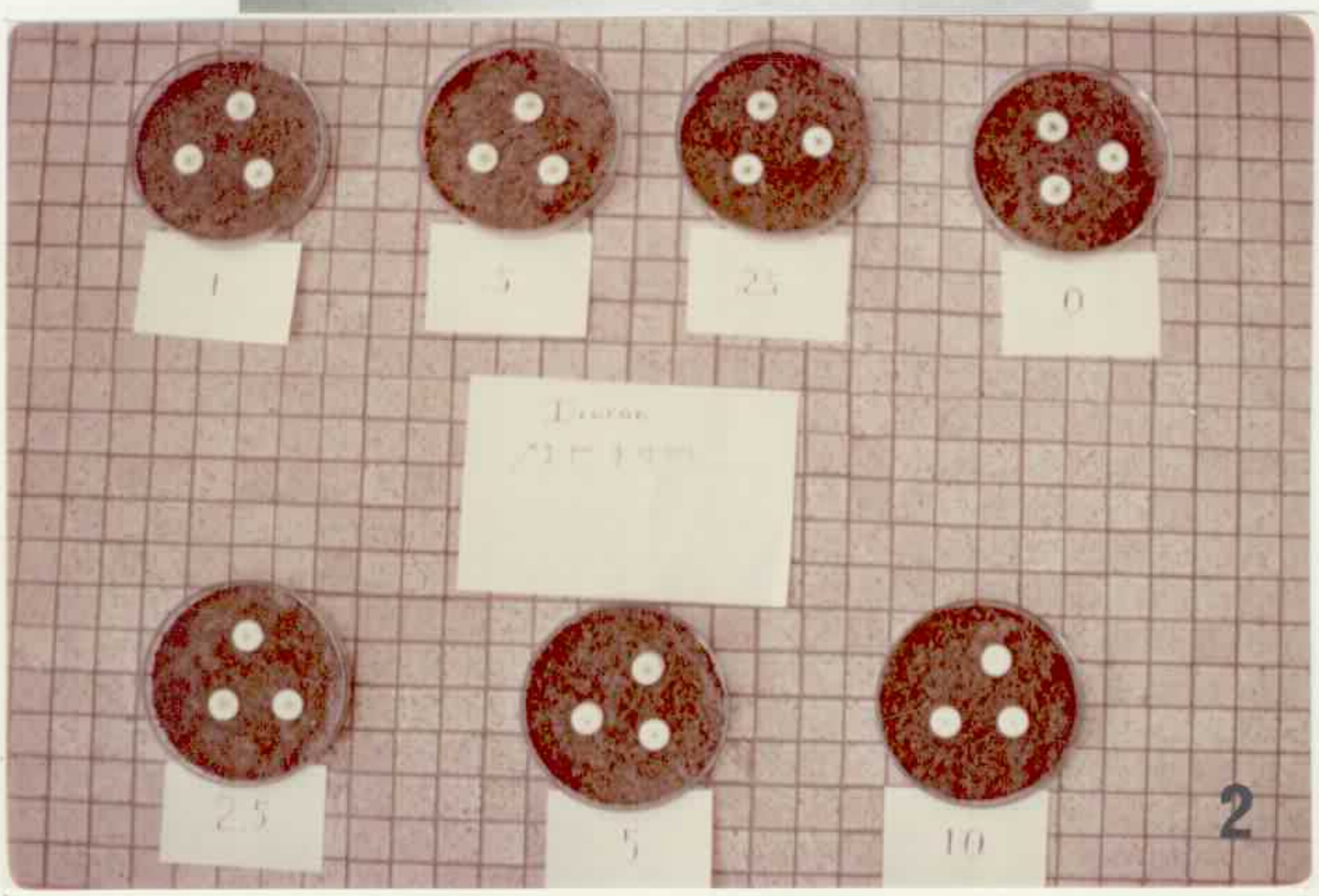
Plate 6.1.1 — Standard series with 10 grams of sand and 5 ml diuron solution. Inset - Note difference between algal growth on control disc and that on disc at 0.2 ppm diuron.

Plate 6.1.2 — Series with 10 grams of soil (U_2) and different concentrations of diuron ($\mu g/g$ of soil).

6.1



1

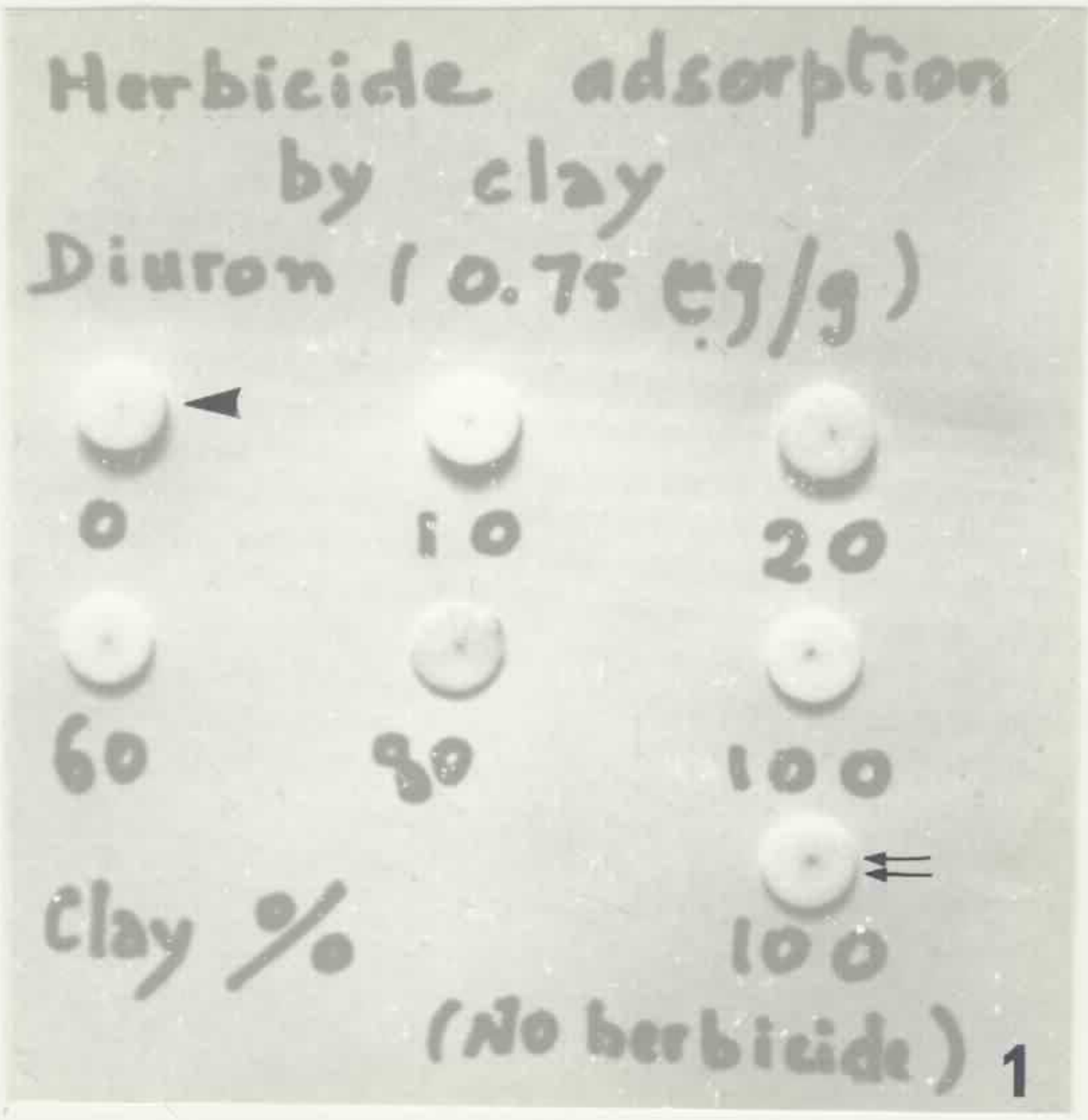


2

Plate 6.2

Plate 6.2.1 Discs show effects of different amounts of clay on toxicity of diuron to Chlorella NMI. Note that amount of diuron used almost completely inhibited algal growth. (arrow). Disc (double arrows) from dish containing 100% clay without herbicide is included to show that clay does not inhibit algal growth.

Plate 6.2.2 Comparison of algal growth on discs from standard series and that on discs from sand-clay series.


Herbicide adsorption
by clay
Diuron (0.75 p.p.m.)
0
10
20
60
80
100
Clay %
100
(No herbicide)
1

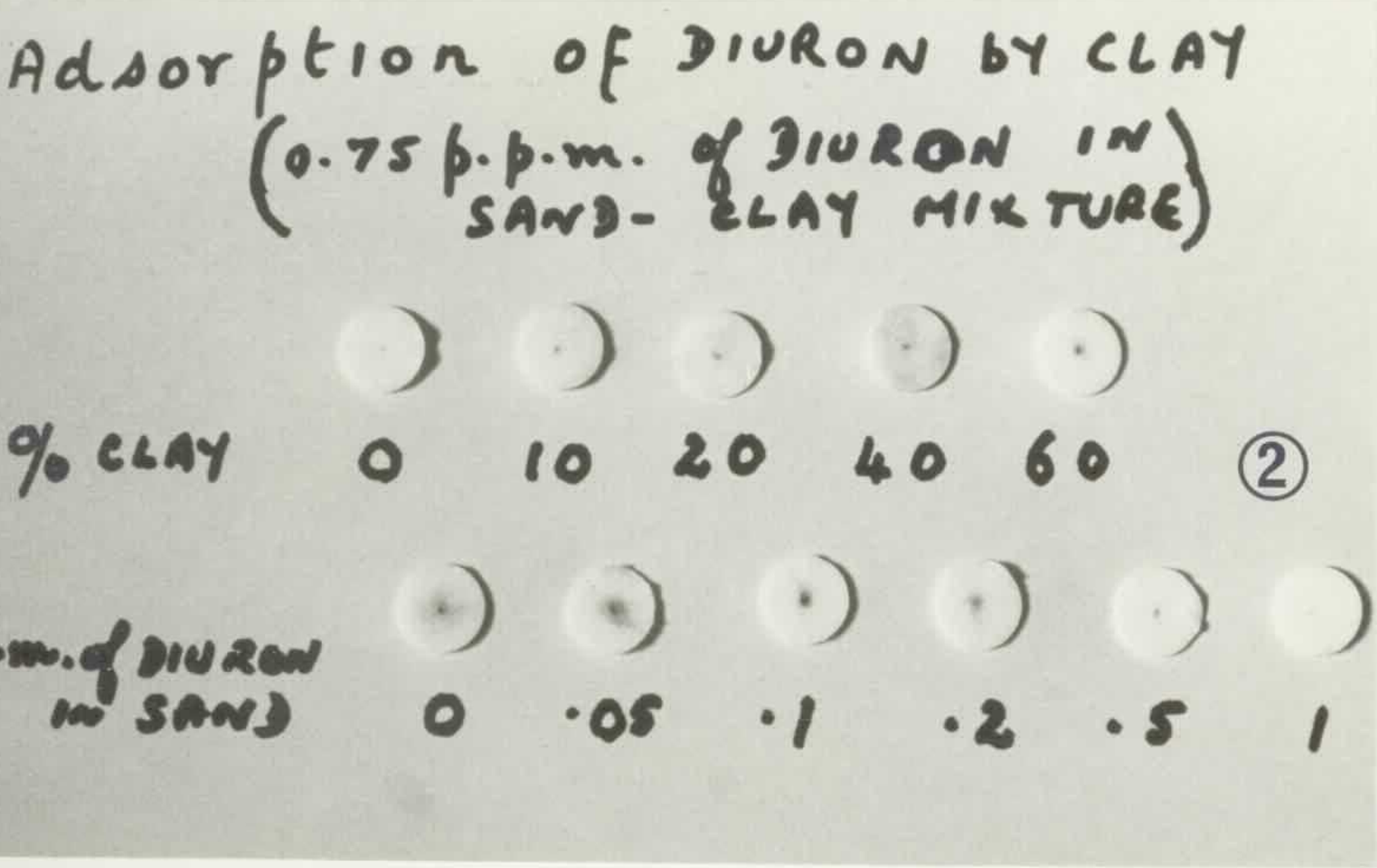
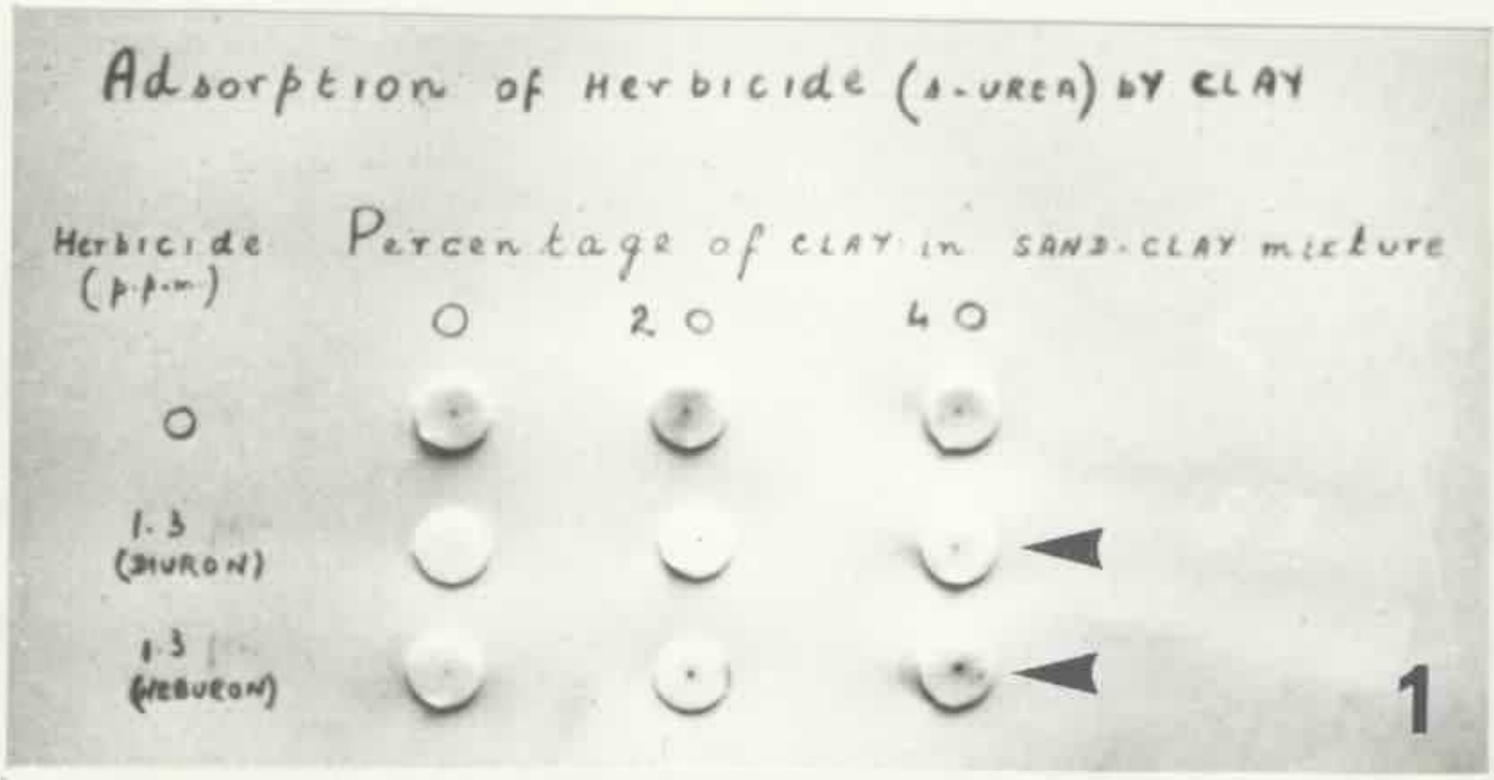
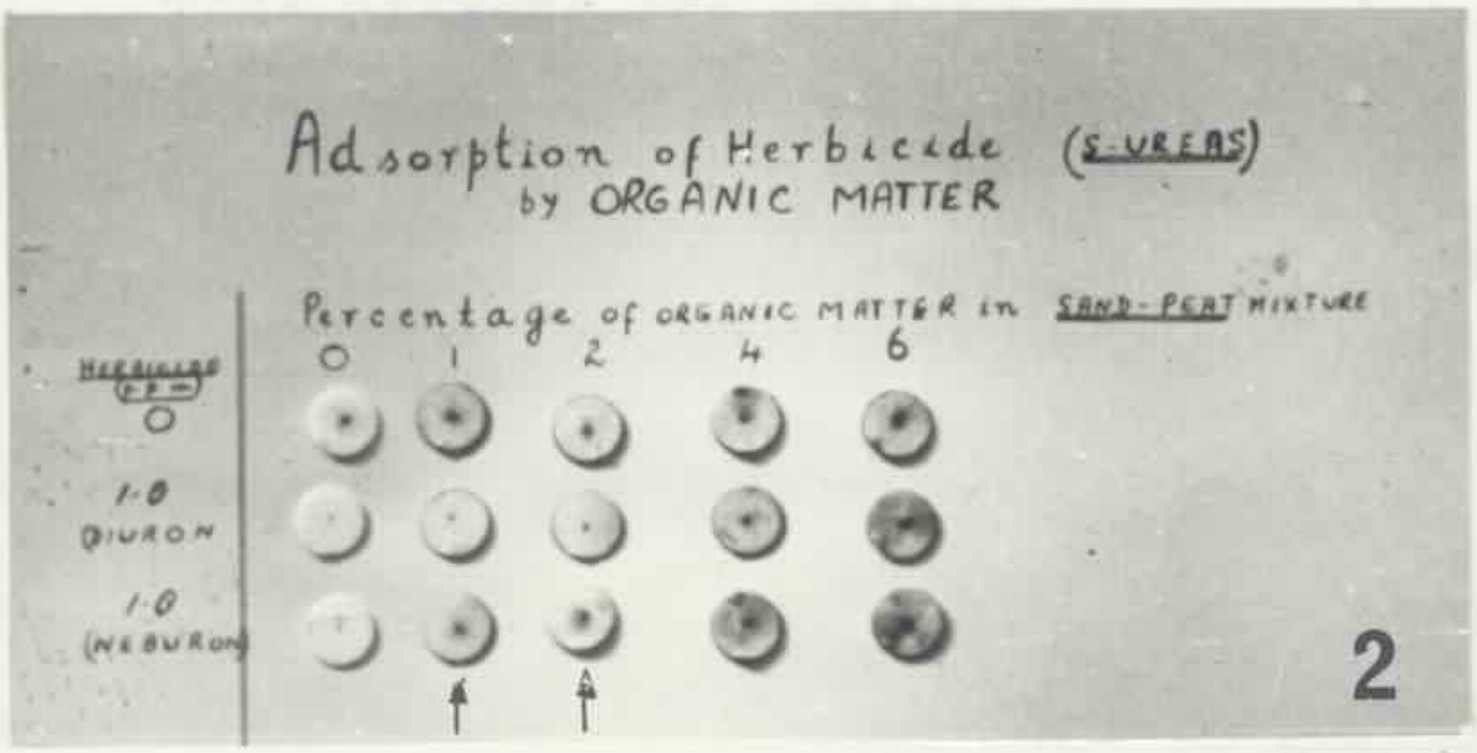

Adsorption of DIURON BY CLAY
(0.75 p.p.m. of DIURON IN
SAND- CLAY MIXTURE)
% CLAY 0 10 20 40 60
②
p.p.m. of DIURON
in SAND 0 .05 .1 .2 .5 1

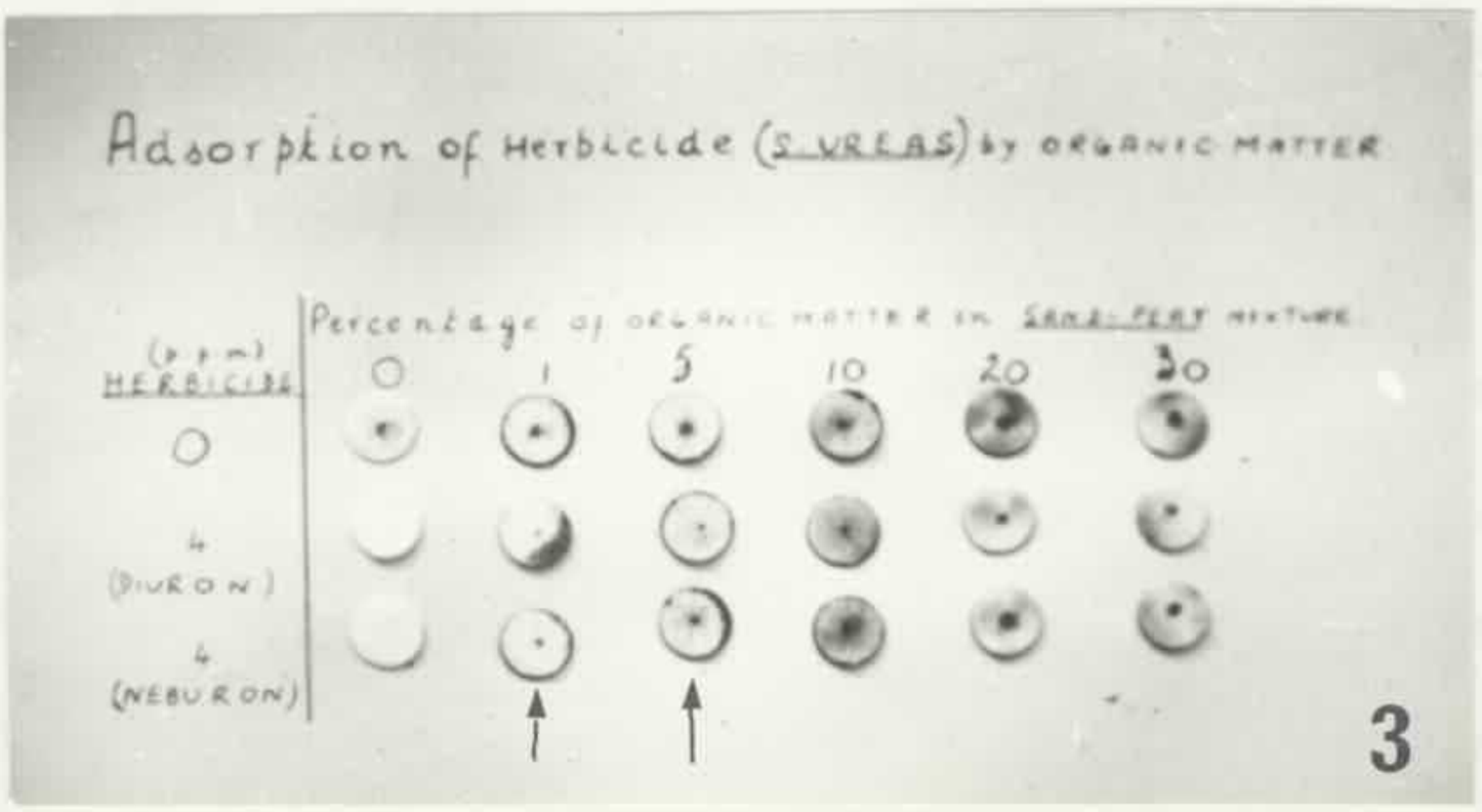
Plate 6.3

Plate 6.3.1 Difference in algal growth in diuron and neburon series (arrows) indicates the difference in amounts of active herbicide remaining in solution after adsorption by clay.

Plates 6.3.2 and 6.3.3 Greater adsorption of neburon than diuron by peat is indicated by the amount of algal growth on discs (arrows).


Adsorption of Herbicide (s-urea) by clay
Herbicide (p.p.m)
Percentage of clay in sand-clay mixture
0
20
40
0
1.3 (diuron)
1.3 (neburon)
1


Adsorption of Herbicide (S-ureas) by ORGANIC MATTER
Percentage of organic matter in sand-peat mixture
0
1
2
4
6
1.0 diuron
1.0 (neburon)
2


Adsorption of Herbicide (S ureas) by organic matter
(p.p.m) Herbicide
Percentage of organic matter in sand-peat mixture
0
1
5
10
20
30
0
4 (diuron)
4 (neburon)
3

CHAPTER 7

ADSORPTION OF PHENYLUREA HERBICIDES IN AUSTRALIAN SOILS

7.1 INTRODUCTION

It is known that adsorption by soil is one of the main factors which influence the effectiveness of phenylurea in the soil. The extent to which these herbicides are adsorbed in soil determines the herbicide concentration in soil solution. This affects the initial phytotoxicity in the soil.

Examination of various soils to determine the soil constituents which affect the activities of different herbicides has been carried out in many countries. This examination consisted of studies of the relationship between the individual or combined soil factors and the quantity of herbicides required to produce a certain degree of biological activity (e.g. 50% inhibition of growth of test organism) (Sheets and Crafts, 1957; Upchurch, 1958; Sheets, 1958; Yuen and Hilton, 1962; Hance, 1965). From these studies it has been noticed that a high statistical correlation existed between the soil organic matter and herbicide activity, and the former has been regarded as the main factor responsible for the inactivation of the phenylurea herbicides in soil. From the data obtained in these particular soils certain

theories about the behaviour of these herbicides were advanced and are acceptable for the soils and herbicides studied. However, different types of soil organic matter have been found to have different adsorption characteristics (Hance, 1965). Also it has been shown that unlike the phenylurea herbicides soil organic matter was not necessarily the dominant factor in inactivation of s. triazine herbicides in Australian soils, (Atkins and Tchan, 1967). In Australia, phenylurea herbicides are being increasingly used for selective control of weeds in crops, for general vegetation control, aquatic weed control and for brush control. But, so far there is very little information concerning the behaviour of these herbicides in Australian soils. Conclusions, derived from investigations in other countries about the behaviour of phenylurea herbicides in soil might not be valid under Australian soil and environmental conditions.

Therefore, bioassays using the algal technique and higher plants (oats) were carried out (1) to study the effect of different Australian soils on the initial toxicity of diuron to the test organisma; (2) to relate various soil properties to the phytotoxicity of diuron; and (3) to investigate the suitability of the rapid algal assay in comparison to the lengthy plant assay in herbicide adsorption studies.

7.2 Oats Bioassay

Fourteen soils collected from various localities in New South Wales and South Australia were used. The soils were chosen to represent a wide range of clay and organic matter contents. Some of the physical properties of the fourteen soils are recorded in Table 1. Algerian oats were used as indicator plants and stock solution of diuron was prepared from an 80% wettable powder formulation.

Six concentrations of the herbicide were set up in duplicate series to assay the concentration of diuron (µg/g. dry soil) required to reduce the fresh weight of oat plants by 50% (ED_{50}).

The trial was set up as described in Section 2.5.4 To compare the effect of different soils on ability of diuron to inhibit shoot growth of oats, ED_{50} values were calculated for each of the duplicate series.

A one-way analysis of variance of the data was carried out to test for differences in ED_{50} values. Significance was determined at 1% and 5% levels. Correlation and regression analyses were done using the data for the ED_{50} values and the different soil properties. Independent variables which were not significant at the 1% level were not used in the regression analysis.

The significance of the ED_{50} values of diuron in the different soils was shown in analysis of variance (Table 9).

Table 9

1. <u>One way Analysis of variance</u> (ED_{50} for oats in 14 soils)

Source of variation (S.O.V.)	Degrees of freedom D.F.	Sum of Squares S.S.	Mean Squares M.S.	F.
Between soils	13	86.274	6.636	37.611
Error	14	2.470	0.176	
Total	27	88.744		

Standard Deviation (S.D.)	0.420
Coefficient of variation (C.V.)	14.787
Standard error of the mean (S.E.)	0.297

L.S.D. (0.05)	0.901
L.S.D. (0.01	1.250

The influence of different soils on the initial phytotoxicity of diuron, expressed as ED_{50} value, was indicated in Table 10.

A high positive correlation (r = 0.870) significant at 1% level was obtained between diuron ED_{50} value and organic carbon content of these soils. Other soil properties such as pH (r = -0.151), sand (r = -0.424), silt (r = 0.546), and clay (r = 0.340) were not significantly correlated with the ED_{50} value of diuron. A regression equation of the form y = a + bx was fitted to the data of ED_{50} values and organic carbon content of the 14 soils. The calculated regression equation was y = -0.145 + 1.3847x (Appendix C).

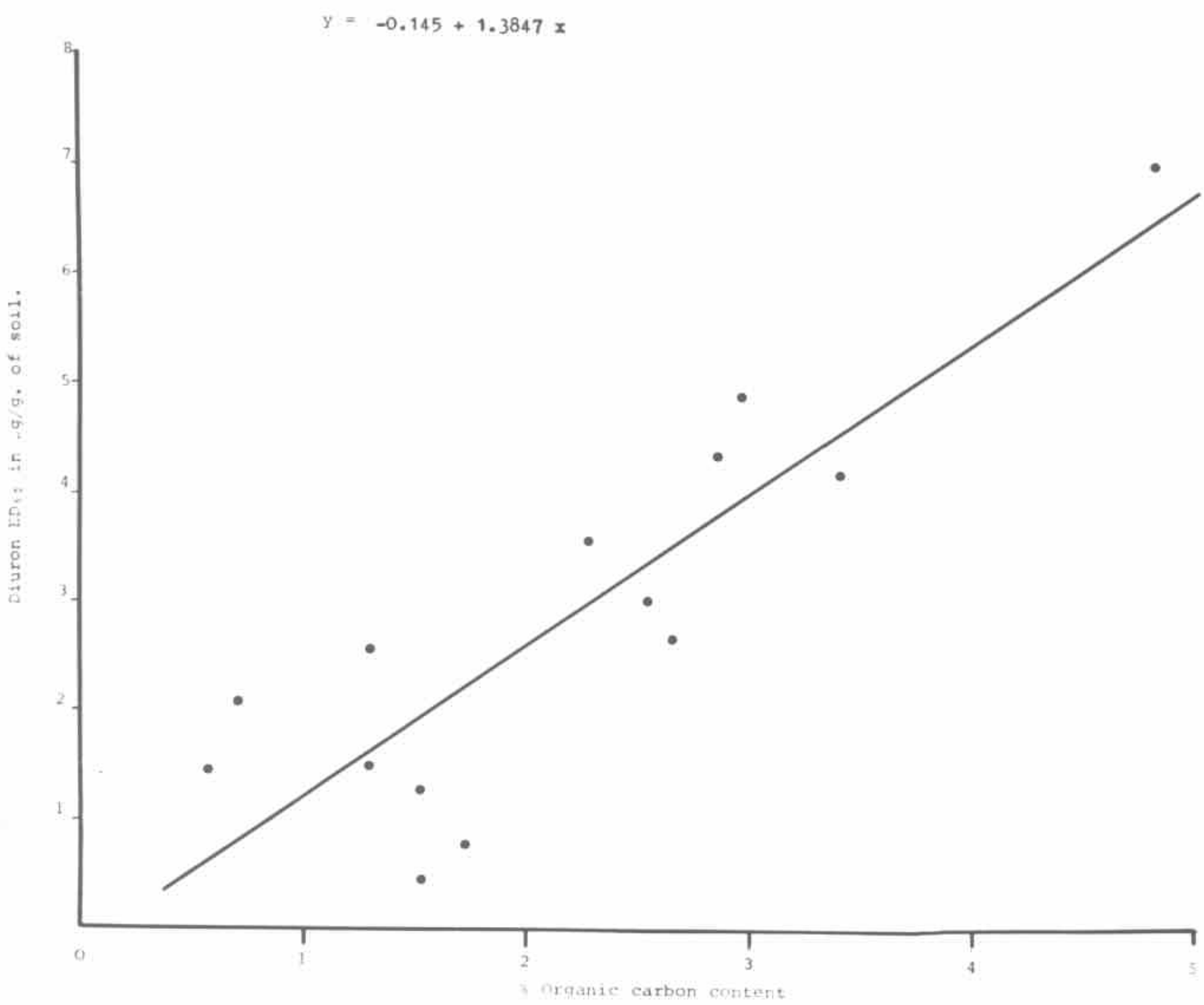
Figure 5 represents the experimental data points (organic carbon content of the 14 soils against the diuron ED_{50} values) and the regression line. The points were close to the regression line. Hence this line could be used to indicate the variation in diuron ED_{50} value with increase in the soil organic carbon content.

Table 10

Effect of different soils on the phytotoxicity of diuron (oats trial).

	ED_{50} values (µg diuron/g. soil)
Wolverton A	2.68
" B	2.53
Cobbity A	2.08
Mount Tomah	7.02
Cobbity B	4.90
Prospect	4.17
Robe	3.00
Seddon	1.28
Blewitt Springs	0.79
Cobbity D	0.48
" E	1.39
Wolverton E	3.58
Turretfield	1.50
Ashbourne	4.36
L.S.D. (0.05)	0.90
L.S.D. (0.01)	1.25

FIG. 5. Correlation of diuron ED_{50} for oats and percent organic carbon content with regression line.



7.3 Algal Assays

The initial toxicities of diuron in twenty soils were investigated. Some of the physical properties of these soils are recorded in Table 1. Triplicate series of 5 concentrations of diuron were used to determine the amount of herbicide required to inhibit algal growth by 50% (ED_{50} values). The algal assays were also used to investigate adsorption of diuron by the various soils. Concentration of diuron chosen to give 0, 0.05, 0.1, 0.2, 0.5, 0.8 and 1.0 ppm in algal nutrient medium were added to acid washed sand and included in the algal assays. At diuron concentration causing 50% inhibition of algal growth, the amounts of herbicide adsorbed by the different soils were then calculated as in Section 2.5.

Correlation, regression and variance analyses were made using the data for the ED_{50}, adsorption and different soil properties.

The influence of soil type on the initial phytotoxicity of diuron, expressed as ED_{50} values, and on diuron adsorption by soil expressed as a percentage of added herbicide, are summarised in Table 11. A summary of one-way analyses of variance of the data is given in Appendix 6. Table 12 shows the simple correlation coefficients among some soil properties, adsorption values, and ED_{50} values obtained by the algal assays. ED_{50} values were most closely correlated

with adsorption values (r = 0.980), and percentage of organic carbon (r = 0.836). Other soil properties (pH and sand) were not significantly correlated with ED_{50} values or adsorption values. Clay and silt values, however, showed a low but significant correlation with ED_{50} and adsorption values. Partial correlations were calculated for the three soil properties, organic carbon, clay and silt, with ED_{50} values and adsorption values. When the effect of any two of these properties was held constant, the partial correlation of the third property, (1) with ED_{50} was for clay 0.32; silt, 0.010; organic carbon, 0.77; and (2) with adsorption percent was for clay, 0.27; silt 0.062; organic carbon, 0.66. These three soil properties were also subjected to a multiple regression analysis. Multiple correlation coefficients (R^2) were determined when each of the two properties, clay and silt, or both properties were added to organic carbon or percentage adsorption. The R^2 values were used to establish whether or not the addition of other factors can account for more variability than has already been assessed by using soil organic carbon or percentage of adsorption alone (r^2). Table 13 summarises the results for simple correlation and multiple correlation coefficients. The values obtained (r^2 x 100 or R^2 x 100) indicated the variation in ED_{50} or adsorption percent (dependent variable) accounted for by the variation in the independent variables.

Table 11

Effect of different soils on the phytotoxicity and adsorption of diuron (Algae trial).

Soils	ED_{50} (µg diuron/g soil)	Adsorption (% of amount added)
Wolverton A	5.43	41.07
" B	4.35	26.44
Cobbity A	4.30	25.58
Mount Tomah	7.29	56.10
Cobbity B	6.77	52.73
Prospect	6.18	48.22
Robe	5.47	41.49
Seddon	3.40	5.88
Blewitt Springs	3.03	7.59
Cobbity D	3.45	7.25
" E	3.80	15.79
Wolverton D	6.90	53.62
" E	5.20	38.46
" F	4.88	34.43
Waite	4.80	33.33
Turretfield	4.41	27.44
Ashbourne	6.15	47.97
Monarto	5.23	38.81
Parafield	4.10	21.95
Mount Burr	3.09	9.38
S.E. of Mean	0.16	2.16
L.S.D. (0.05)	0.46	6.17
L.S.D. (0.01)	0.62	8.26

Table 12

Simple correlation coefficients (r) between diuron ED_{50}, percent adsorption, and five soil properties

Factors correlated	Soil Adsorption %	Organic Carbon %	Clay %	Silt %	Sand %	pH %
ED_{50} (algae)	0.980**	0.870**	0.349	0.546*	-0.424	-0.151
Soil adsorption		0.762**	0.452*	0.579*	-0.507*	-0.159
Organic carbon			0.314	0.601*	-0.433	-0.290
Clay %				0.591**	-0.885**	-0.410
Silt %					-0.832**	-0.533*
Sand %						0.469*

** - 1% level of significance

* - 5% level of significance

Table 13

Percent (r^2 x 100 or R^2x 100) of the variation in ED_{50} or soil adsorption values of diuron, predicted from soil properties.

Dependent Variable	Independent variables used for prediction	r^2 x 100 or R^2 x 100
ED_{50}	Adsorption %	96
	Organic carbon	70
	Organic carbon + clay	74
	Organic carbon + silt	71.2
	Organic carbon + clay + silt	74
Adsorption %	Organic carbon	58
	Organic carbon + clay	63.1
	Organic carbon + silt	60.3
	Organic carbon + clay + silt	63.2

Regression equations of the form $\hat{y} = a + bx$

where $\hat{y}$ = expected ED_{50} values

and x = soil organic carbon content or adsorption %

were fitted to the data from the 20 soils (Table 11.

The calculated regression equations (Appendix) were

$\hat{y} = 3.0477 + 0.9145x$ for ED_{50} and soil organic carbon

and $\hat{y} = 9.8897 + 10.6903x$ for ED_{50} and adsorption %.

Figures 6 and 7 represent the experimental data points and the regression curves corresponding to the above equations.

From graph 6, the distribution of data suggested that prediction of ED_{50} values from organic carbon content below 0.8% than in soils with higher levels of organic carbon.

Figure 7, however, showed a closer distribution of the experimental data around the regression line.

FIG.6. Correlation of diuron ED_{50} values for algae and percent organic carbon content with regression line.

$y = 3.0477 + 0.9145x$

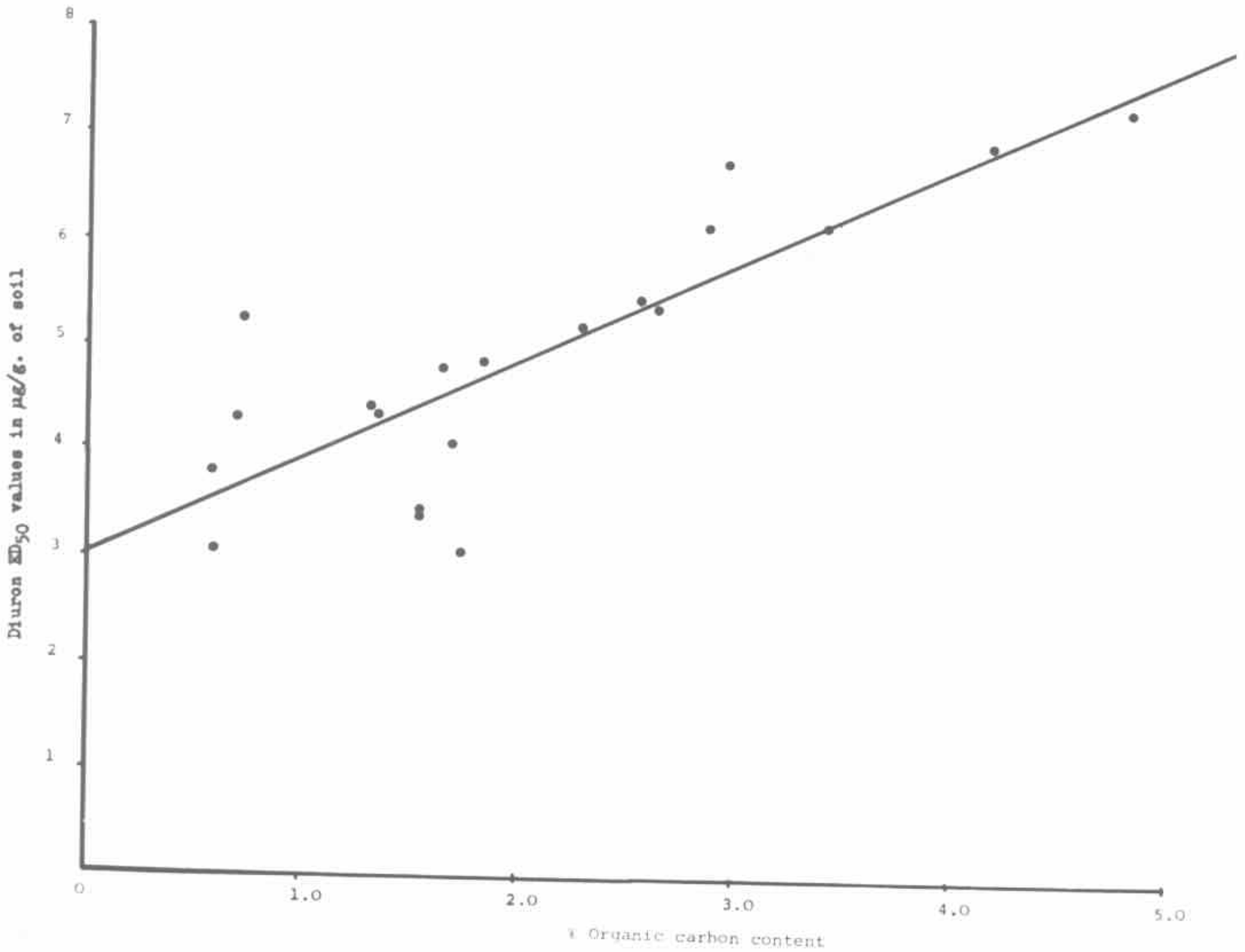
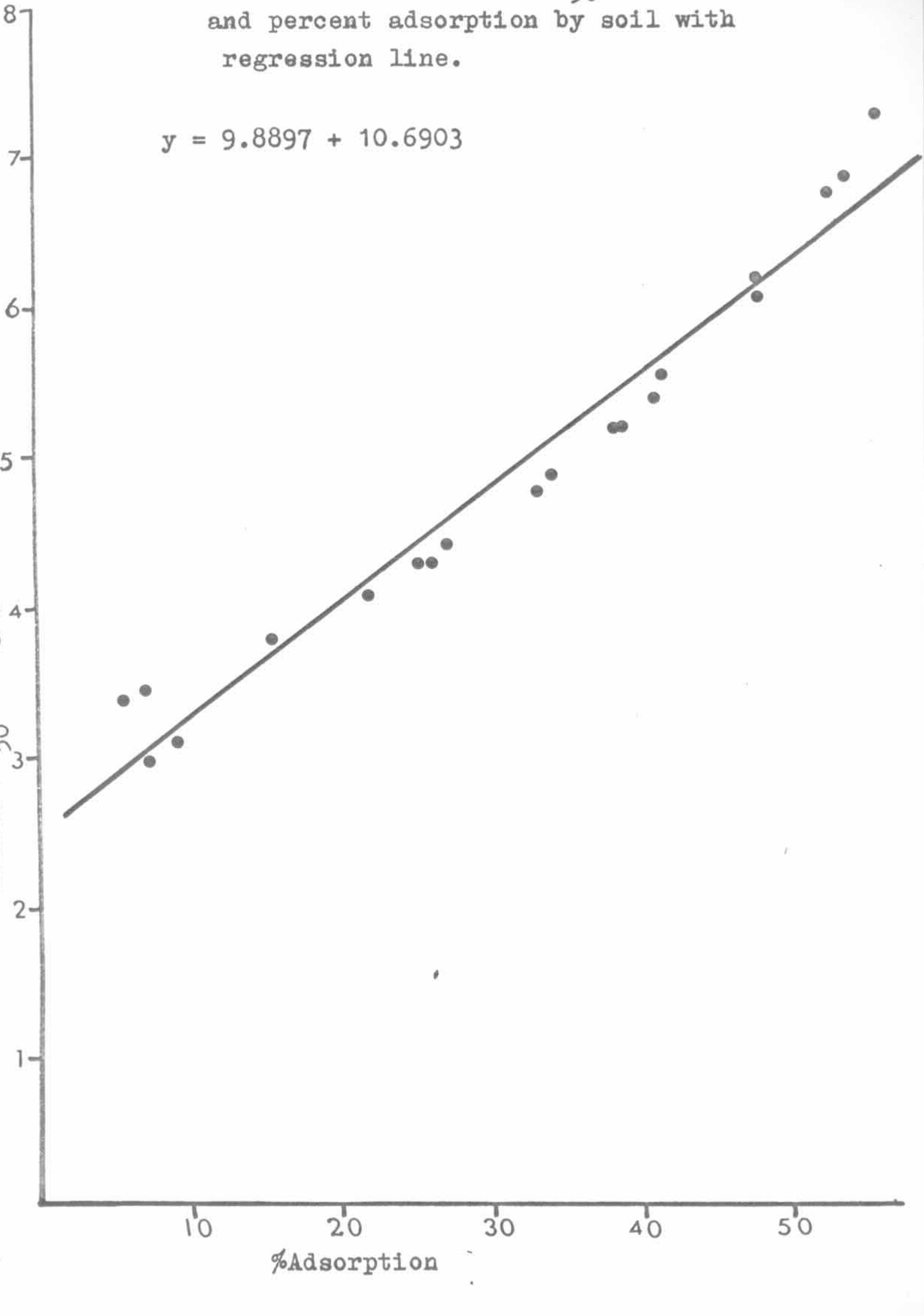


FIG.7. Correlation of diuron ED_{50} for algae and percent adsorption by soil with regression line.



7.4 Oats assay against algal assay

The diuron ED_{50} values obtained from the trials with oats and algae in fourteen soils (Table 14) were analysed to assess the validity of using algae as test organisms for the bioassay of diuron in soils. There was a high significant correlation (r = 0.931) between oats pot trial and algal assay for the 14 soils under the experimental conditions. A regression equation of the form $y = ab^x$ or $\log y = \log a + x \log b$, was fitted by the method of least squares to the data from the 14 soils.

The significance of the regression equation is shown in the Regression Analysis (Table 15). The calculated regression equation was $y = 0.5062 + 0.0609\ x$.

where y = the expected diuron ED_{50} value for algae and x = the diuron ED_{50} value as determined by the oats pot trial.

Figure 8 represents the experimental data points and the regression line corresponding to the above equation.

Table 14

Comparison between ED_{50} values obtained by oat seedlings and algae trials.

Soils	Oats (ED_{50})	Algae (ED_{50})
Wolverton A	2.68	5.43
" B	2.53	4.35
Cobbity A	2.08	4.30
Mount Tomah	7.02	7.29
Cobbity B	4.90	6.77
Prospect	4.17	6.18
Robe	3.00	5.47
Seddon	1.28	3.40
Blewitt Springs	0.79	3.03
Cobbity D	0.48	3.45
" E	1.39	3.80
Wolverton E	3.58	5.20
Turretfield	1.50	4.41
Ashbourne	4.36	6.15
S.E. of Mean	0.30	0.26
Coeffident of variations	14.8	5.26
L.S.D. (0.05)	0.90	0.44
L.S.D. (0.01)	1.25	0.59

Table 15

Oats assay against algal assay

* Transformation applied to data.

Variable

x No transformation

y Log_{10}

Log algal assay against oats assay.

Correlation coefficient (r) 0.93106

Regression analysis

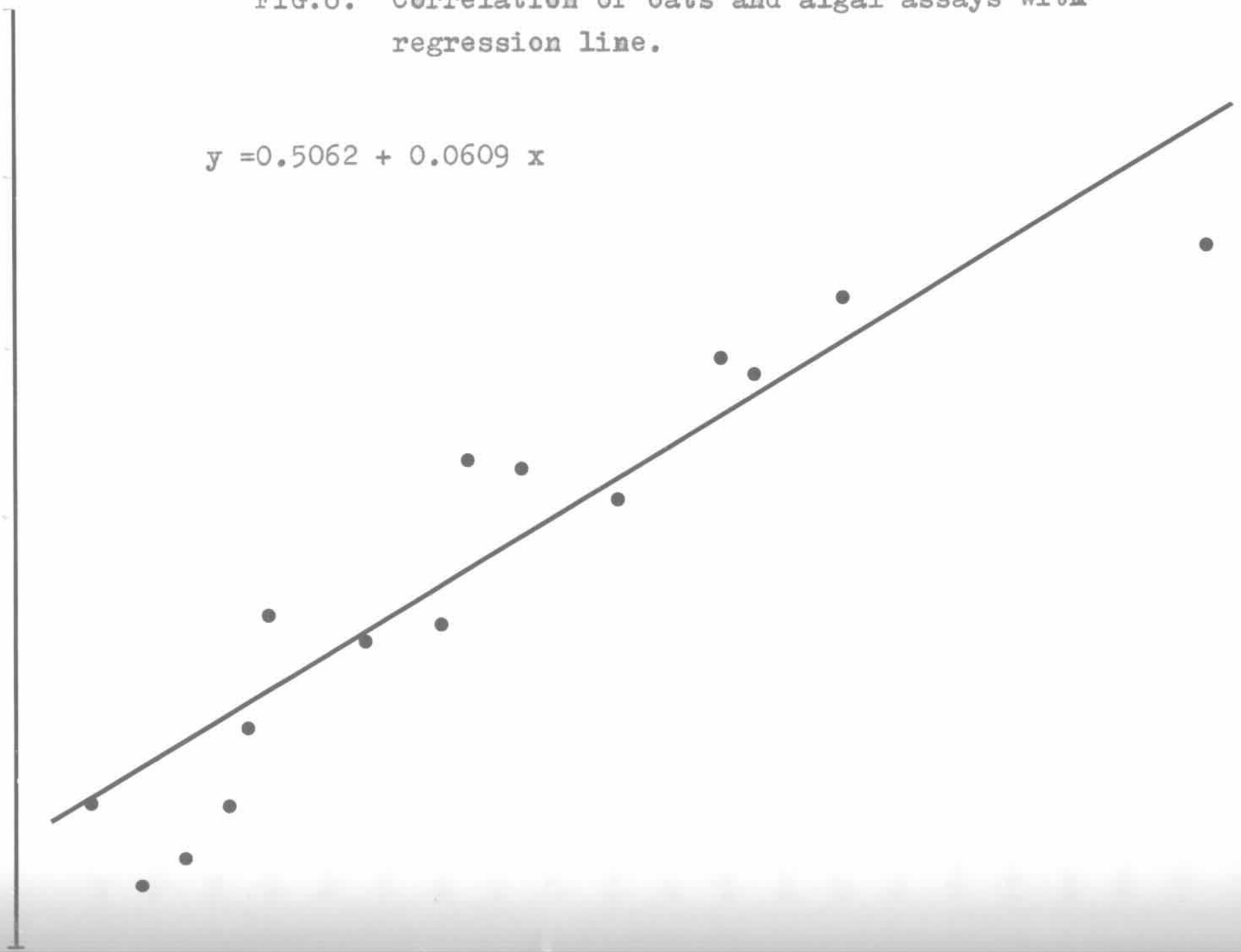
Source	d.f.	S.S.	M.S.	V.R.
Regression	1	0.1602	0.1602	78.146
Deviations	12	0.0246	0.0020	
Total	13	0.1848		

Regression coefficients	Standard errors
0.5062	0.02301
0.0609	0.0069

Regression equation (x, y as transformed)

$$\hat{y} = 0.5062 + 0.0609\,x.$$

FIG.8. Correlation of oats and algal assays with regression line.



7.5 Discussion

Sheets, (1958), investigated the initial phytotoxicities of 4 phenylurea herbicides in 17 soil types. He found that the ED_{50} was positively correlated with percentage of organic matter and clay and cation exchange capacity. Upchurch (1958), who studied the initial phytotoxicity of diuron in 12 soil types from North Carolina, found a high correlation between organic matter, cation exchange capacity and diuron toxicity. However, no significant correlation between percentage of clay and diuron toxicity was observed. Upchurch and Mason (1962), also failed to detect such correlation between diuron toxicity and clay content, but showed high positive correlations of GR_{50} (amount of herbicide required for 50% reduction in dry shoot weight) with soil organic matter and cation exchange capacity. Although it seemed that a difference in correlations of diuron toxicity with soil properties existed from one region to another, the authors agreed that amongst all soil factors the soil organic matter was the major contributor to the reduction of diuron toxicity in soil. Similar investigations were made in this study and it would be possible to discuss the data obtained in relation to soil factors in (1) oats bioassay, and (2) algal bioassay.

(1) Oats bioassay

The influence of different soils on diuron phytotoxicity was clearly demonstrated by the results (Table 10). The

lowest ED_{50} was in a sandy soil (Cobbity D) and the highest in a loamy clay soil (Mount Tomah). The high positive correlation between diuron ED_{50} values and organic carbon content agreed with the findings of other workers (cf. introduction). The lack of correlation with clay, although its content ranged from 0.5 to 61%, was in agreement with the conclusions of Harris and Sheets (1965), Upchurch and Mason (1962) but differed from those of Sheets (1958).

Under the conditions of the experiment, it could be concluded that organic carbon content should be considered in schemes to predict diuron dosage requirements in Australian soils.

(2) Algal bioassay

Significant differences were observed in amounts of diuron required to cause 50% inhibition of algae in different soils. Significant differences were also found in amounts of diuron adsorbed by the various soils (Table 11).

Diuron ED_{50} was positively correlated with percentage of organic carbon ($r = 0.836$), and to a lesser degree with silt ($r = 0.593$) and clay ($r = 0.456$). The highest correlation was between adsorption values and diuron ED_{50} ($r = 0.980$). But adsorption was significantly correlated with organic carbon content ($r = 0.762$). This was

found by Sherbourne and Freed (1954) and Harris and Sheets (1965). Highly significant correlations were also observed among the soil properties (Table 12). Such relationships are frequently observed. Sheets et al. (1962), pointed out "that caution must be used in interpreting the results as causative relations". Inaddition to the interrelations of soil properties, other factors which were not considered in the present study could influence herbicide toxicity in soil. For example, cation exchange capacity has been found to play a role in adsorption of phenylurea herbicides (Upchurch and Mason, 1962). Partial and multiple regression analyses indicated that for prediction of diuron ED_{50} values, the use of silt and clay contents, in addition to organic carbon, was of little advantage, although a very slight improvement was obtained when clay factor was taken into consideration (Table 13). However, an adsorption index accounted for far more variability in the ED_{50} values (96%) than would the use of organic carbon with clay and silt (74%) (Table13).

In the soils studied, adsorption on to clay particles did not seem to affect markedly the phytotoxicity of diuron. The high correlation between soil organic carbon content and the herbicide toxicity was important.

Within the localities studied, phenylurea herbicide recommendations based on soil organic matter content can be made. One must emphasise, however, that at a soil organic carbon level below 0.8% the recommendation based on the carbon content might be unreliable.

It appears that adsorption is the main characteristic which regulates herbicide phytotoxicity in most soils so far investigated, therefore it is reasonable to assume that an adsorptivity index would correlate better with herbicide application rates than the organic carbon content. The present results confirmed this assumption (Table 13). The use of an adsorptive index for prediction purposes would be more reliable since different types of organic matter have different adsorption characteristics (Hance, 1965). Table 13 showed that while percent adsorption of diuron could account for 96% of the variation in ED_{50} values, organic carbon content accounted for only 58% of the variation in adsorption of the herbicide by soil. Furthermore, for the s-triazines Atkins and Tchan (1967) had found a poor relationship between somazine ED_{50} and organic matter content in Australian soils.

(3) Oats assay against Algal assay

On statistical grounds, the algal method would be able to replace the lengthy pot trials for the bioassay of phenylurea herbicides in soil, provided the conditions were similar to those of the present experiments. The high degree of correlation ($r = 0.931$) between the higher plants and algal trials provided a sound basis for the use of the algal method for recommendation of herbicide dosage in the field. In addition, the algal technique could be extended to other herbicides which are toxic to the algae, and to various soil-herbicide studies, which were previously done with oats as assay plants.

7.6 SUMMARY

1. The oats assay shows that organic carbon content is the only soil property which is significantly correlated (1%) to ED_{50} (r = 0.870) for diuron.

2. A high correlation (r = 0.836) between organic carbon content and diuron ED_{50} is also shown in the algal assay. However, a higher correlation (r = 0.980) is observed between diuron adsorption and ED_{50}.

3. Multiple regression analysis shows that use of clay and silt in addition to organic carbon does not significantly increase the predictive value of organic carbon alone, for determining rates of application of diuron.

4. Adsorption values account for 96% of the variability in ED_{50} as compared to 74% accounted for by organic carbon, clay and silt together.

5. Organic carbon content may not always be reliable as an indicator for phenylurea herbicide recommendation in Australian soils, particularly in soils with very low organic carbon content. Diuron adsorption values, as determined by the algal method, would be a reliable index for prediction of herbicide dosage.

6. On statistical grounds, the rapid algal assay could be used in place of the lengthy pot trials in soil adsorption studies and for dosage recommendations of herbicides (photosynthesis inhibitors).

CHAPTER 8

PREDICTION OF RATES OF APPLICATION OF HERBICIDES BY ALGAL METHODS

8.1 INTRODUCTION

In pre-emergence weed control practices, the ideal rate of herbicide application is a dose which gives adequate weed control over a long period of time. It should also allow a margin of safety for the crop or succeeding crops on a given soil type and under the given environmental conditions. In practice, however, herbicide injury to sensitive crops does occur as a result of excessive application, or of residues remaining after a previous treatment, or of cumulative effect of the herbicides in repetitive applications (Bruns et al, 1962; Burnside et al 1963; Holly and Roberts, 1963; Fink and Fletchall, 1963, 1969; Fenster et al, 1965; Lee, 1966; Arle et al, 1965; Dawson et al, 1968). Swain (1970) has indicated that if possible the dosage of herbicide should be reduced as much as possible but still be able to control the weeds. The difficulty, however, resides in the lack of a suitable method to assess this optimal dose.

For many years workers have tried to find ways to determine a safe and efficient application rate on a given soil, which avoids the persistence of residues harmful to succeeding crops. The most elaborate method evolved was

to carry out field trials for a given soil in a particular climate to determine the optimum application rate. Such trials have been performed on various soil types and repeated annually under different seasonal conditions. From these data, commercial recommendations are made for different soils. It is assumed that margins between rates adequate for weed control and rates that are safe from risk to the crop are usually wide enough for general use. Often, however, some soils are treated for which the margin of safety of the recommended dose is too narrow, and injury to crops has occurred.

From studies on initial and residual phytotoxicities of phenylureas in soils, most workers agree that organic matter content seems to be the most important factor influencing phytotoxicity of these herbicides in soil (Chapter 7).

As a result, organic matter content has been considered to be the best single soil property on which to base herbicide dose recommendations (Doherty and Warren, 1969; Sund, 1964; Harris and Sheets, 1965). Hance *et al* (1968) pointed out that adsorption measurement appeared to have slightly greater predictive value than organic matter content under glass house conditions, although the correlation between organic matter content and adsorption was high.

In view of the high predictive value obtained with an adsorption index for diuron phytotoxicity in soil (Chapter 7), the algal techniques have been adapted to give a method for determining optimal application rates of herbicides for weed control. Because of the importance of wheat to the Australian economy, this crop was chosen in field trials.

In the past, post-emergence herbicides have provided such an easy and successful method of weed control in cereal crops that little attention has been given to the use of pre-sowing or pre-emergence herbicides in Australia. In recent years, annual and perennial grasses and broad-leaf weeds have become major problems for the Australian wheat growers (Swan and Furtick, 1962; Connor, D.J. 1965). Although diallate and triallate have proved successful in the control of wild oats and rye grass in wheat fields, there remains a need for other herbicides which can provide good control of broad-leaf weeds, with a wide margin of safety for the wheat crop.

It is evident that weed competition can be most damaging in the very early stages of the life of the crop. In some circumstances the weeds are not harmful when they compete more successfully with crops for water, nutrients and light. In some circumstances they may be harmful as hosts of pests and diseases (Van der Zweep, 1971).

It is reasonable, therefore, to expect that pre-emergence weed control will generally result in improved crop yields. Weeds that emerge after a crop is established cause less damage than weeds which emerge with the crop (Burnside and Wicks, 1967; Knake and Slife, 1968). Simazine, as a pre-emergence herbicide, has been used on a relatively wide scale with some success in wheat cultivation in Europe. Its selectivity, however, was not adequate under all conditions (Holroyd, 1966).

In their studies with -chloro-6-t-butyl-o-acetotaluidite (Monsanto CP31675), Holly et al (1966) pointed out that surface application or incorporation of the chemical in the soil played an important part in its effectiveness as a herbicide in wheat crops. They proposed that soil incorporation of some pre-emergence herbicides gave greater reliability and uniformity of action than surface application. Mechanical incorporation of the herbicides into the soil helps to reduce loss of the herbicide from the soil surface, and allows the herbicide to come into close contact with the germinating seeds or emerging weed seedlings. As diuron and simazine are fairly insoluble in water and are adsorbed very strongly in soils (Upchurch and Pierce, 1957; Harris and Warren, 1964; Harris and Sheets, 1965) they are resistant to leaching and move only very slightly in the soil (Sheets, 1959; Weldon and Timmons, 1961, Roadhouse and Birk, 1961).

This tendency of these herbicides to remain in the zone of incorporation of the soil together with the fact that many common weeds germinate from a depth of less than 2 inches (5.1 cm) (Chancellor, 1965) leads to a good control of weeds. The crop plants are more tolerant to such treatment because the seeds are placed below the herbicide active zone, and hence their roots are not exposed to the herbicide. This method has the advantage of allowing the weeds to absorb the herbicide at germination, as their roots are primarily distributed in the upper layers of the soil. As they emerge from the soil and leaves are formed, the herbicide interferes with their photosynthetic activities, thus causing death of the weeds.

Because of the above considerations, field trials were set up with diuron and simazine, as pre-planting and incorporated herbicides, to assess the reliability of calculations made to predict the optimal application rates in winter wheat from data obtained by the algal techniques. At the same time, information would be obtained on the performance of the recommended rates as compared to lower and higher doses under field conditions in various site - weed situations.

8.2 Laboratory assays

8.2.1 Determination of adsorption of herbicides by the algal techniques.

The amounts of herbicide causing 50% and 100% inhibition of algal growth were determined by the paper disc and liquid algal techniques for washed sand and each of the soils from the chosen localities. The data were used to calculate the quantity of herbicide adsorbed by soil at 50% and 100% algal inhibition (Sections 6.3 and 6.6). The ratio of amount of herbicide adsorbed by soil at 100% inhibition to amount adsorbed at 50% inhibition was determined for each soil. An example of the determination is given in Appendix 1. It was found that soils from the different sites gave similar ratios. Tables 16 and 16a summarise the results. The adsorption value at 100% algal inhibition of the paper disc data was used to predict the dose for field trial.

Table 16

Year	% inhibition of algal growth	Adsorption Studies of diuron by paper-disc and liquid algal techniques					
		Disc technique			Liquid algal technique		
		Amount of diuron (µg) adsorbed/gram of soil	Ratio of amount adsorbed 100% inhibition / 50% inhibition	Mean Ratio	Amount of diuron (µg) adsorbed/gram of soil	Ratio of amount adsorbed 100% inhibition / 50% inhibition	Mean Ratio
1969	Castle Hill						
	50%	2.9	3.70		1.7	3.0	
	100%	10.70			5		
	Camden						
	50%	2.2			1.4		
	100%	8.1	3.66	3.65	4	2.86	2.95
	Narrabri						
	50%	1.75	3.60		1.0	3.0	
	100%	6.3			3.0		
1970	Castle Hill						
	50%	3.3	3.62		1.4	2.6	
	100%	11.9			3.64		
	Camden						
	50%	3.1	3.36				2.65
	100%	10.5		3.50			
	Narrabri						
	50%	3.0	3.50		0.9	2.7	
	100%	10.51			2.43		
1971	Castle Hill						
	50%	3.4	3.50				
	100%	11.9					
	Camden						
	50%	3.1	3.45	3.47			
	100%	10.7					

Table 16a

Adsorption studies by paper disc technique for simazine

Year	Site	% Inhibition of algal growth	Disc technique		
			Amount of simazine (μg) adsorbed/ gram soil	Ratio of simazine adsorbed 100% inhibition / 50% inhibition	Mean Ratio
1969	Castle Hill	50%	4.44		
		100%	18.10	2.5	
	Camden	50%	3.4		
		100%	8.10	2.42	2.40
	Narrabri	50%	4.4		
		100%	11.0	2.3	
1970	Castle Hill	50%	6.0	2.30	
		100%	13.8		
	Camden	50%	4.5	2.53	2.38
		100%	11.4		
	Narrabri	50%	4.4		
		100%	11.0	2.30	
1971	Castle Hill	50%	4.55	2.40	
		100%	12.0		2.51
	Camden	50%	4.05	2.62	
		100%	10.8		

Table 17

Dosage determination of herbicide

Year	Herbicides	Camden		Castle Hill		Narrabri	
		Disc Data in ug/g	Recom-* mended dose in lb/ac.	Disc. data	Recom-* mended dose in lb/ac.	Disc data	Recom-* mended dose in lb/ac.
1969	Diuron	8.10	1.05	10.7	1.25	6.3	0.75
	Simazine	8.10	1.05	11.0	1.25	11.0	1.25
1970	Diuron	10.5	1.20	11.9	1.40	10.5	1.20
	Simazine	11.4	1.30	13.8	1.60	11.0	1.20
1971	Diuron	10.7	1.20	11.9	1.40	-	-
	Simazine	10.8	1.20	12.0	1.40	-	-

* Adjusted recommended rates with depth.

8.2.2 Determination of doses of herbicides for field application

As the laboratory tests were made with soil under a high moisture level (saturation capacity), the rule proposed by Geissbuhler et al (1963) for phenylurea herbicide application in the field was adapted to these studies. The rule states that the dose should be one-tenth of the amount of herbicide adsorbed (in µg/g. of soil) espressed in Kg per ha. to a depth of 5 cm. The recommended dose was calculated by using the quantity of herbicide adsorbed (in µg/g. of soil) at 100% inhibition of algae in the paper disc technique. in the present studies, however, the herbicide was incorporated to a depth of 6.6 cm., accordingly an adjustment to the recommended rate was made relative to the depth of incorporation (Table 17).

8.3 FIELD TRIALS

8.3.1 Setting up of field trials

The trials were set up in Camden, Castle Hill and Narrabri. Doses at and on either side of the calculated optimum rate were included in the trials. In 1969 and 1970, four rates of application of diuron and simazine were used, triallate was included as a comparative herbicide, because of the expected predominance of triallate-sensitive weed species, wild oats (Avena sp.) and rye grass (Lolium sp.). Duplicate plots (9 feet by 5 feet or 2.7 by 1.5 m.) were used for each treatment, with one control plot for each

herbicide. The plots were separated by strips 3 feet (0.91m.) wide. The chemicals were applied, before crop planting, with knapsack sprayers on prepared soil. The soil surface was free of debris or plant residue at the time of treatment. The herbicides were immediately incorporated in the top 2-3 inches (5-7.6 cm.) of soil with a power-driven rotary cultivator with a depth control device. Winter wheat (variety Timgalen) was sown in rows 7 inches (17.8 cm.) apart, and about 3-4 inches (7.6-10 cm.) deep, at a seed rate of 30-40 lb. per acre (44 Kg/ha).

The details of site, date of treatment, dates of planting and harvesting are given in Table 18.

8.3.2 Assessment of Trials

All treatments were scored visually on a crop tolerance and weed susceptibility basis and compared with the untreated control. The wheat was cut by hand, threshed and the yield expressed as percentage of control.

8.3.3 Determination of herbicide residues

Soil samples from the trial sites were taken during the growth period or at harvest time for herbicide residue determinations. The samples were collected at 0-7.6 cm. layer from each plot, bulked, taken to the laboratory and air dried. The samples were sieved, mixed thoroughly and then stored in polythene bags at room temperature. The amount of herbicide in each sample was determined using the liquid algal bioassay (Section 2.5.1).

TABLE 18. Herbicide treatments at the different sites

SITE	DATE OF SPRAYING	DATE OF PLANTING	DATE OF HARVEST	RECOMMENDED HERBICIDE RATE* lb/ac. (a.i.)	
				Diuron	Simazine
Camden	18.6.69	2.7.69	7.12.69	1.05	1.05
Castle Hill	12.8.69	5.9.69	-	1.25	1.25
Narrabri	30.7.69	31.7.69	3.12.69	0.75	1.05
Camden	12.6.70	17.6.70	18.12.70	1.20	1.30
Castle Hill	19.5.70	17.6.70	4.12.70	1.40	1.60
Narrabri	5.6.70	7.6.70	14.12.70	1.20	1.20
Camden	18.5.71	14.6.70	29.12.71	1.20	1.40
Castle Hill	12.5.71	17.6.70	14.12.71	1.20	1.40

* 1 lb/ac. = 1.12 Kg/ha.

8.34 Field Trial Results

A. 1969

The site in Narrabri was almost free of weeds. Two months after spraying with herbicide only a few wild oats were present. A single oat plant appeared in each of the control plots, and 0.32 and 0.86 lb/ac.* simazine treated plots and 2 plants in the 0.11 lb/ac. diuron treated plots. In the triallate treatments, one oat plant was present in each of the 0.125 and 0.25 lb/ac. plot. There were a few secondary germinating wireweeds and vetch in most of the plots. No summer weeds were found in the plots at that time.

At Camden, the site was covered with summer growing weeds before it was prepared for herbicide treatment. The incorporation of herbicide into the seed bed had cleared the weeds and allowed the wheat to grow for some time before new weeds appeared. Throughout the growing period the weeds were dominated by the crop canopy.

The siteat Castle Hill was heavily infested with weeds before it was prepared for the field trial.

Rates of diuron and simazine at and below 0.8 lb/ac. gave no control or only marginal control of weeds in Camden and Castle Hill. The recommended rates of diuron and simazine gave good control of weeds over the first 3-4 months

* 1 lb/ac. = 1 Kg/ha.

after spraying. Satisfactory activity of the two herbicides (>70% - North and Proctor, 1966) was still evident five months after spraying. The major original dicotyledonous weeds were controlled. Although secondary germination of weeds could be observed, the number was insignificant and the weeds were stunted and were dominated by the crop canopy.

Table 19 summarises the overall weed control score in the three localities. Control of some dominant individual weed species was assessed by counting. For this purpose Castle Hill was chosen because weed infestation was particularly heavy. The results are expressed as percentage control with comparison to the untreated plots (Table 20). The effect of the herbicides on weeds was fully expressed, as the untreated plots showed particularly heavy weed growth, with several weeds visible above the crop (Plate 8).

In some plots, the recommended doses caused slight scorching of the upper leaves in the wheat. These symptoms were apparent 4-5 weeks after planting, but they disappeared later. There was no sign of crop depression or thinning. At doses above the recommended rates, better weed control was achieved (90-92%), but injury to wheat plants occurred in some plots. Reduction in early crop vigour and crop stand were observed at Camden. In Narrabri rates of 1.15

and 1.35 lb/ac. of diuron and simazine respectively affected slightly the establishment of the wheat crop.

Although no marked increases in yield were noted at Camden and Narrabri at the different treatments, it was apparent that plots at the recommended rates showed a slight improvement (Table 21).

B. 1970

The field trials were repeated at Castle Hill, Camden and Narrabri. Table 18 gives the laboratory results and the predicted rates for diuron and simazine Lower herbicide doses differing by small amounts from the recommended rates were chosen to give a more accurate assessment of weed control and selectivity in winter wheat. Again, the site at Narrabri was devoid of weeds during the trial period. The degree of overall weed control was assessed 85 and 125 days after spraying at Castle Hill and Camden respectively (Table 19). The results (Table 21) showed that in general improved yields were obtained at the recommended rates, but a marked reduction was caused at the higher doses of both diuron and simazine. It was noted that at Castle Hill, where weed infestation was severe, reduction in yield was also obtained at the lower level of herbicide application.

C. 1971

The field trials were set up at Castle Hill and Camden to obtain more information about the yield performance of the recommended diuron and simazine rates as compared to higher doses. A complete randomised block design with five replications was used. The yield data was used in a variance analysis to compare treated and untreated plots (Table 22). Overall weed control, crop tolerance scores and yield data are summarised in Table 23.

The results showed that there were no significant differences between herbicide treated and untreated plots at Camden. Furthermore, no significant differences in crop yields were noted for different herbicide doses. At Castle Hill, however, where weed infestation was very heavy, the data indicated that the herbicide treatments were effective and significantly increased the yields of wheat under the conditions of the experiment (Table 22). No differences in crop yields for different doses of diuron and simazine were noted, but diuron and simazine at the recommended rates were particularly effective against the dominant broad leaf weeds (cafe weed and potato weed).

D. RESIDUE ANALYSIS

Table 24 summarises the results of algal bioassays of soil samples from the experimental sites for herbicide residues.

No active residues were detected at Narrabri and Camden in 1969, when samples for analysis were taken at harvest time, nearly six months after herbicide spraying. In 1970, soil samples were taken 160 days after herbicide treatment. The residue analysis showed that levels of active diuron and simazine residues were less than 0.01 ppm. for plots which received the recommended rates. A slightly higher amount of simazine residue was detected for the plot which received the higher doses. At Camden, soil samples were taken 3 months after treatment. Levels of active residues were again less than 0.01 ppm.

Table 19

Visual assessment of wheat and weed growth - Castle Hill (1970 trial).

Effects of the different herbicide treatments 133 days after spraying or 104 days after planting	Effects of the different herbicide treatment 189 days after spraying or 160 days after planting.
The wheat crop was about 18 inches high.	The wheat crop was about 14 days from harvest. The crop was mature but the stalks were not yet brittle.
SIMAZINE	
Control (untreated)	
There were tall rye grass plants and broad-leaf weeds in all the control plots, with a dense secondary germination of weeds below. Rye grass seemed to be competing better than wheat. The rye grass plants were very luxuriant.	Mature rye grass plants were present in the plots. There was a large number of cape weeds too. Other weeds were below the crop and rye grass canopy, probably due to weaker competitive power.
1.00 lb/ac.	
Only slight control of original weeds was evident, There was a heavy secondary germination of broad-leaf weeds. A number of rye grass plants and summer grasses was present.	The plots were not different from the untreated plots.

1.40 lb/ac.

Slight control of original weeds was noticeable. Secondary germination of broad-leaf weeds had started, but they were unidentifiable because of their small size. There were still some tall rye grass plants.

There seemed to be fewer weeds present compared to the previous treatment.

1.60 lb/ac. (recommended dose)

There was good control of original weeds, but a few rye grass plants were still visible. Summer grasses and some broad leaf weeds had germinated.

The very few rye grass present were far frombeing mature. There was no increase in the secondary germination of broad leaf weeds. Those present were stunted. There was an increasing number of summer grasses.

2.00 lb/ac.

Good control of original weeds was observed. The only old weeds noticed were a few small rye grass plants. Summer grass had started to germinate.

Rye grass was well controlled. Summer grasses continued to grow. The wheat crop appeared somewhat poor. No broadleaf weeds were present.

DIURON

Control (untreated)
As described above.

0.80 lb/ac.

Weed control was very poor. Heavy infestation of rye Rye grass and Cape weed present grass had caused the wheat were quite lush, to the extent crop to lodge. Broadleaf that the wheat crop was not weeds other than Cape weed, as strong as it should be. seemed to have been Secondary germination of smothered out or at least broadleaf weeds and summer remained stunted by the rye grasses was evident. grass and wheat crop stands.

1.20 lb/ac.

Slight control of rye grass was observed, but control of broadleaf weeds was more marked.

The rye grass infestation was thinner than at the lower dose.

1.40 lb/ac. (recommended dose)

Good weed control was obvious. However, a few rye grass plants and Cape weeds were present. Secondary germination of new weeds was apparent. Summer grasses could also be observed in the plots.

A few rye grass plants were present, but they were far from being mature. The few broadleaf weeds and summer grasses had remained stunted

1.80 lb/ac.

Good weed control was effected. The wheat crop appeared a little retarded as compared to the other plots. Few small secondary weeds were seen.

Very few rye grass plants were present. The wheat crop seemed to be slightly affected. Other weeds had been almost eradicated.

TRIALLATE

Control (untreated)
As described above

0.50 lb/ac.

There was practically no control of rye grass. Tall broadleaf weeds were also present. Beneath the rather dense canopy of wheat and rye grass, secondary weeds were present.

The plots were not different from the untreated plots.

0.75 lb/ac.

Weed control was as poor as in the previous treatment.

No visual differences from the plots treated with lower doses could be detected.

1.00 lb/ac.

There was evidence of rye grass control in the plots. Fewer original broad leaf weeds were present. Germination of secondary broadleaf weeds was unaffected by the herbicide treatment.

Although a number of rye grass plants were present, the wheat crop stood out. The secondary broad leaf weeds seemed to be under biological control by the crop dense canopy.

1.25 lb/ac.

Very good control of rye grass could be noted. A few broadleaf weeds were also present. Secondary germination of weeds had started.

No rye grass was present.

TABLE 19a. Weed control in Castle Hill and Camden (1969-70)

TREATMENT	1969 RATES In a.i lb/acre	1969 WEED CONTROL %	1970 RATES In a.i lb/acre	1970 WEED CONTROL %
Castle Hill (85 days after spraying)				
DIURON	0.80	--	0.80	--
	1.05	34	1.20	63
	1.25	77	1.40	65
	1.40	92	1.80	81
SIMAZINE	0.80	17	1.00	--
	1.05	40	1.40	54
	1.25	80	1.60	76
	1.80	90	2.00	84
TRIALLATE	0.125	--	0.50	--
	0.25	--	0.75	--
	0.30	--	1.00	--
	0.75	--	1.25	--
Camden (125 days after spraying)				
DIURON	0.32	0	0.70	-
	0.70	33	0.90	42
	1.05	65	1.20	70
	1.35	90	1.50	92
SIMAZINE	0.36	0	0.90	-
	0.70	40	1.10	60
	1.05	70	1.30	75
	1.35	80	1.50	90
TRIALLATE	0.125	--	0.50	--
	0.25	--	0.75	--
	0.50	--	1.00	--
	0.75	--	1.25	--

TABLE 20. Control score of some dominant weed species at Castle Hill - 85 days after spraying (1969).

WEED SPECIES	Weed control expressed as % of control in untreated plot											
	SIMAZINE in lb/ac. (a.i.)				DIURON in lb/ac. (a.i.)				TRIALLATE in lb/ac. (a.i.)			
	0.80	1.05	1.25	1.80	0.80	1.05	1.25	1.40	.125	.25	.50	.75
Cryptostema calendula (Cape weed)	33	16	66	83	0	33	83	83	-	-	-	-
Galinsoya parsiflora (potato weed)	0	20	94	94	0	40	73	73	-	-	-	-
Lobium perenne (Rye grass)	33	87	94	94	0	33	87	94	0	33	55	89
Poa annua (Winter grass)	0	40	80	--	0	--	50	--	-	--	--	--
Cotula sp. (Carrot weed)	0	50	80	100	0	--	60	80	-	--	--	--
Overall control of the dominant weeds	3.8	41	85	94	0	21	76	84	-	--	--	--

TABLE 21. Yields of wheat in 1969 and 1970

TREATMENT	1969 RATES IN lb/ac.(a.i)	1969 YIELDS AS % OF UNTREATED CONTROL	1970 RATES IN lb/ac.(a.i)	1970 YIELDS AS % OF UNTREATED CONTROL
Castle Hill				
DIURON	0.80	----	0.80	90.6
	1.05	----	1.20	107.7
	1.25	----	1.40	107.0
	1.40	----	1.80	103.0
SIMAZINE	0.80	----	1.00	83.7
	1.05	----	1.40	103.9
	1.25	----	1.60	99.2
	1.80	----	2.00	82.0
TRIALLATE	0.125	----	0.50	----
	0.250	----	0.75	----
	0.500	----	1.00	----
	0.750	----	1.25	----
Camden				
DIURON	0.32	98.3	0.70	105.0
	0.70	----	0.90	89.0
	1.05	111.5	1.20	114.4
	1.35	103.3	1.50	82.8
SIMAZINE	0.36	102.6	0.90	97.3
	0.70	102.5	1.10	101.2
	1.05	100.00	1.30	86.1
	1.35	101.4	1.50	65.8
TRIALLATE	0.125	98.5	0.50	109.9
	0.250	100.0	0.75	96.3
	0.500	100.0	1.00	121.3
	0.750	101.3	1.25	101.0
Narrabri				
DIURON	0.11	102	0.80	89.0
	0.22	105	1.05	103.4
	0.75	96.6	1.20	106.9
	1.15	98.0	1.50	----
SIMAZINE	0.32	93.3	0.80	95.4
	0.86	90.6	1.10	91.3
	1.05	108.0	1.20	----
	1.35	105.0	1.50	97.3
TRIALLATE	0.125	92.6	0.50	106.7
	0.25	101.0	0.75	88.7
	0.50	97.3	1.00	96.9
	0.75	99	1.25	100.0

Table 22

Analysis of Variance - Camden field trial (1971)

Dry weight of harvested wheat (in grams)

Treatments	Replications					Treatments mean
	1	2	3	4	5	
Control	766	603	697	513	748	665.4
Diuron (1.2lb/ac)	630	822	556	784	785	693.4
Diuron (1.8lb/ac)	566	896	660	521	632	655.0
Simazine (1.4lb/ac)	798	682	659	538	480	631.4
Simazine (2.0lb/ac)	643	618	633	406	568	573.6

Source of variation	d.f.	s.s	m.s.	F.	
Replication	4	83592.2			
Treatments					
Control v/s chemicals	1	2926.8	2926.8	0.24	n.s.
Among chemicals	3	37743.0	12581.0	1.03	n.s.
Error	16	195124.6	12195.3		
Total	24	319386.6			

Standard deviation	110.43
Coefficient of variation	17.15
Standard error of mean	49.39

Table 22a.

Analysis of Variance - Castle Hill field trial (1971)

Dry weight of harvested wheat (in grams)

Treatments	Replication					Treatments mean
	1	2	3	4	5	
Control	1040	906	738	849	851	877
Diuron (1.2lb/ac)	1031	907	1043	860	1405	1049
Diuron (1.8lb/ac)	1126	816	896	1123	1155	1023
Simazine (1.4lb/ac)	977	1284	1054	874	1275	1095
Simazine (2.0lb/ac)	1108	830	873	959	922	938

Source of Variation	d.f.	s.s.	m.s.	F.
Replication	4	156592		
Treatments				
Control v/s chemicals	1	88685	88685	4.05 !
Among chemicals	3	63410	21137	0.97
Error	16	349946	21872	
Total	24	658633		

Standard deviation	159.14
Coefficient of variation	15.98
Standard error of the mean	71.17

! - significant at 10% level.

TABLE 23. Overall weed control, crop tolerance and yields of wheat in 1971

TREATMENT	RATES IN lb/ac (a.i)	WEED CONTROL %		CROP TOLERANCE % 130 days after spray	YIELDS % OF UNTREATED CONTROL
		70 days after spray	130 days after spray		
Castle Hill					
DIURON	1.2	50	62	100	124.8
	1.8	80	76	92	106.2
SIMAZINE	1.4	80	68	90	119.6
	2.0	100	88	80	116.6
Camden					
DIURON	1.2	--	74	96	100.4
	1.8	--	84	92	93.6
SIMAZINE	1.4	--	84	86	96.8
	2.0	--	94	80	82.4

Table 24. Herbicide residues in treated plots from the field trials (1970) estimated by the liquid algal technique

Site	Herbicide	Original treatment in lb/ac.	O.D. of chlorophyll extracts	% of control
Castle Hill	Diuron (157 days after spraying)	0.00	0.090	100
		0.80	0.092	102 *
		1.20	0.092	102
		1.40	0.110	122
		1.80	0.094	104
	Dimazine (165 days after spraying)	0.00	0.095	100
		1.00	0.098	103
		1.40	0.098	103
		1.60	0.102	107
		2.00	0.093	98 **
Camden	Diuron (120 days after spraying)	0.00	0.095	100
		0.70	0.105	110
		0.90	0.092	97
		1.20	0.102	107
		1.50	0.103	108
	Simazine (120 days after spraying)	0.00	.088	100
		0.90	.087	100
		1.10	.090	102
		1.30	.084	95
		1.50	.078	90

* Stimulatory response indicates traces of active herbicide residues

** Toxic response indicates harmful residues.

8.4 Discussion

1. Narrabri

For the Narrabri trials, although no statistical analysis was carried out, it was apparent that there were no differences in yiels in both 1969 and 1970. (Table 19). The Narrabri trials were initially set up to study the effects of diuron and simazine on wild oats and ryegrass, but the absence of these weeds in the plots did not allow for an assessment of weed control. However, the absence of injury to the wheat crop and the unaffected wheat yield at the recommended doses showed that dosages up to 0.75 lb/ac for diuron and 1.05 lb/ac for simazine were tolerated by wheat under the conditions of the experiment. With doses above the recommended rates, injury, a retardation in early growth and a slight thinning of the crop were observed in some plots. However, the disappearance of some seedlings was compensated for by improved tillering, and the final yield was not affected (Table 19). The residue analysis of soil treated with the recommended dose showed that levels of active diuron and simazine residues were less than 0.01 ppm, 165 days after treatment. This indicated that there would be no risk in replanting the area after harvest.
In 1970, a slight carry-over of simazine was detected when the plots received the higher doses (1.5 lb/ac). Clay and Ivens (1966) had reported that residues of the order of 0.1 to 0.2 lb/ac would be sufficient to cause serious injury to small seeded arable crops.

Therefore, it seemed likely that at doses above the recommended rates, there would be a danger of residual effects in replanting the area 6 months after spraying with simazine (1.5 lb/ac).

2. Castle Hill and Camden

Diuron and simazine applied at the calculated optimal rates, gave good control of the weeds present in the plots, with no deleterious effect on the growth or yield of the wheat crop.

In Castle Hill, where weed infestation was dense, the recommended rates of diuron and simazine consistently maintained weed control (Table 20) until the crop canopy was fully developed. (Plates 8.1 and 8.2). At these rates, the wheat showed an early maximum vigour, as it was free from weed competition for moisture, nutrients and sunlight. It developed a dense canopy of foliage which later restricted the growth of secondary weeds, and thus made unlikely a rapid return to high weed density. After 3 - 4 months, the wheat canopy was so dense that it exercised effective biological control of emerging weeds. The presence of weeds observed at this stage indicated that the residual effect of the added herbicide was reduced and possible damage to a later crop was not to be expected.

The trials clearly showed that herbicide doses above recommended rates, although providing excellent weed control (90%), did not significantly increase yields above those from

plots treated with the recommended rates, which provided only 70% weed control. (Table 21). Furthermore, harmful residue effects after harvest were noted at the higher doses. This was illustrated by the absence of weed at harvest time in the plots with high herbicide treatment in the 1971 field trials (Plate 8.3). These results indicated that the use of a dose which could maintain 70% weed control over an adequate period during the early growth of the crops could bring distinct advantages and should be preferred to a dose giving complete weed control.

Rainfall has been generally considered of importance for most pre-emergence herbicides to be effective. It (1) moves the herbicide into the soil thus reducing loss from the soil surface, (2) brings the herbicide into contact with germinating weed seeds, (3) provide adequate moisture for absorption of the herbicide by the weed seedlings (Stickler et al, 1969). It had also been shown that both simazine and diuron were more toxic under high than under low moisture conditions. In the present investigation, however, the herbicides were mechanically incorporated to an appropriate depth. The only function of rainfall then might be to provide optimum soil moisture for absorption of the herbicide by weed seelings. The results at Castle Hill, which had a severe weed infestation, showed an additional advantage in the use of diuron and simazine as incorporated pre-planting herbicides. The chemicals could be incorporated into the soil

from a period of 24 hours to 4 weeks prior to wheat planting. This would allow a certain flexibility in the choice of suitable soil moisture conditions for herbicide application under the prevailing climatic conditions.

The field trials also showed that any yield increase, resulting from the removal of weed competition during the early stages of winter wheat growth, was not the only benefit. The herbicide treatment retarded weed growth, causing a delay in seed formation of the weed plants. The absence of weed seeds at harvest time would greatly reduce grading costs and a higher return would be obtained for a high quality product. The successful biological competition of the dense crop against the young weeds would ensure a stunted weed growth, and thus increase the ease of mechanical harvesting. Furthermore, after crop harvesting, it would be possible to remove the weed plants before their seed setting, by mechanical cultivation. In Australia, labour, power, and machinery costs approximate one third of the total cost of cereal production, so that these benefits would be potentially of considerable importance.

It could be concluded that the recommended rates of herbicide application derived from adsorption values obtained by the algal techniques were reliable and effective in the field trials. Furthermore, the algal techniques could be used to detect active residues in soils which had been previously treated with simazine or diuron.

The techniques would take into account such residues, and prevent adverse additive effects in subsequent herbicide recommendations for the same soil.

8.5 Summary

1. The algal techniques (paper disc and liquid) are suitable to determine the adsorption of diuron and simazine by soils at the three trial sites.

2. The adsorption data by paper disc technique are used for herbicide dosage recommendations (diuron and simazine). The results of the field trials (weed control and wheat yields) over the three years at the three sites confirm the recommendation procedures as correct and reliable.

3. The trials at Castle Hill emphasise the advantage of incorporation and pre-planting treatments. The timing of herbicide can be adjusted to match favourable soil-moisture conditions.

4. Pre-emergence treatments with the herbicides provide sufficiently long-lasting control to protect the wheat crop from weed competition during the early growth stages. At later stages of crop development the toxicity of the herbicides is reduced, but the dense crop canopy is able to compete successfully against the emerging weeds.

5. The selectivity of the herbicide at the recommended doses is consistently high, and good control of broad-leaf weeds is achieved.

6. The residual activity of diuron and simazine is short and at the recommended rate is unlikely to exceed six months. There is therefore no risk of herbicide damage to succeeding crops.

Plate 8.1

Field trial with diuron

Castle Hill (60 days after sewing).

Plate 8.1.A — Control plot. Wheat plants are indicated by arrows.

Plate 8.1.B — Diuron applied at 1.05 lb/ac. Wheat plants are indicated by arrows.

Plate 8.1.C — Diuron applied at the recommended rate (1.25 lb/ac.)

Plate 8.1.D — Diuron applied at a dosage above the recommended rate (1.40 lb/ac.)

Plate 8.2

Field trial with simazine

Castle Hill (60 days after sowing)

Plate 8.2A — Control plot. Wheat plants are indicated by arrows.

Plate 8.2.B — Simazine applied at 1.05 lb/ac.

Plate 8.2.C — Simazine applied at the recommended rate (1.25 lb/ac).

Plate 8.2.D — Simazine applied at a dosage above the recommended rate (1.80 lb/ac.)


B


A



8·2






D


C

PART II

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introductory Remarks

The modes of action of herbicides have been intensively examined in recent years from physiological and biochemical standpoints. However, progress towards an understanding of the mechanisms of action of the herbicides, has been very slow because of the complexicity of the problems.
It is reasonable to assume that the application of any chemical might affect more than one vital process in the plant. The most sensitive process, which is affected first or at the lowest concentration of the chemical, is usually considered as the primary site of action. A number of metabolic functions in plants cells have been found to be affected by herbicides. Many of these herbicides exert at least part of their phytotoxic action by inhibiting the Hill reaction in photosynthesis, (Moreland, 1967; Good, 1961; Kerr and Wain, 1964 b; Baldwin et al, 1968). Some herbicides interfere with respiration, and thus with the mitochondrial system in plant cells (Kerr and Wain, 1964 b; Jones and Watson, 1965; Negi et al, 1968). Other herbicides are able to inhibit cell division (Hilton et al, 1963; Brian, 1964), or affect the metabolism of nucleic acids (Moreland, 1967).

The classification of herbicides as inhibitors of growth, cell division, or photosynthesis is an over-simplification. For example, carbamates were first classified as inhibitors of cell division, but it has been recently shown that some of the carbamates may in fact inhibit photosynthesis (Herrett and Kramer, 1966).

Among the several metabolic processes that are affected by herbicides, photosynthesis is sensitive to a large number of chemicals currently used for weed control, e.g. phenylureas, s-triazines, uracils, amides, benzimidazoles, benzonitriles and carbamates.

On account of the high toxicity and long residual activity of some herbicides which inhibit photosynthesis (e.g. phenylurea herbicides), an understanding of their mode of action may help in the improvement of a safer and a more economical use of these chemicals.

1.2 Electron Microscopic and Biochemical Studies

Although the phenylurea herbicides have been used in a number of biochemical studies, a clear and simple picture cannot be drawn of their mode of phytotoxic action. (c.f. Review, Section 1.2 of Part 1). A study of the ultrastructural effects of the phenylurea herbicides on algal cells, particularly on the chloroplasts, might contribute to the understanding of their mode of action.

Cells can be examined in the electron microscope only after they have been fixed, and it is not possible to follow the changes in the same cell with time. Thus, the electron micrograph presents a static picture of one plane through the cell, stopped at a given moment in time. Examination of serial sections, however, has increased the amount of information which can be obtained from single sections. Electron microscopy clearly shows the distribution and organisation of cell substance, but usually gives only limited information about the chemical nature and functions of the different parts of the cell. However, specific substances (e.g. labelled chemicals) used in the electron microscopy have made it possible to locate certain reaction sites within the cells. Biochemical studies, on the other hand, provide information on physiological changes and biochemical reactions, but do not always give the location of active sites or show structural changes and interaction of organelles within the cell. Therefore, sequences of reactions can be biochemically investigated and studied in detail, but the way in which those different reactions are interrelated in the cell is not revealed. Thus, while the biochemist provides an understanding of cell chemistry, the electron microscopist gives a detailed picture of the fine structure of the cell.

In using both approaches, it is possible to relate distinct biochemical funstions to structures seen in the electron microscope, and a better understanding of cell organization and functions can be obtained.

1.3 Electron Microscopy in Herbicide Study

Although the gross morphological changes in plants treated with herbicides have been reported in a number of papers, Wardlaw, C.W., 1953; Gorter and Van Derzweep, 1964; Kiermayer, 1964; (Fisher et al, 1968; Rodebush and Anderson, 1970) there is relatively less information at light microscopy level. Only recently, has electron microscopy been used to investigate ultrastructural changes in cells of treated plants. These studies have mostly been with herbicides inducing chlorosis in plants. Usually whole plants are used as test materials (Ashton et al, 1963, Bartels and Pegelow, 1968; Baur et al, 1969; Guillot-Salomon, 1966; Hill et al, 1968; Hallam, 1970). The use of higher plants poses many difficulties in the study of mode of action of those herbicides which primarily interfere with photosynthesis. Because of the transport of these herbicides from the roots to the leaves and the translocation through non-photosynthetic to the photosynthetic sites, it is not always possible to separate the influence of the effects occurring in the adjacent cells from those in the photosynthetic cells. In addition, in some monocotyledonous and dicotyledonous plants photosynthetic cells in the mesophyll and in the vascular bundle sheath parenchyma of the leaf have different types of chloroplasts. These differ in size, and in the extent of granal stacking.

Shumway and Weier, 1967; Laetsch et al, 1966; Laetsch, 1968; Walker and Crofts, 1970; Downtown et al, 1969; Karpilov et al, 1971; Chang and Troughton, 1972).

Consequently, interpretation of observations after herbicide treatment can be difficult.

The use of unicellular algae (Chlorella sp) which are physiologically similar to higher green plants, removes the problems involved in translocation and types of chloroplasts. Modifications in structure under herbicide treatment can be easily followed. The test alga (Chlorella NMI) offers some further advantages, as it has been previously used for the bioassay of photosynthetic inhibiting herbicides (Atkins and Tchan, 1967; Pillay and Tchan, 1969; Part 1), has a much quicker response to the herbicide treatment than higher plants, and can be obtained in large numbers in the same physiological state from synchronous cultures.

The present work describes the ultrastructural changes occurring in the algal cells, after treatment with herbicides under different experimental conditions. These results, combined with the existing biochemical data, would lead to a better understanding of the modes of action of the herbicides.

CHAPTER 2

GENERAL MATERIALS and METHODS

2.1 Cultivation of the Algae

The unicellular algae, Chlorella sp. (NMI) and Euglena sp., were kept as pure stock cultures in small McCartney bottles on a mineral nutrient agar slope.

The growth medium for Chlorella NMI is given in Part I. (Section 2.1.1) This medium was supplemented with a combined vitamin B, and B_{12} solution for Euglena cultures. The pH was adjusted to 5.5 with O.IN HCl.

The culture media were autoclaved at 15 lb/sq inch for 15 minutes. For Euglena, the solution of vitamins was sterilized separately by millipore filtration, and added aseptically to the liquid medium.

The inoculum for the experiments was prepared as described in Part I (Section 2.1.4)

2.2 Measurement of Pigments

The algal cells were harvested by centrifugation, usually at 12,000 rpm during 10 minutes in a Sorvall RC-2 Centrifuge with SS - 34 rotor at $0^{\circ}C$. The supernatant was discarded and the algal pellet suspended in 80% acetone in distilled water and left for 3 hours or overnight in the dark at $8^{\circ}C$.

The optical densities of the acetone extract at 645 mu (D_{645}), at 663 mu (D_{663}) and at 460 mu (D_{460}) were measured in a Unicam SP 600 spectrophotometer in a 1 cm light path.

The concentrations of this extract in chlorophyll "a" and in chlorophyll "b", expressed in grams/litre, were calculated by applying the following formulae (Arnon, 1949):

$$\text{Concentration of chlorophyll "a"} = 0.0127\ D_{663} - 0.00269\ D_{645}$$

$$\text{Concentration of chlorophyll "b"} = 0.0229\ D_{645} - 0.00468\ D_{663}$$

The total concentration in carotenoids of the acetone extract, expressed in grams/litre, was calculated from the following formula, established according to the coefficients determined by Mackinney (1941):

$$\text{Concentration of carotenoids} = D_{460} - \frac{[(\text{C.chl "a"} \times 3.19) + (\text{C.chl "b"} \times 130.3)]}{200}$$

C. Chl = concentration of chlorophyll, in g/l

It is assumed that the approximate coefficient determined by Mackinney to estimate the total amount of carotenoids of Malva could be utilised for the Chlorella control and for the Chlorella treated with herbicides. The composition in carotenoids of the Chlorophyces is in fact similar to that of the higher plants: β-carotene and lutein representing the major proportion. (Strain, 1958; Nakayama 1962)

2.3 Cell Counts

Counts of algal cells were made in a Petroff-Hauser counting chamber under phase microscopy. Twelve samples from each of duplicate experimental vessels were counted to determine cell numbers. Cultures showing high cell density levels were diluted before counting. Statistical analysis of the measurements showed no significant differences in cell number/ml between duplicated experimental vessels. (Appendix E)

For the Euglena cultures, the cells were immobilized before counting by the addition of a drop of 5% glutaraldehyde.

2.4 Electron Microscopy Techniques

2.4.1 Solutions used in the fixation procedures

1. Buffer solution (Ryter and Kellenberger, 1958)

Solution A	Sodium veronal	2.94 g
	Sodium acetate (hydrated)	1.94 g
	Sodium chloride	3.40 g

Made up to 100 ml with distilled water.

Solution A	20 ml
Distilled water	52 ml
0. IN HCl	28 ml
M $CaCl_2$	1 ml

pH 6.0

2. Uranyl acetate solution

0.5% uranyl acetate in solution 1

3. Osmium tetroxide (OsO_4) solution

1% osmium tetroxide in solution 1

4. Phosphate buffer solution - (0.1M)

Solution A - $Na_2HPO_4 \cdot 2H_2O$ 3.61 g in 100 ml dist. H_2O

Solution B - $NaH_2PO_4 \cdot H_2O$ 2.76 g in 100 ml dist. H_2O

49 ml solution A + 51 ml solution B

Made up to 200 ml with distilled water, pH 6.8

5. Cacodylate buffer

Sodium cacodylate 300 ml (6.42 g in 300 ml dist. H_2O)

0.1N HCl 143 ml

Made up to 600 ml with distilled water

pH 6.0 - 6.1

6. Glutaraldehyde stock solution at 0°C

Stock solution of glutaraldehyde (25%) was purified by adding a small amount of barium carbonate. A precipitate formed, and after centrifugation the clean supernatant was removed and used for the fixative. Purification was necessary as the pH of the 25% glutaraldehyde would fall during storage due to impurities or oxidation. When the pH falls below 3.5, the quality of fixation is poorer (Sabatini et al, 1963).

7. Glutaraldehyde solution

The fixative was prepared by adding 25% glutaraldehyde stock solution to the cacodylate buffer to give a final concentration of 5% glutaraldehyde.

8. Veronal acetate solution (Palade, 1952)

Sodium veronal	2.89 g
Sodium acetate (hydrated)	1.90 g
Distilled water	100 ml

pH 7.3-7.4

9. Potassium permanganate ($KMnO_4$) solution.

The permanganate solution was prepared with 0.12 g of potassium permanganate ($KMnO_4$) in 10 ml of solution (8) or in 10 ml distilled water. Solutions were prepared fresh before use.

10. Embedding medium (Araldite)

Casting resin	20 ml
Hardener	20 ml
Accelerator	0.6 ml
Dibutyl pthalate	1.5 ml

Dehydration and embedding

Different procedures were used to fix the algal cells. After the final washing, the cells were resuspended in a small amount of 1.5% agar. The small agar blocks with fixed cells were stained with 0.5% uranyl acetate solution for 2 hours. Dehydration was then carried out in a graded series of water

-alcohol. The agar blocks were left for 5 minutes in each of 30%, 50%, 70%, 80% alcohol solution, followed by 10 minutes in 90% and two changes of 15 mins. each in 100% alcohol. The agar blocks were cut into very small pieces, soaked in three changes of araldite for one hour each. The specimens were transferred into small gelatin capsules containing araldite, left overnight at room temperature and then polymerised at 60°C for 36-48 hours.

Sectioning and electron microscopic examination

Sections were cut with a Porter Blum utramicrotome MT-2 using glass knives, and collected on nitrocellulose coated grids. The sections were stained with lead citrate (Reynolds, 1963). Specimens were examined and photographed in a Philips EM 200 or a Siemens Elmiskop 1 operating at 60 or 80 Kv.

CHAPTER 3

FIXATION PROCEDURES FOR ELECTRON MICROSCOPY

Direct osmium tetroxide (Os O_4) fixation was first used in animal cells (Palade, 1952, 1956). It not only achieved good preservation of form, but also acted as a stain by increasing the apparent density of the structures. It was found, however, that the quality of preservation varied with time of exposure, and more important, penetration of the fixative in the tissues was slow. Long periods of fixation in Os O_4 might result in production of artifacts and leaching out of proteinaceous materials from the tissues (Bahr, 1955; Dallam, 1957; Luft and Wood, 1963; Bennet & Luft, 1959).

As the rate of penetration of Os O_4 in plants was even slower than for animal material, preservation was disappointing in early attempts with plant tissue.

On the other hand, potassium permanganate (KMn O_4) was found to penetrate plant tissues rapidly and to preserve membranes. The micrographs were diagrammatic in appearance and therefore appealing. As a result, $KMnO_4$ was extensively applied in electron microscope studies of plant material. During exposure to $KMnO_4$, however, many constituents of the cell were not preserved. It extracted RNA and a part of the protein from tissues, therefore ribosomes could not be found after fixation. (Marinozzi, 1964 a.b; Bradbury and Meek, 1960). Depending on the time the fixative was allowed to

act (1 hr), the nucleus could be identified by its profile and nuclear envelope, but it lacked internal structure. With shorter periods of fixation, structural differences in the nucleus were preserved. Numerous observations had indicated that DNA was preserved by fixation in $KMnO_4$ (Luft, 1956; Bradbury and Meek, 1960), $KMnO_4$ had been found to stabilise the nucleoplasm of bacteria in a manner comparable to the Kellenberger et al (1958) procedure. (Kellenberger, 1962). In their studies of nucleic acids of mitochondria in chick embryo, Nass and Nass (1963) (1965) found that $KMnO_4$ stabilised DNA as a branching network of fibrils.

Recently, it was shown that double fixation using glutaraldehyde and OsO_4 considerably reduced loss of proteins from tissues and gave excellent preservation of cell constituents. (Marinozzi, 1963, a; 1964 b; Dales 1963).

The present study is focused on ultra structural changes in uni-algal cells with particular attention to chloroplasts when treated with herbicides. It is known that certain procedures of fixation with OsO_4 and $KMnO_4$ usually fail to preserve adequately the finer structures within the chloroplast (Gunning, 1965).

It is important to decide on a fixation procedure capable of preserving the stroma region of the chloroplast of Chlorella NMI

Therefore studies in the preservation of fine structure have been carried out, by comparing different fixation procedures using osmium tetroxide (OsO_4), potassium permanganate ($KMnO_4$) and glutaraldehyde (Glut.).

Observations are summarised in Table 1.

TABLE 1 Comparison of fixation procedures for Chlorella NMI

	FIXATIVE	BUFFER	pH	TIME (hours)	TEMP	POST-FIXATIVE	BUFFER	pH	TIME (hours)	TEMP	0.5% Uranyl acetate (2 hours)	Lead Citrate (15 min)	REMARKS
1	5% Glutaraldehyde	Cacodylate	6.0	2½	4°C	1% OsO_4	Veronal acetate (Ryter et al)	6.0	12	4°C	-	+	Good fixation of cell organelles. Ground substance of endoplasm granular, with particles resembling ribosomes. Nucleolus observed.
2	1% OsO_4	Phosphate	6.8	24	4°C	-	-	-	-	-	-	+	Poor fixation.
3	6% Glut.	Cacodylate	6.0	2½	4°C	1% $KMnO_4$	Veronal acetate (Palade)	7.3	1/2	4°C	-	-	Good fixation of cell membranes. Granular endoplasm, but not homogenous. Severe plasmolysis.
4	2% OsO_4	Phosphate	6.8	24	4°C	-	-	-	-	-	-	+	Moderately well fixed. Sometimes unspecific deposition of dark granules in cell.
5	5% Glut.	Cacodylate	6.0	2½	4°C	2% OsO_4	Phosphate	6.8	12	4°C	-	+	Same as 4
6	1% OsO_4	Veronal acetate (Ryter et al)	6.0	24	22°C	-	-	-	-	-	+	+	Same as 1, with better fixation of nuclear material.
7	0.6% $KMnO_4$	Veronal acetate (Palade)	7.3	1/4	22°C	-	-	-	-	-	+	+	Good fixation of cell membranes. Nucleolus observed. Plasma membrane not discernible. Plasmolysis frequent but not severe.

	FIXATIVE	BUFFER	pH	TIME (hours)	TEMP	POST-FIXATIVE	BUFFER	pH	TIME (hours)	TEMP	0.5% Uranyl acetate (2 hours)	Lead Citrate (15 min)	REMARKS
8	2.5% Glut.	Cacodylate	6.0	2	22°C	1% OsO_4	Michaelis	6.0	20	22°C	+	+	Good fixation of cell organelles. Membranes of mitochondria, nuclei and E.R. better defined than in 1 and 6. Double membrane of plasma lemma observed. Structures possibly microtubules in nucleus. DNA fibrils and ribosomes observed in both chloroplasts and mitochondria.
9	2.5% Glut.	Cacodylate	6.0	2	22°C	0.3% $KMnO_4$	Aqueous Sol.	7.0	1/6	22°C	+	+	Membrane systems of cell wall preserved. Mitochondria with smooth membranes. Cristae long. Mosaic pattern of electron dense and electron transparent areas in nucleus. Electron transparent areas contain fibrillar material Similar areas in chloroplast and mitochondria.

MICROSCOPIC STUDIES OF CHLORELLA NMI and EUGLENA

4.1 Synchronization of Chlorella NMI culture

4.1.1 Introduction

In a normal uni-algal culture, cell divisions occur randomly, and at one time usually contains cells at different stages of the life-cycle (Plate 4.A). In such a mixed population, individual algal cells may respond differently when treated with herbicides. This difficulty may be overcome by the use of a synchronous culture of the test alga. Thus a possible correlation between cytological and biochemical changes as a result of herbicide treatment may be studied. The following describes the method for inducing synchrony in Chlorella NMI.

4.1.2 Materials and Methods

A combination of the techniques for synchronization of Chlorella developed by Tamiya et al, (1953) and by Pirson and Lorenzon (1958) was adopted. A culture of Chlorella NMI, made as homogeneous as possible by gentle centrifugation, to remove the heavier cells, was separated from a normally growing population. This culture was then subjected to two light-dark periods (16 hours light - 8 hours dark). The resulting culture was centrifuged at 1000 g for 5 mins and the cells from the supernatant, were harvested at 1,000 g for 20 min. The packed cells obtained were washed once, resuspended in fresh medium and used to produce a synchronous culture of Chlorella NMI.

300 ml of fresh medium in a 750 ml Erlenmeyer flask was inoculated with the algae to an approximative cell concentration of 2×10^6 cells/ml. The flask was mechanically shaken in a constant temperature water-bath ($28 \pm 1^{\circ}c$) and subjected to 16 - hour light and 8 - hour dark cycles. The culture was diluted daily by fresh medium to an approximative cell number of 2×10^6 per ml as indicated in Table 2. The light intensity used was 8 kilolux from incandescent lamps. The culture was continuously aerated with 5% CO_2 - air mixture, at a rate of 50 c.c/min, as regulated by an air-flow Gap-meter. During the period of synchronous culture cell diameters were measured, with an ocular micrometer. Cell numbers were estimated using a Petroff-Hauser counting chamber.

Photomicrography

Photomicrographs were taken with a Leitz Ortholux microscope fitted with a camera, using Ilford Pan F film, a 40X or 100X objective, and a 12X ocular. The exposure time was automatically determined by 'Wild' photometer.

4.1.3 Observations

In the synchronising procedure, the algal mother cell produces eight autospores. This indicates that alga NMI has a basic characteristic of dividing into eight cells.

TABLE 2

Changes in cell number occurring during light-dark cycle (16:8 hours)

Time interval Light-dark cycle	I 1 - 24 hrs	II 24 - 48 hrs	III 48 - 72 hrs
Initial No of cells/ml	2.12×10^6	2.06×10^6	2.00×10^6
No of cells/ml after one cycle	17.5×10^6	17.0×10^6	17.74×10^6
Average division number (n)	8.34	8.25	8.87

TABLE 3

Changes in cell diameter of alga NMI during light-dark cycle (16 : $\overline{8}$ hours)

Time after dark period (hours)	Range of diameters of algal cells (in μm)	Dominant cell size (in μm)
1	1.8 - 2.7	2.0
4	3.6 - 4.6	3.6
10	5.0 - 5.8	5.5
15	7.3	7.3

Chlorella sp used by other workers produced 4, 8, 16 autospores(Tamiya et al, 1961; Wanka and Mulders (1967). After three light-dark cycles, satisfactory synchrony is obtained. The average division number is not absolutely constant, but fluctuates between 8.2 and 8.9. Similar fluctuations were reported by Lorenzon and Ruppel (1960), and Tamiya et al (1961).

The cell diameter varies according to age. Cells released from the mother cell during the dark period, have diameters ranging from 1.8 to 2.7 µm (Plate 4.B.).

After 4 hours in light the diameter of cells increase to about 3.6 - 4.6 µm. After 10 hours, larger single cells, before nuclear division , reach diameters of 5.0 to 5.8 µm. Ripened cells with autospores have diameters of about 7.3 µm. Table 3 represents data taken at several stages of cell development.

It is evident from the observations that the small cells, derived in the dark period, (Plate 4.B.) ripen in the light. There are no cell divisions during illumination, only increase in cell volume occurs. Cell division takes place during the dark period, and cell number is increased 8 - fold. With the technique used, the alga NMI can be maintained in synchronous culture for days.


A


B

4.1.4 Discussion

Leaves of higher plants have been generally used in studies of effects of chemicals on plant cells. However, the presence of old and new cells in the same leaf, makes it difficult to obtain chloroplasts which are physiologically homogeneous in a leaf. This lack of homogeneity constitutes a problem more particularly in ultrastructural studies. But, synchronous culture of Chlorella NMI, provides an excellent material for ultrastructural studies because of the high homogeneity in structure, growth and development.

4.2 Fine structure of Chlorella NMI and Euglena

4.2.1 Introduction

The toxic effects of herbicides, causing chlorosis of leaves in higher plants, are most probably directed against chloroplasts, photosynthetic pigments and cell growth. It is expected that these herbicides interfere with the protein synthetic systems of the chloroplast and cytoplasm, as well as with other organelles and cell division. As indicated earlier, investigation of their mode of action at an ultrastructural level may yield more information with single algal cells, (Chlorella NMI and Euglena sp) than with higher plants. However, the present knowledge of ultrastructure of Chlorella cells is unsatisfactory due to the common use of potassium permanganate as fixative in the electron microscopic studies (c.f. Chapter 3). It is, therefore, essential that the ultrastructure of the test organism be well preserved and characterised before the effects of herbicides on them can be accurately determined.

In recent years, much evidence has been accumulated indicating the presence of a chloroplastic protein synthetic system distinct from the cytoplasmic protein synthetic system in plants and algal cells.

(Kirk, 1964; Smillie et al,1967; Ray and Hanawalt, 1965; Selsky, 1967).

Both DNA, RNA have been demonstrated in chloroplasts of plants and algae, (Brawerman, 1962, 1963; Kislev et al, 1965; Eisenstadt and Brawerman, 1964; Gunning, 1965; Sagan et al, 1965; Ris and Plaut, 1962). However, the distribution and organization of DNA and presence of polyribosomes by electron microscopy have not been reported in Chlorella and Euglena.

In view of the above considerations, this study is confined to the electron microscope examination of Chlorella NMI and Euglena cells, with particular emphasis on the fine structure and nucleic acid contents of the chloroplasts.

4.2.2 Materials and Methods

The Chlorella NMI and Euglena cells were grown in Erlenmeyer flasks in the defined mineral media and aerated with CO_2 enriched air. (Section 2.1). Samples were taken from 2 - day cultures for cytological studies.

Preliminary work indicated that the removal of starch grains from Chlorella NMI and paramylon granules from Euglena was necessary to facilitate the detection of fine structures. Therefore, algal samples, taken from an actively growing culture, and left in the dark for 24 hr. at room temperature (22^{o}c) were also used in this study.

Light Microscopy

The Chlorella NMI cells were fixed in alcohol-acetic acid (3:1) for 10 minutes. Drops of the fixed cells were transferred to clean glass slides, washed with absolute ethanol and then brought to water through a series of alcohol-water. The slides were subjected to hydrolysis for 15 minutes with IN Hcl at 60°C., and stained with a Feulgen-fluorescent dye for 60 minutes in the dark. Control slides were stained without hydrolysis. The slides were washed in distilled water, and bleached in a mixture of 1 part sodium metabisulphite, 3 parts IN Hcl., and 3 parts distilled water for 10 minutes with two changes. The slides were washed in a stream of tap water, and the stained cells mounted in glycerine.

Preparations were observed by both phase contrast and fluorescence microscopy with a Leitz Wetzlar Ortholux microscope using 100 x oil immersion objective. For fluorescence, illumination was provided with a Leitz Weltzar illuminator equipped with a HB 200 high pressure mercury arc lamp and a 3 mm Leitz BG 12 blue exciting filter. The microscope eyepieces were fitted with circular orange bloc-king glass filters No. K.530. The phase and fluorescence images were recorded on Kodak Super XX film at magnitude of 600 diameters.

Electron Microscopy

The cells were fixed according to procedures 8 and 9 (Table 1). The algal cells were prefixed at room temperature in 2.5% glutaraldehyde in sodium cacodylate buffer (0.45M, pH 6.2) for 2 hours, washed in the buffer for 24 hours (3 changes), and followed by:

(a) post fixation in 1% OsO_4 in Michaelis buffer (pH 6.0) overnight at room temperature for Chlorella MNI cells, and for 3 hours at room temperature for Euglena cells. The cells were washed three times in a 3 - fold dilution of the buffer, and resuspended in a small amount of 1.5% agar (50°C).

OR

(b) Post - fixation in freshly prepared 0.3% unbuffered $KMnO_4$ solution for 10 minutes. The cells were washed five times in a 3 - fold dilution of Palade's acetate buffer, and resuspended in a small amount of 1.5% agar (50°C).

The small agar blocks from fixations (a) and (b) were stained with 0.5% uranyl acetate solution for 2 hours.

Dehydration, infiltration, embedding, sectioning and staining with lead citrate were as described in Section 2.4. Specimens were examined in a Philips EM200 or a Siemens Elmiskop 1 operating at 60 or 80 Kv.

Digestion with deoxyribonuclease

Chlorella NMI cells were fixed in 10% formalin in veronal acetate buffer (pH 6.2) for 10 minutes, washed in distilled water and digested with deoxyribonuclease (Sigma), 1 mg/ml veronal acetate buffer, at 40°C for 4 hours. Control sample was incubated in the buffer only. The cells were pre-fixed in glutaraldehyde and post - fixed in $KMnO_4$ as in (b). The fixed cells were resuspended in 1.5% agar and prepared as described in Section 2.4 for electron microscopic examination.

4.2.3 Results

A. Chlorella NMI

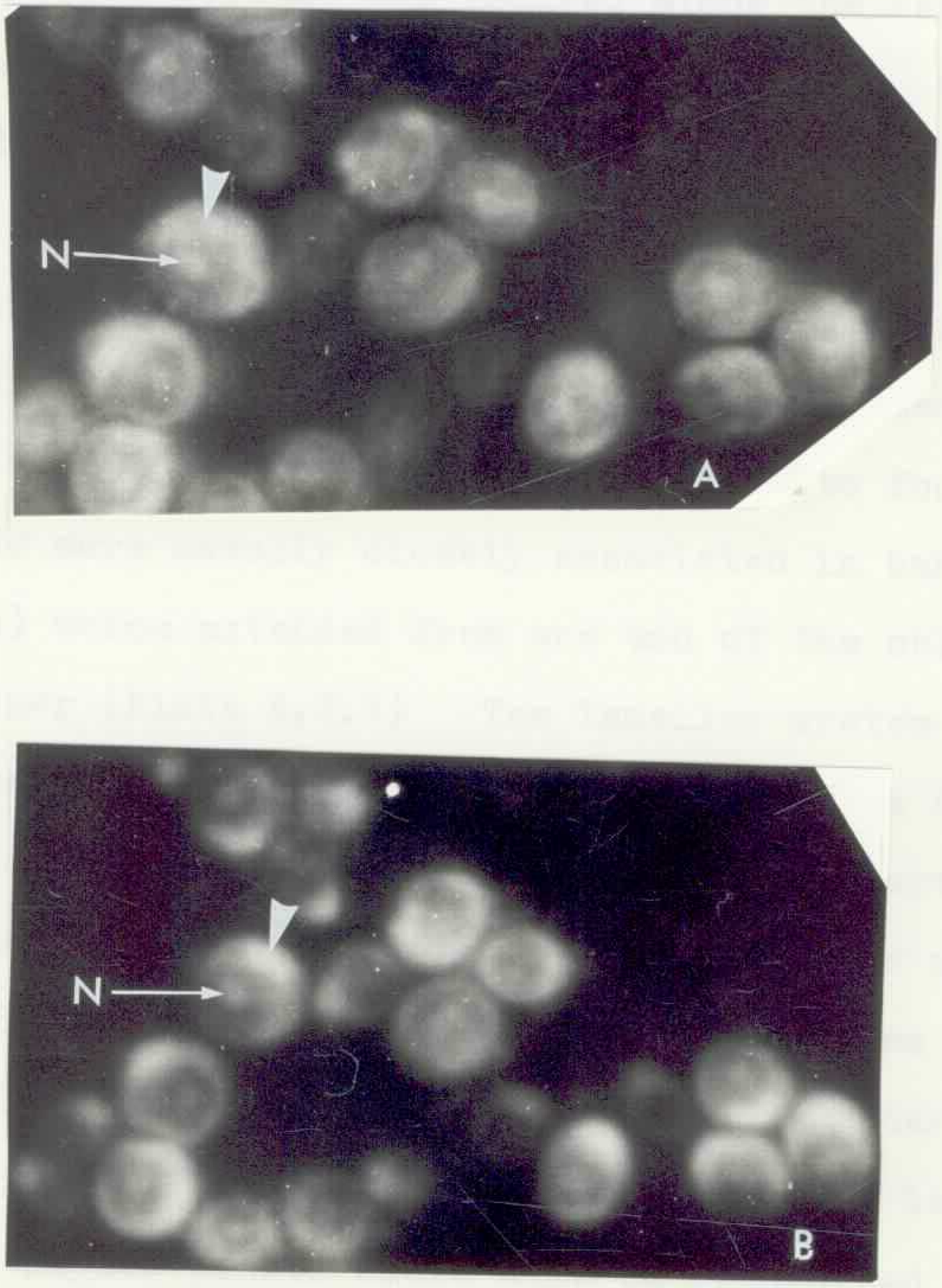
The same field of Chlorella NMI cells, stained with the fluorescent - feulgen dye, were photographed under phase-contrast (Plate 4.1.A) and fluorescence microscopy (Plate 4.1.B). Comparison of the internal structures of the cells as shown by phase contrast (Plate 4.1.A) and the DNA - containing structures in the cells fluorescing under U.V. light (Plate 4.1.B) clearly demonstrated the presence of DNA in chloroplasts of the alga.

Under the electron microscope, a mature cell was seen to contain a single cup - shaped chloroplast, with a well - defined pyrenoid, a large nucleus with an associated Golgi apparatus, and small mitochondria.

Plate 4.1.A Feulgen-fluorescent dye stained Chlorella NMI cells under phase contrast microscopy. Chloroplast (unlabelled arrow), nucleus (N)

Plate 4.1B Feulgen-fluorescent dye stained Chlorella NMI cells under fluorescent microscopy. Note fluorescence in regions identical to those identified as chloroplast and nucleus in Plate 4.1.B

4·1



A starch sheath was found around the pyrenoid and starch granules were located between the chloroplast lamellae (Plate 4.1.1)

Chloroplast

The chloroplast was located along the periphery of the cell (Plate 4.1.1). The chloroplast membrane consisted of two dense lines separated by a zone of low electron density (Plate 4.2.1). The lamellar system was well defined and consisted of orientated thylakoids (Menke, 1962), always lying with their long dimension parallel to that of the chloroplast. Two to four thylakoids were usually closely associated in bands (lamellae) which extended from one end of the chloroplast to the other (Plate 4.2.1) The lamellar system lacked the typical grana or tight stacks of thylakoids found in higher plants. However, in certain areas, parts of the thylakoids associated face to face with similar areas of other thylakoids to form 'grana-like' structures which were similar in appearance to the grana in higher plants (Plate 4.2.1). The 'stacking' pattern of the lamellar system in higher plants could also be recognized in Chlorella NMI (Plate 4.2.1). Occasionally, thylakoids from one band might cross the matrix to join an adjacent band. Small electron - opaque globuli were occasionally found in the matrix of the chloroplast (Plate 4.5.1)

Nucleic acids in the Chloroplast

Areas of low electron density were present in the ground substance between the lamellae of the chloroplast (Plate 4.3.a). These areas were not bounded by membranes and contained fibres (Plate 4.3.1A). Although there was no organised distribution of these low density areas, they were present in the vicinity of the pyrenoid and were also randomly scattered within the chloroplast. (Plates 4.3.1). Fibrils were not observed in the low electron dense areas after digestion with deoxyribonuclease. (Plate 4.4).

These areas in the OsO_4 fixed cells contained fine fibrils, prior to enzymatic treatment (Plate 4.2.1). At a higher magnification, unattached filaments, and numerous DNA structures attached to the lamellar membranes were well resolved. These included (1) linear, (2) single looped, (3) double looped configurations. (Plate 4.2.2).

Ribosomes and polyribosomes were frequently seen in close proximity or adhering to the DNA fibrils near the lamellar membranes. Free ribosomes were also observed in the vicinity of pyrenoid and in the stroma regions between the lamellae. (Plate 4.2.2).

Pyrenoid

The chloroplast usually contained one pyrenoid (Plate 4.1.2), but occasionally two pyrenoids might form on a single chloroplast (Plate 4.5.3). The pyrenoid was composed of a granular, homogeneous matrix surrounded by starch deposits (Plate 4.5.4). In general, a single lamella made up of two closely associated thylakoids transversed the pyrenoid (Plate 4.5.1). Sometimes two separate lamellae could be observed.(Plate 4.5.2). A number of DNA fibrils, with associated ribosomes were always present in the vicinity of the pyrenoid. (Plate 4.5.4).

Golgi apparatus

There was only one Golgi apparatus localised in a perinuclear region. It was composed of 4 to 6 cisternae aligned in parallel to form a stack or bundle. The cisternae were straight or only slightly curved. They were separated by intracisternal spaces. (Plate 4.6.1).

In agreement with the concept proposed by Mollenhauer and Whaley (1963), the Golgi apparatus appeared to be composed of two poles or faces. A forming face of cisternae with small diameters and a maturing face of broad and often dilated cisternae. The cisternae appeared swollen around the rims, with vesicles attached to them or found as if they had been budded off from the rim (Plate 4.6.1). Plates 4.6 (2 to 4) showed sections

of the Golgi body cut at different levels. The number of free vesicles in the space between the nucleus and the forming face of the Golgi body and the evaginations of the outer membrane of the nuclear envelope indicated that the nucleus provided membrane material to the Golgi body. (Plate 4.7).

Nucleus

The nucleus occupied a position facing the concave side of the chloroplast. It was bounded by an envelope composed of two membranes separated by an intervening space. (Plate 4.1.2). The continuity of the envelope was interrupted sometimes by pores, the lumen of which seemed to contained some organized material. (Plate 4.1.4). These pores might probably allow passage of material between the nucleus and the cytoplasm (Plate 4.1.3).

The nucleus contained a prominent nucleolus, consisting of particles about the size and density of the cytoplasmic ribosomes (Plates 4.1.1, 4.1.3).

In OsO_4 fixed cells, the nuclear material was homogeneous (Plate 4.1.1). In (Plate 4.1.3), however, the nuclear material appeared in two forms, a relatively dense area, presumably representing inactive chromatin (heterochromatin), and less dense active chromatin. The heterochromatin was found around the periphery of the nucleus, and also in small chumps scattered throughout the nucleoplasm.

Such distinct regions in the nucleus could also be distinguished in the Glut. $KMnO_4$ fixed cell (Plate 4.3.1). The outer membrane often gave rise to blebs, especially in the region adjacent to the forming face of the Golgi body. (Plate 4.7).

Mitochondria

These organelles were limited by two distinct membranes. The invaginations of the inner membrane into the mitochondrial matrix formed cristae. (Plate 4.5.5). The shape of the mitochondria was variable, and there was no apparent association of the mitochondria with other organelles. The mitochondria tended to lie along the periphery of the young cell (Plate 4.1.2).

Fine DNA fibrils resembling those in the chloroplasts and dense particles resembling ribosomes were also observed in glutaraldehyde - OsO_4 fixed cells (Plate 4.5.6) However, in $KMnO_4$ fixed cells only the areas of low electron density could be distinguished (Plate 4.3).

Vacuole

In the young cell, a distinct vacuole was usually absent. As the cell aged, however, a large vacuole was seen surrounded by a membrane (tonoplast). (Plate 4.5.4).

4.2.4 Discussion

The electron microscopic observations show that, although the Chlorella chloroplast lacks the typical grana found in higher plants, stacking of thylakoids occurs in the alga to form grana-like structures. These are very similar in appearance and construction to the grana of higher plants, however, in Chlorella the stacking occurs by the lateral associations of 2 to 6 thylakoids. The results indicate that stacking may be an essential element of the lamella system of Chlorella.

Based on chloroplast lamellae organisation Manton (1966), has suggested that Chlorella may represent an intermediate form between "lower" and "higher" plants. She proposed that higher plants have grana and Chlorophyll b, and lower plants have grana free chloroplasts or free thylakoids and accessory pigments different from Chlorophyll b.

Nucleic Acids

In the present electron microscope study, the removal by deoxyribonuclease digestion of fibrils, associated with the low density areas in the ground substance of the chloroplast, indicates that they are likely to be DNA fibrils. This observation is supported by the light microscope evidence obtained with Feulgen fluorescent dye staining of DNA in the chloroplast (Plate 4A).

Gibbs (1968), Bisalputra and Bisalputra (1969) have shown the existence of a regular pattern of internal organisation of DNA containing areas in brown algae. In Chlorella NMI however, no such pattern is detected. A number of DNA containing areas occur in sections of chloroplasts. They are found in the vicinity of the pyrenoid as well as between the lamellae (Plate 4.2.1). Their distribution is much more conspicuous than that reported by Ris and Plaut (1962) for Chlamydomonas.

Different configurations of DNA units in chloroplast and relationship between these units and internal chloroplast membranes have been reported in plants and algae (Bisalputra, 1967; Woodcock and Fernandez, 1968; Werz and Kellner, 1968; Bisalputra and Burton, 1969).

The present results clearly demonstrate that chloroplast DNA of Chlorella NMI exist as a number of short single units, (Plate 4.2.2). These units are not restricted to the low density areas, but are also found in electron dense areas, where their presence may sometimes be obscured by the ribosomes. This suggests a wide distribution of DNA in chloroplast. However, a few areas containing more DNA appear to be randomly distributed through the chloroplast matrix and around the pyrenoid region. These areas may represent sites of greater activity in the chloroplast.

The presence of linear, single looped and double looped configurations of DNA units (Plate 4.2.2) support the conclusion of Bisalputra and Burton (1969) that the chloroplast has its own self-duplication mechanism. Furthermore, this study shows an intimate association of DNA units not only with the lamella membranes but also with the pyrenoid. Plates 4.2.2 and 4.5.4 show attachment of DNA fibrils to the lamellae at several locations. The direct associations of ribosomes to the DNA fibrils and their close proximity to the lamella membrane (Plate 4.2.2) suggest the possibility of formation of ribosomes at the site of attachment of the DNA units. Gibbs (1967) using Ochromonas danica,

had shown by autoradiography that during synthesis of chloroplast RNA, the newly formed RNA appeared over the regions of the chloroplast DNA. After two hours the RNA was distributed throughout the chloroplast. Scott and Smillie (1968) have presented evidence that chloroplast DNA coded for the RNA of chloroplast ribosomes. These findings receive support by the presence of ribosomes and polyribosomes close to DNA fibrils as well as free within the matrix in Chlorella NMI. The close association of DNA fibrils with the lamellar membranes, ribosomes and polyribosomes suggest the presence of a protein synthetic system closely linked with the lamellae of the chloroplast. This visual indication of an association between the lamellar membranes and a protein synthetic in the chloroplast makes it easier to understand the biochemical finding of the interrelationship between lamellae and pigment formation andprotein synthesis in chloroplast (Sokawa and Hase, 1967; Stern et al. 1964 a,b).

Golgi body

Plates 4.6.1, 2, 3 and 4 suggest a rather complex structure of the Golgi body. They indicate the presence of a compact central region, which gives rise to the form of cisternae seen in sectioned material (Plate 4.6). Short tubules project all around the rims of the plates. The tubules do not anastomose as in the meristematic cells of Anthoceros (Manton, 1960), or cells from the stem of Allium cepa (Cunningham et al, 1966). Vesicles are attached to them or are released by budding at their tips. The connection between the Golgi body and the nucleus of Chlorella NMI is clearly shown in Plates 4.7.1, 2, 3 and 4. The Golgi body is consistently oriented close to the nuclear envelope. The involvement of the nucleus appears to be the synthesis of membrane which is first seen as small blebs associated with the outer layer of the nuclear envelope. The blebs constrict from the remainder of the envelope and detach as isolated vesicles, which are then incorporated into the structure of the Golgi body at the formative face.

Bouck (1965) found that in the order Fucales, there was a tendency for the Golgi body to be associated

with the endoplasmic reticulum as well as with the nuclear membrane, and he proposed that this tendency might be of some evolutionary significance. The present study shows that, while the Golgi body is located near the nucleus, raw material for its development is provided by the outer nuclear membrane. In more highly evolved organisms, several Golgi bodies are present in a cell, and these are usually found away from the nucleus. In these cases, raw material is probably provided by the endoplasmic reticulum (Kessel, 1971).

Mitochondria

There are a number of electron microscope reports concerning nucleic acids in mitochondria of animal cells, but very few in plant cells. DNA fibrils have been reported in mitochondria of a flagellate (Ris, 1962), chick enbryo (Nass and Nass, 1963), and ribosome in mitochondria of rat liver (Watson and Aldridge, 1964). The present study shows both DNA fibrils and ribosomes in mitochondria of Chlorella NMI cells, and provides additional evidence to the view that mitochondria may be able to synthesise proteins independently of nuclear control, and be self-reproducing.

4.2.5. Results.

B. Euglena

The fine structure of the normal green Euglena has been described in various papers (Gibbs, 1960; Wolken, 1961; Leedale, 1963; Buetow, 1968).

To facilitate interpretation of changes accompanying treatment with herbicide, a brief description of the species used in the present study was thought necessary, but with particular reference to the existence of the components of a protein synthetic system in the Euglena chloroplast.

Plate 4.8 shows photomicrographs of Euglena cells in different stages of the life cycle. The Euglena cell had 10 to 12 elongated chloroplasts. (Plate 4.9.2) and usually contained a number of paramylon bodies (Plate 4.9.1). Plate 4.10.1 represents an electron micrograph of a Glut - OsO_4 fixed cell. Each of the chloroplasts contained 10 to 20 bands of lamellae, made up of a variable number of thylakoids (2 to 6), (Plate 4.11.1). Each chloroplast contained a single pyrenoid. In a transverse section, the pyrenoid was observed to extend across the width of the chloroplast, and to have a sheath of paramylon on each side. Plate 4.12.1 shows a micrograph of such a section from a cell fixed in $KMnO_4$ only. The pyrenoid matrix was different from that of the remainder of the chloroplast, and was crossed by widely spaced bands of lamellae, consisting of two thylakoids only.

Nucleic acids

In cells fixed with $KMnO_4$ only, the chromatinic areas of the nucleus were well contrasted (Plate 4.12.1). Regions of low electron density, presumably containing DNA were present within the matrix of the chloroplast, mitochondria and nucleolus. Fibrils within these areas could be distinguished in the mitochondria and nucleolus, but were difficult to detect in the chloroplast.

The stabilisation of the fibrils in the nucleus by $KMnO_4$ was clearly illustrated in Plate 4.12.2. The fibrils in low density areas in the nucleolus were morphologically similar to those in the nucleus (Plate 4.12.2).

After deoxyribonuclease digestion, the fibrils were no longer visible in the nucleus, which appeared as a structure of homogeneous matrix. A similar homogeneous ground substance could be observed in the mitochondria and the areas between the chloroplast lamellae (Plate 4.13.1).

Pre-fixation by glutaraldehyde followed by $KMnO_4$ preserved the DNA fibrils in the chloroplast. These fibrils were distributed throughout the matrix between the lamellae, and at least some were seen attached to the lamellar membranes (Plate 4.13.2).

Ribosomes, removed in the $KMnO_4$ fixed cells, were well preserved by the glutaraldehyde - OsO_4 fixation. Polyribosomes were often seen attached to the lamella membranes and free in the stroma region of the chloroplast (Plate 4.14.1).

Pellicle

The pellicle was quite flexible and changed shape during movement (Plate 4.9.2).

The pellicle was composed of (1) the plasmalemma, (2) ridges and grooves, (3) four microtubules, and (4) a subpellicular tubule of the endoplasmic reticulum. Two of the microtubules within the ridge are close together with one touching the cell membrane (Plate 4.11.2)

Stigma or eyespot

The eyespot was found outside the chloroplast, in contrast to the eyespot found within the chloroplast membrane in the motile green chlamydomonas. (Walne, *et al* 1967) The eyespot was located at one side of the base of the reservoir, facing the paraflagellar body. (Plate 4.14.2) In the living cells, the eyespot consisted of a group of orange-red granules, (Plate 4.9.3), which appeared as dense osmiophilic globules of variable diameter under the electron microscope. Each granule seemed to be surrounded by a membrane. The eyespot, as a whole, had no bounding membranes. The granules appeared to lie free in the cytoplasm in a loosely packed group.

A number of tubules could be observed between the eyespot and the limiting membrane of the reservoir. (Plate 4.14.2).

Golgi apparatus

More than one Golgi body were found in the Euglena cell. Each consisted of 8 to 20 stacked flattened cisternae, with vesicles budding off at both ends (Plate 4.11.3).

Mitochondria

The mitochondria varied in size, shape and structure and were usually found scattered within the cytoplasm (Plate 4.12.1).

4.2.6 Discussion

The electromicrographs, showing sections of chloroplast, Golgi body, sculptured pellicle, eyespot of the Euglena sp. used in the present study, are very similar to those described by Gibbs (1960), and Buetow (1968) for Euglena gracilis. These similarities suggest that the Euglena sp. studied may be a Euglena gracilis strain.

Nucleic acids

The presence of DNA and RNA in the chloroplast of Euglena have been well established by biochemical and autoradiographic techniques (Brawerman, G, 1963;

Brawerman and Eisenstadt, 1964; Ray and Hanawalt, 1964; Sagan et al, 1965). Detailed electron microscopic studies of Euglena cells have been made (Wolken, 1961; Leedale, 1967; Buetow, 1968) and although the presence of ribosomes have been reported, DNA fibrils and polyribosomes have never been demonstrated in the chloroplasts.

In this electron microscope investigation, the presence of DNA fibrils, ribosomes and polyribosomes is convincingly demonstrated in the chloroplast of Euglena. The attachment of DNA and polyribosomes to the lamella membrane suggests that ribosomes in the chloroplast of Euglena are formed at the site of attachment of DNA to the lamella membrane and assembled into polyribosomes. This would also imply that synthesis of proteins by these polysomes takes place at the same site. During the ribosome cycle, the ribosomes and polysomes may become detached from the membrane and move into the stroma region.

This electron microscope demonstration of both DNA and polyribosomes in the Euglene chloroplast confirms that chloroplast contains the components necessary to synthesize most if not all of its proteins. Furthermore, as discussed earlier, for Chlorella

chloroplasts, the present investigation shows the close relationship between a chloroplast protein synthetic system and the lamellae in Euglena.

KEY to labeling

C - chloroplast

La - lamella

f - DNA fibrils

Py - pyrenoid

CW - cell wall

CM - chloroplast membrane

R - cytoplasmic ribosome

Cr - chloroplast ribosome

M - mitochondria

G - Golgi body

N - nucleus

Po - polyribosome

Lo - low density area

Plate 4.1

Plate 4.1.1

Section of a chlorella NMI cell.
The chloroplast (C) is cup-shaped and located around the periphery of the cell. Note the single pyrenoid (P) with starch sheath (S). Starch granules (S) are also found between the lamellae.
The nucleus (N) is well defined with a central nucleolus (Nu), and a perinuclear Golgi body (G). Structure in the nucleus indicated by an arrow may be microtubule.
Glut - OsO_4. x 33,600

Plate 4.1.2

Nuclear pore (arrow), showing presence of material in the lumen.
Glut - OsO_4. x 105,000

Plate 4.1.3

Section of cell, showing the nucleus (N) and Golgi body (G) and mitochondria (M). Note darkly stained areas of heterochromatin (white arrows) in the nucleoplasm. Nuclear pore is indicated by black arrow.
Glut - OsO_4. x 61,800

Plate 4.2

Plate 4.2.1

Section of chlorella NMI showing the peripheral chloroplast. Fine fibrils (f) and ribosomes (Cr) can be seen in the organelle. Thylakoids, associated into bands, (La) are well orientated in one direction, and cross the chloroplast from one end to the other. Plasma membrane (PM) is made of two dense lines with an intervening clear space.
The chloroplast membrane (CM) is also tripartite, but each dense line has a unit membrane structure - (circle in inset 4.2.1A)
Glut - OsO_4. Plate 4.2.1 x 53,000
Plate 4.2.1A x 116,000

Plate 4.2

Plate 4.2.2

Magnified portion of Plate 4.2.2. Note presence of linear DNA fibrils (double arrows), single loop (L) and double loops (LS). White arrows indicate attachment of DNA fibrils to lamellar membrane. Ribosomes (Cr) and polysomes (Po) are found close to or on the DNA fibrils.

Glut - OsO_4. x 105,000

Plate 4.3

Plate 4.3.1

Cell printed for maximum contrast. Note presence of low electron dense areas (Lo) around the pyrenoid and between the lamellae in chloroplast, and in mitochondria (M). Two differently stained areas can be seen in the nucleus (N). Inset (A) shows DNA fibrils in low density area.

Glut - $KMnO_4$. x 60,000

Plate 4.4

Plate 4.4.1

Portion of chloroplast, after deoxyribonuclease treatment and printed for maximum contrast. Note absence of DNA fibrils in low electron dense areas.

Glut - $KMnO_4$. x 60,000

Plate 4.5

Plate 4.5.1

Portion of chloroplast showing pyrenoid crossed by one lamella. Arrow indicates lamella from one 'granum-like' stack crossing over to another.

Glut - OsO_4. x 66,300

Plate 4.5 con't.

Plate 4.5.2 — Micrograph showing three lamellae across the pyrenoid.
Glut - OsO_4. x 88,500

Plate 4.5.3 — Section of chlorella NMI cell, showing chloroplast with 2 pyrenoids.
Glut - $KMnO_4$. x 29,700

Plate 4.5.4 — Portion of chloroplast showing pyrenoid area. Note starch sheath (S) and DNA fibrils (f) around pyrenoid.
Glut - OsO_4. x 22,100

Plate 4.5.5 — Micrograph shows a mitochondrion. Arrow indicates crista formed from invagination of inner membrane.
Glut - OsO_4. x 50,400

Plate 4.5.6 — Section shows DNA fibrils (f) and ribosomes (Mr) in mitochondrion. Note presence of DNA fibrils of similar morphology in chloroplast (arrow).
Glut - OsO_4. x 60,000

Plate 4.6

Plates 4.6. 1,2, 3 and 4 — Golgi body sectioned at different levels. Note absence of cytoplasmic ribosomes in area between nucleus and forming face of Golgi body. (Plates 4.6.1,2).
Glut - OsO_4.
Plate 4.6.1 - x 72,000
Plates 4.6.2,3 and 4 - x 116,000

Plate 4.7

Plate 4.7.1 — Section of chlorella NMI cell illustrates perinuclear location of Golgi body.
Glut - OsO_4. x 61,800

Plates 4.7.2, 4.7.3, 4.7.4 — The nucleus (N) and region of bbebbing of the outer membrane of the nuclear envelope (arrows) are identified in all figures. Constriction of bleb from remainder of the nuclear envelope is indicated by double arrows (plate 4.7.3)
Glut - OsO_4.
Plates 4.7.2 and 3 x 116,000
Plate 4.7.4 x 36,200
Plate 4.7.5 x 88,200

Plate 4.8

Plates 4.8. 1, 2, 3 and 4 — Euglena cell at different stages of cell division.

Plate 4.9

Plate 4.9.1 — Euglena cell grown autotrophically in light for 72 hours. Note number of paramylon bodies (p), nucleus (n).

Plate 4.9.2 — Euglena culture starved for 24 hours to facilitate viewing of chloroplasts. More than 10 elongate chloroplasts are found in the cell. The different shapes of the cells illustrate the flexibility of the pellicle. Note nucleus (n) with well defined nucleolus (nu).

Plate 4.9.3 — Euglena cell fixed in glutaraldehyde. Note packet of dark granules forming the eye-spot (E).

Plate 4.9.4 Particulate appearance of nucleoplasm (arrow) indicates presence of chromatin and heterochromatin areas.

Plate 4.10

Plate 4.10.1 Section of Euglena showing a number of chloroplasts and Golgi bodies. Note presence of several paramylon bodies in the cytoplasm.

Glut - O_sO_4. x 12,000

Plate 4.11

Plate 4.11.1 Section of chloroplast to illustrate grana-like regions containing 2-6 thylakoids, lipid globules (arrow)

Glut - O_sO_4. x 60,000

Plate 4.11.2 Pellicle complex of Euglena cells. Sub- pellicular endoplasmic reticulum (SER), microtubules (T)

Glut - O_sO_4. x 105,000

Plate 4.11.3 Section showing Golgi body. Note vesicles budding off at both ends of the cisternae.

Glut - O_sO_4. x 60,000

Plate 4.12

Plate 4.12.1 — Section of Euglena cell, showing elongate chloroplasts (C) with pyrenoid (Py) and adjacent paramylon sheath (P), mitochondria (M) with low density areas (arrows), Golgi body (G), nucleus (N) with areas containing network of fine and beaded DNA fibrils. Note low density areas with DNA fibrils (arrow) in nucleolus (Nu).
$KMnO_4$. x 12,900

Plate 4.12.2 — Magnified portion of Plate 4.12.1 to illustrate DNA fibrils (arrows) in low density areas in nucleus and nucleolus.
$KMnO_4$. x 26,7000

Plate 4.13

Plate 4.13.1 — Section through part of Euglena cell after deoxyribonuclease treatment. Note homogeneous matrix of nucleus (N), mitochondria (M) and stroma between lamellae of chloroplast (arrow), as compared to the matrix with DNA fibrils in the untreated cell (Plate 4.12.1)
$KMnO_4$. x 20,000

Plate 4.13.2 — Section through chloroplast of Euglena, showing five DNA fibrils (black arrows). Note attachment of DNA fibrils to lamellae (white arrows)
Glut - $KMnO_4$. x 80,000

Plate 4.14

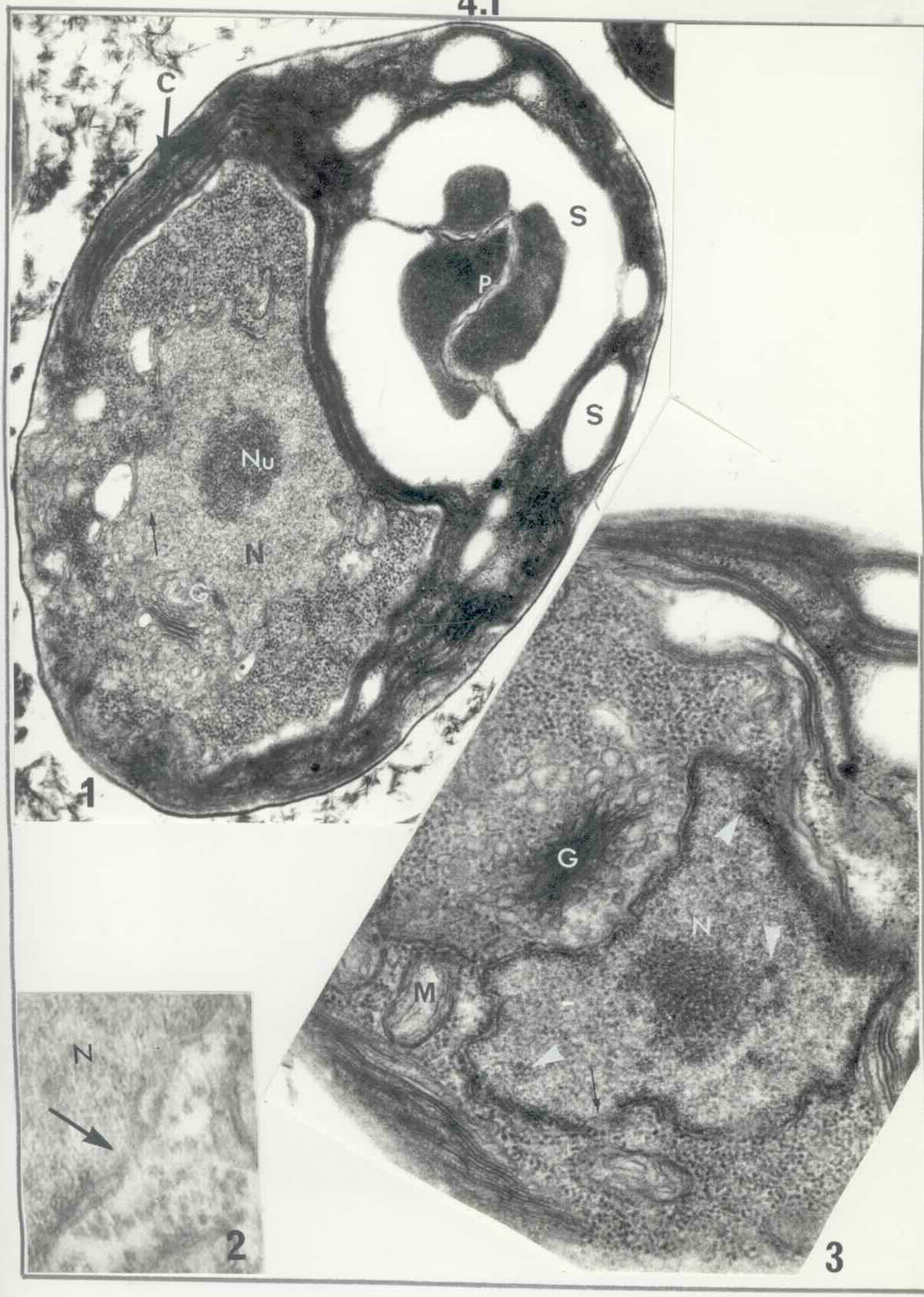
Plate 4.14.1 — Portion of Euglena chloroplast, showing polyribosomes attached to the lamellae (insets A and B)

Glut - O_sO_4. - x 66,750 A.
x 116,900 B.

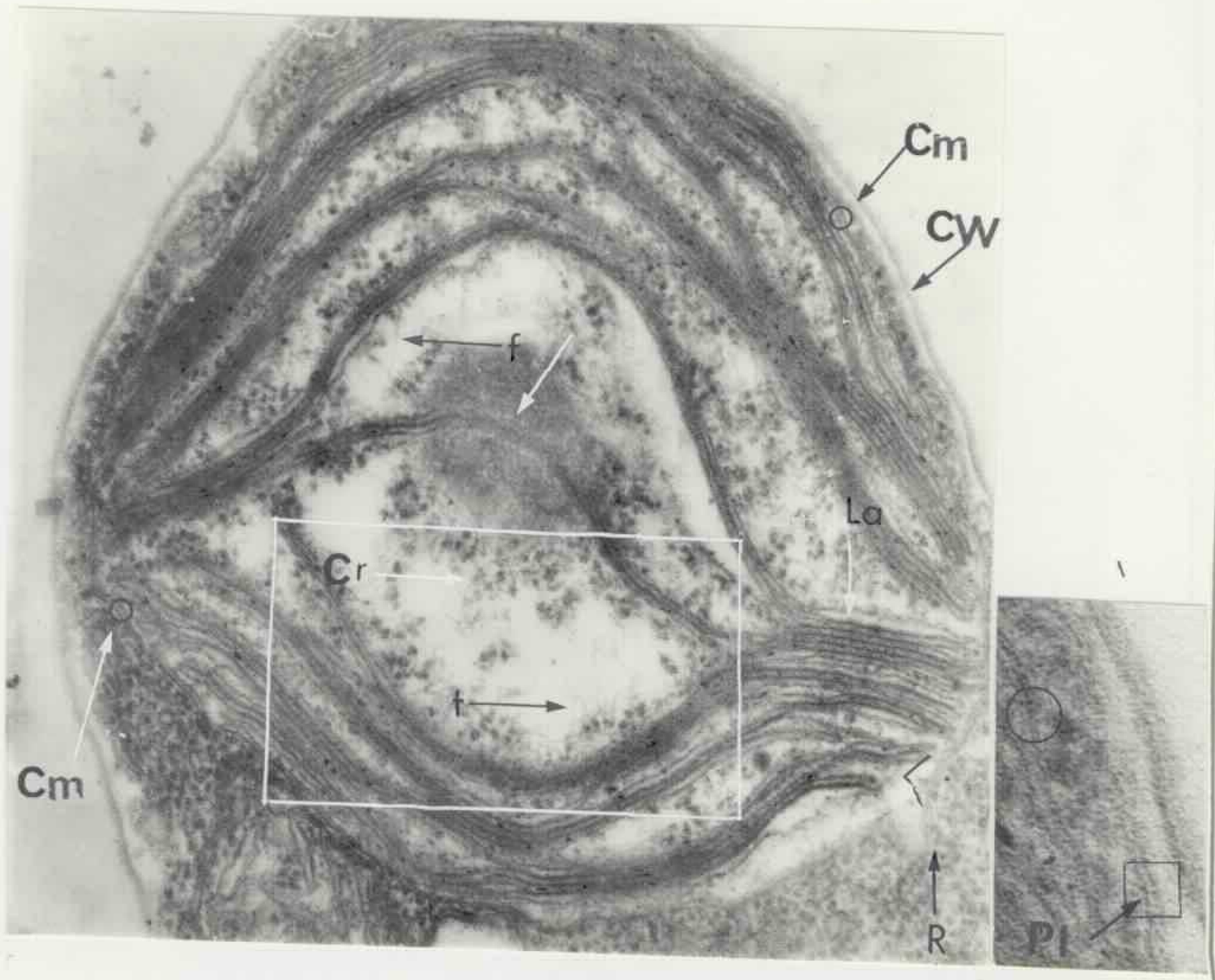
Plate 4.14.2 — Portion of Euglena showing eye spot, adjacent to the reservoir (R).

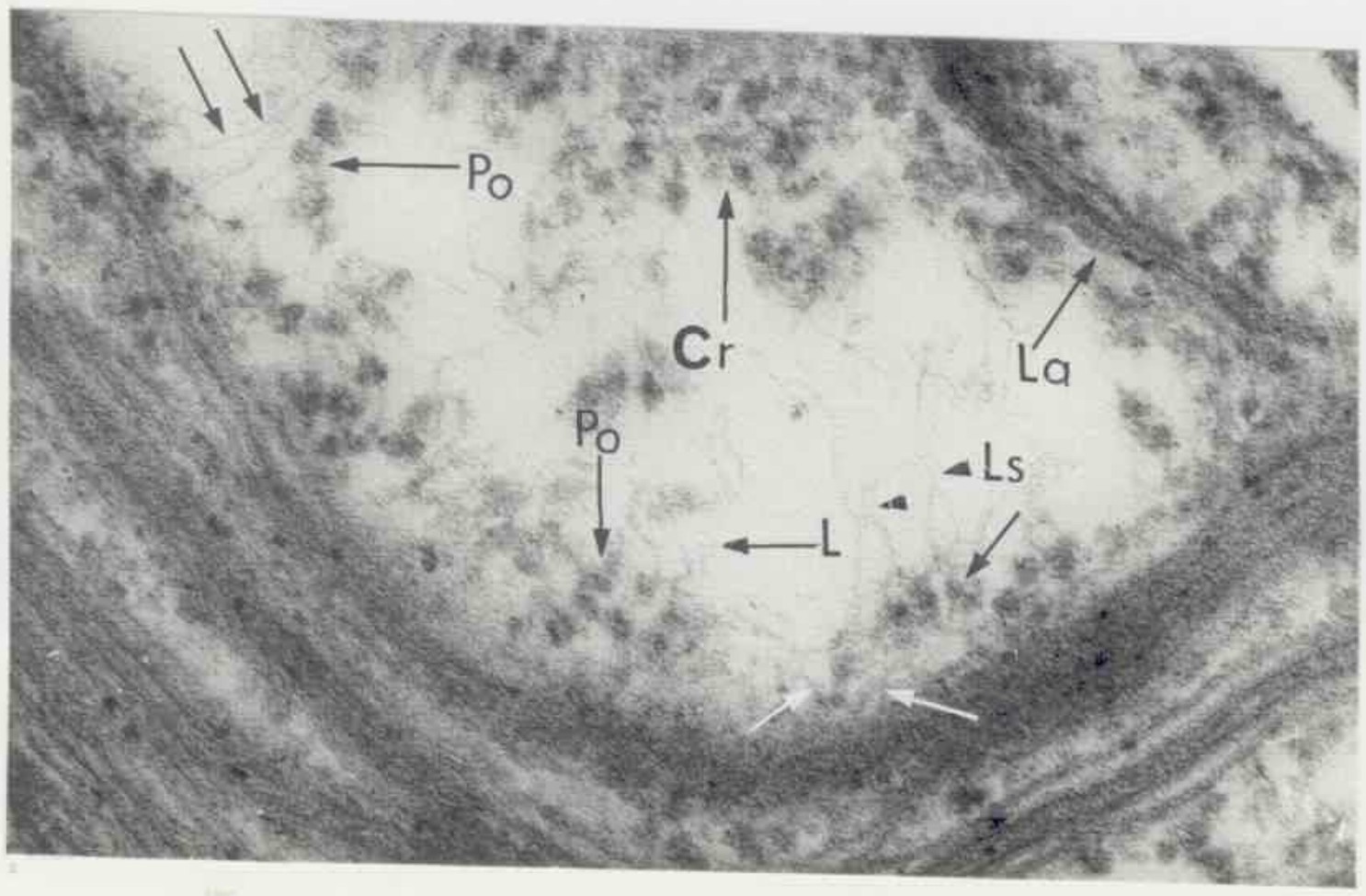
Glut - O_sO_4. x 52,500

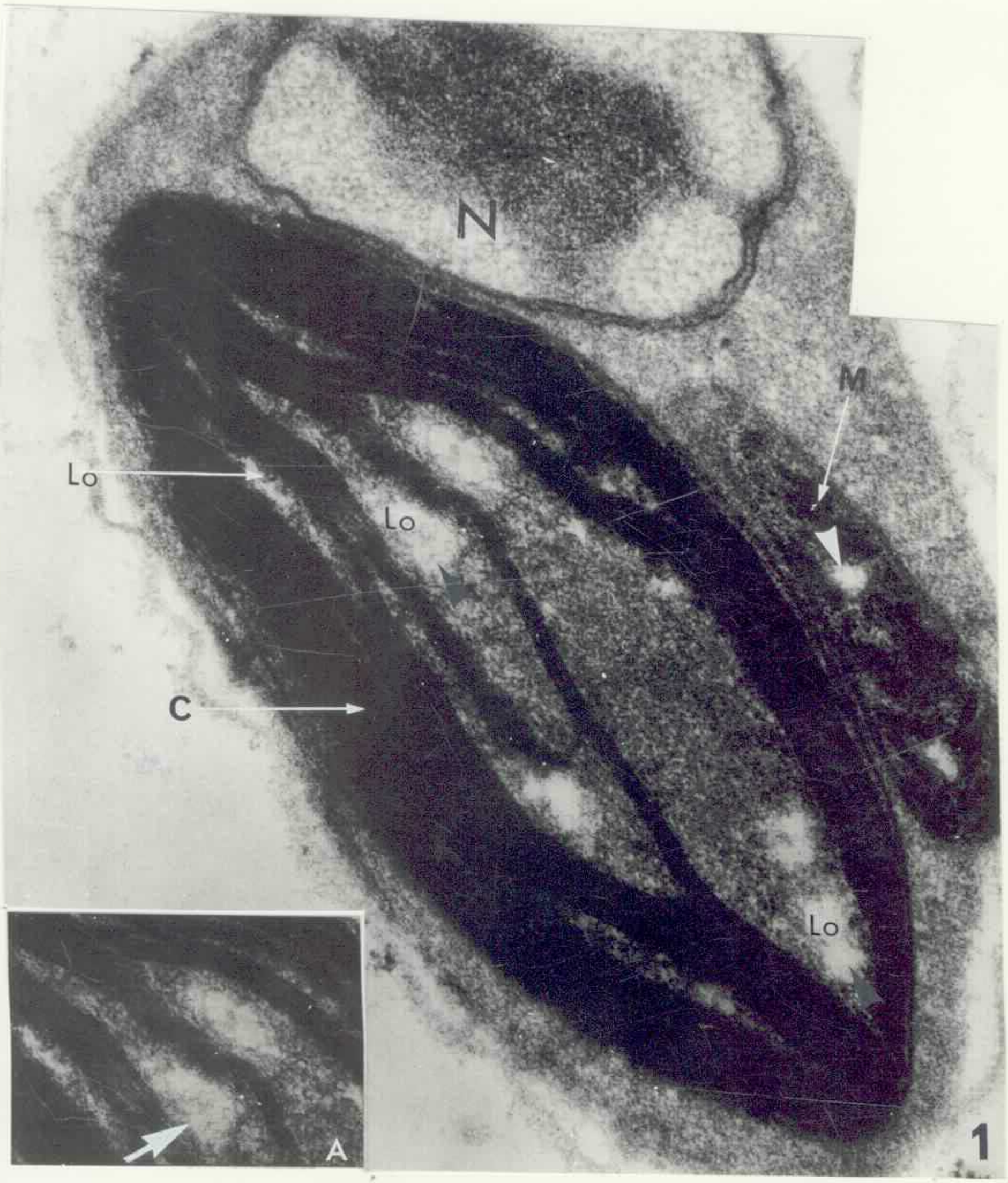
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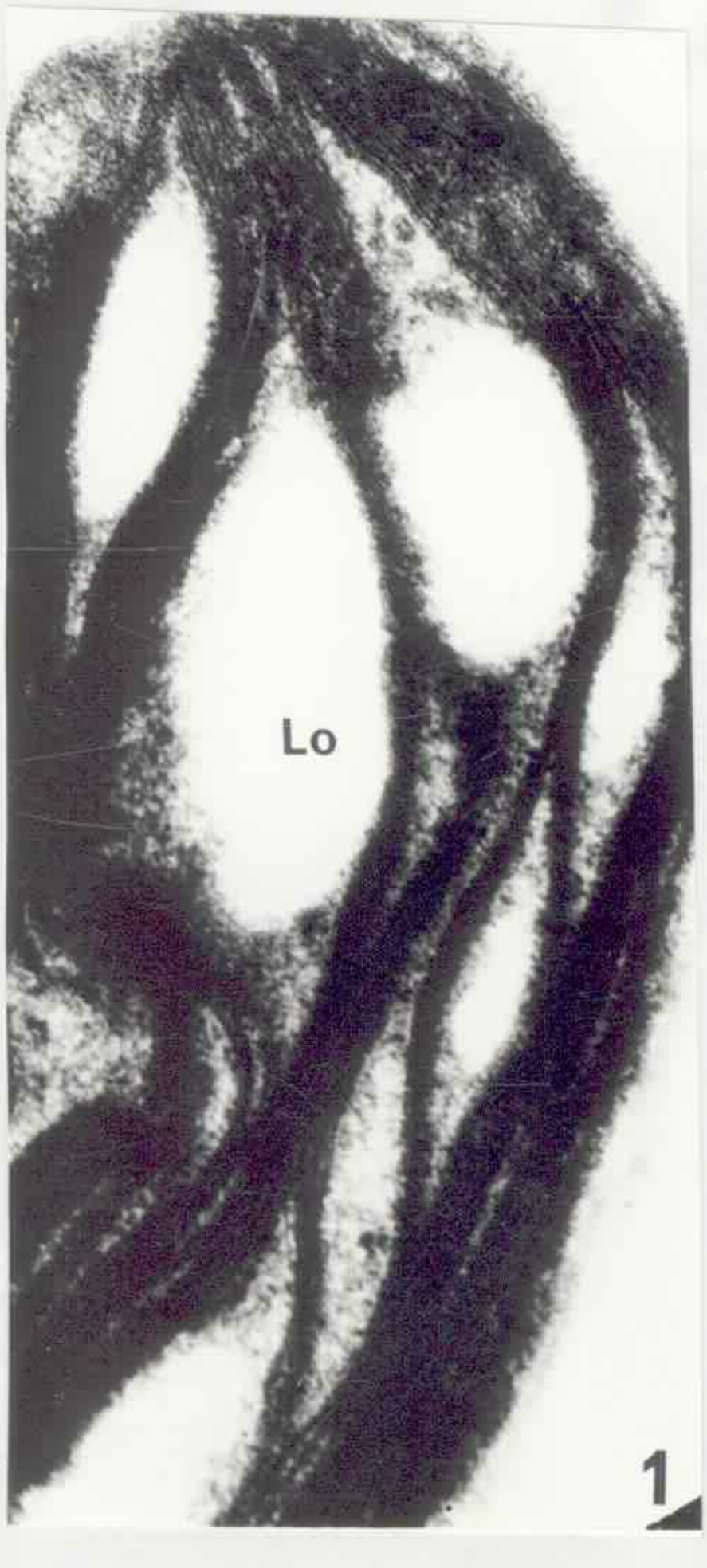
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N
M
Lo
Lo
C
Lo
A
1

4.4



4.5



1



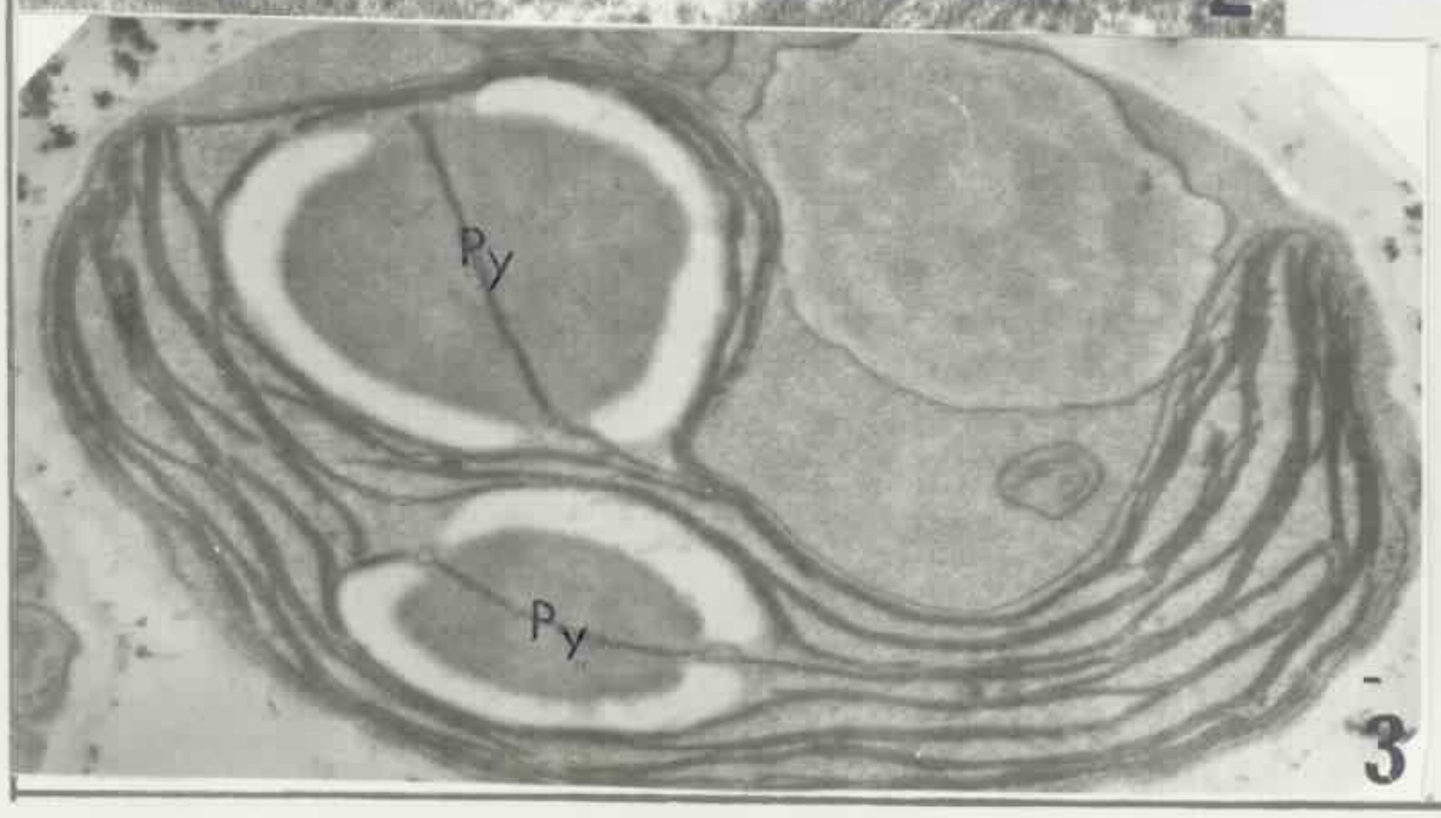
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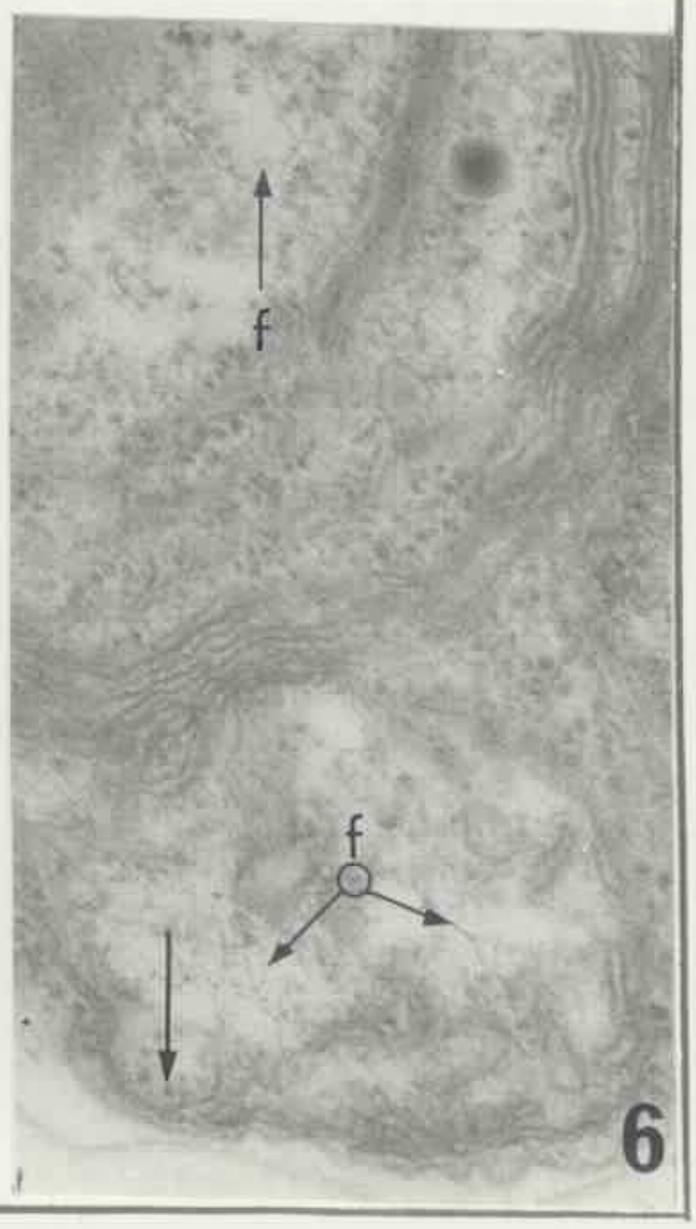
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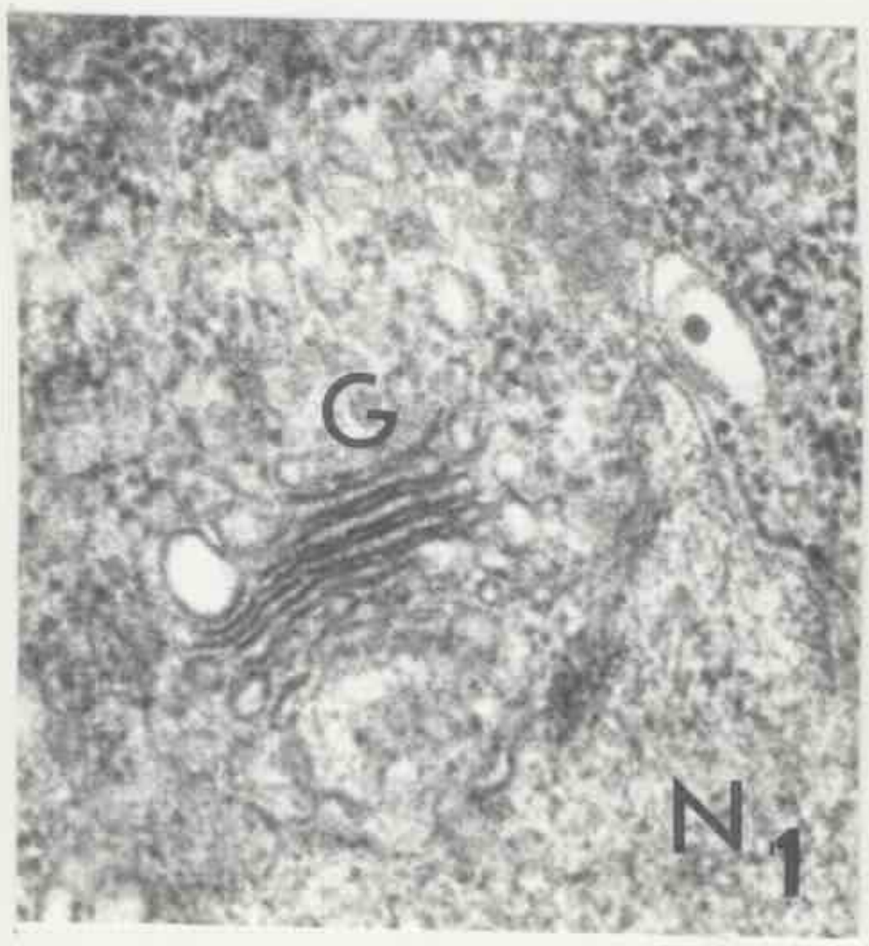


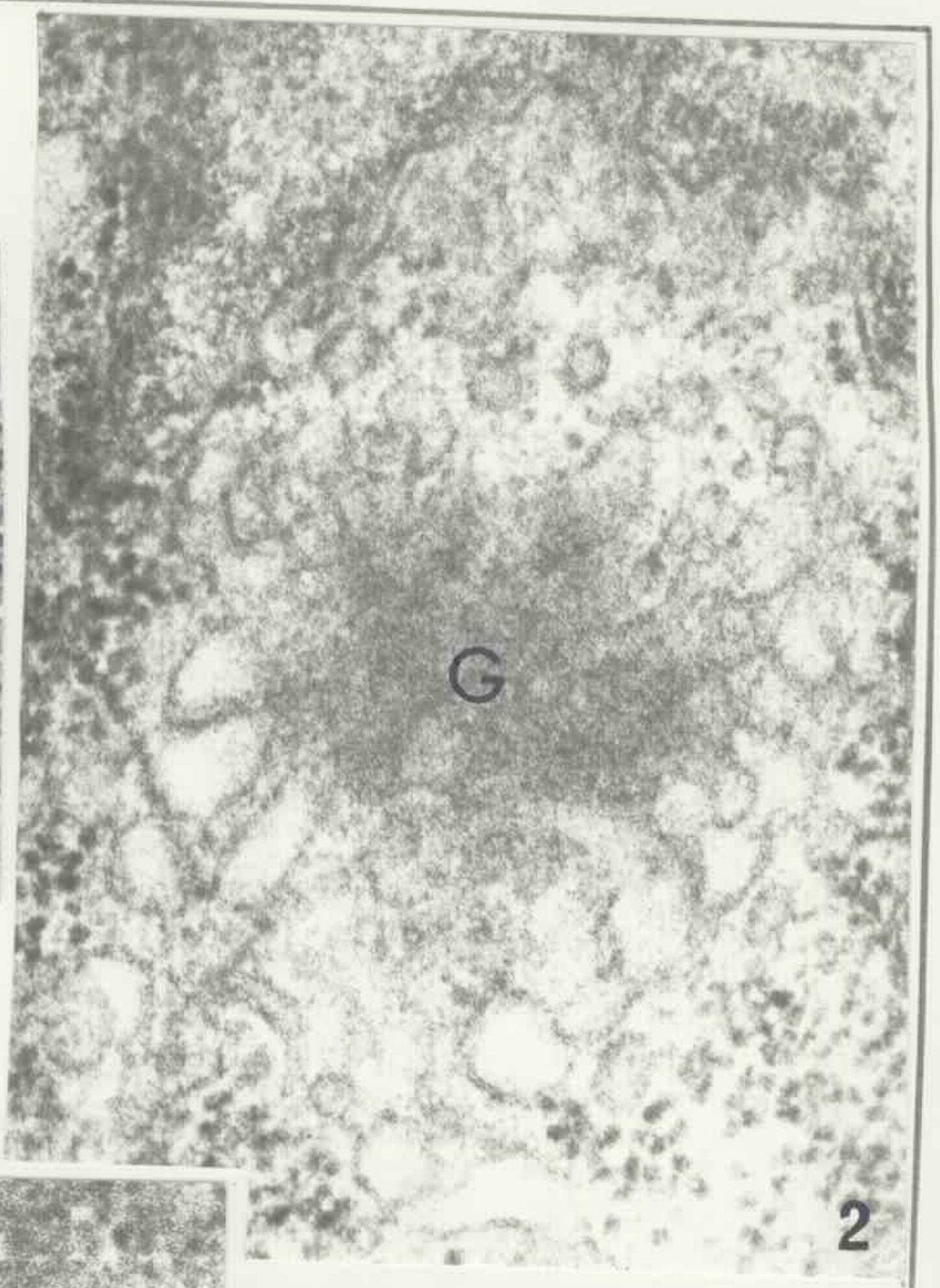
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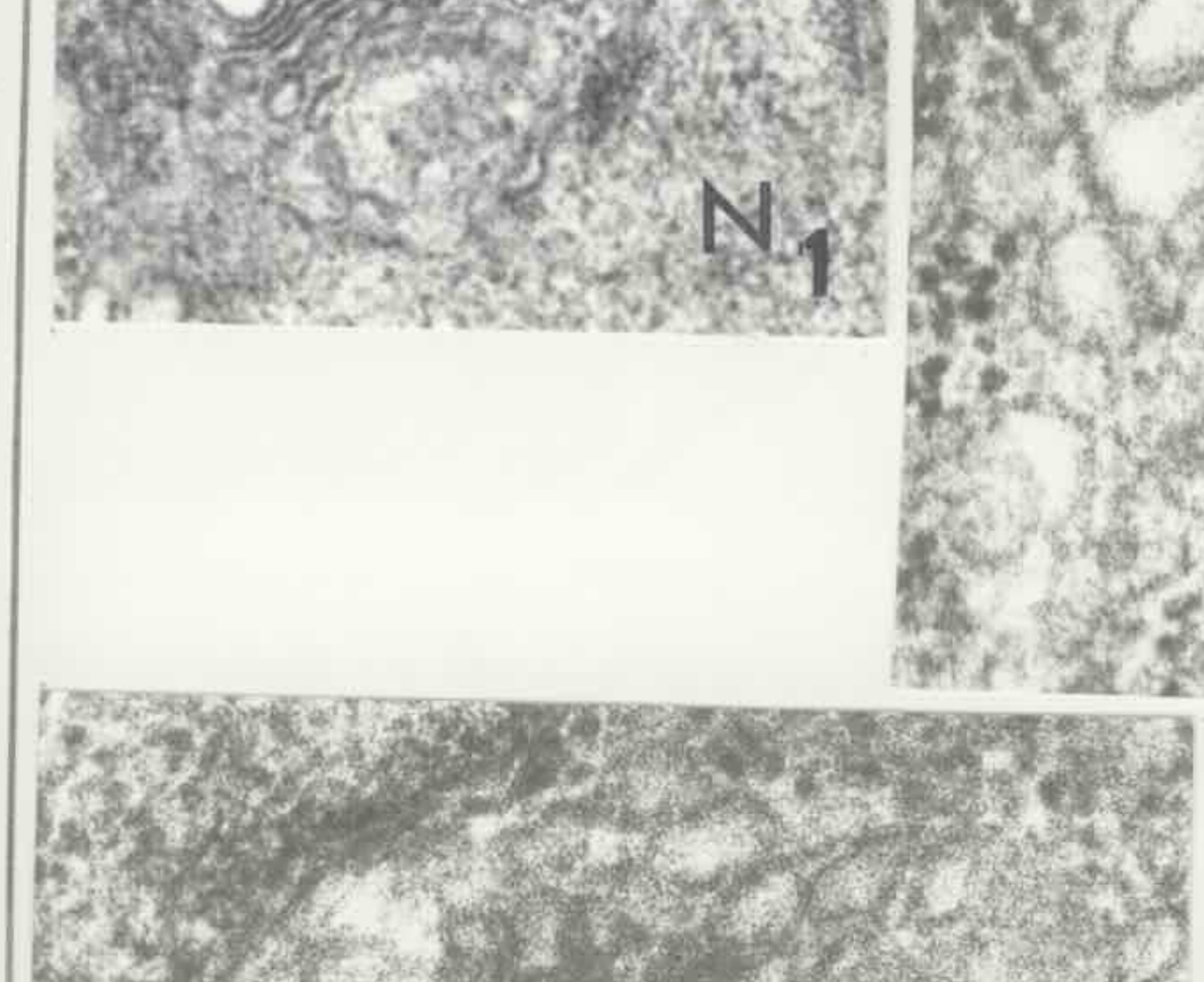


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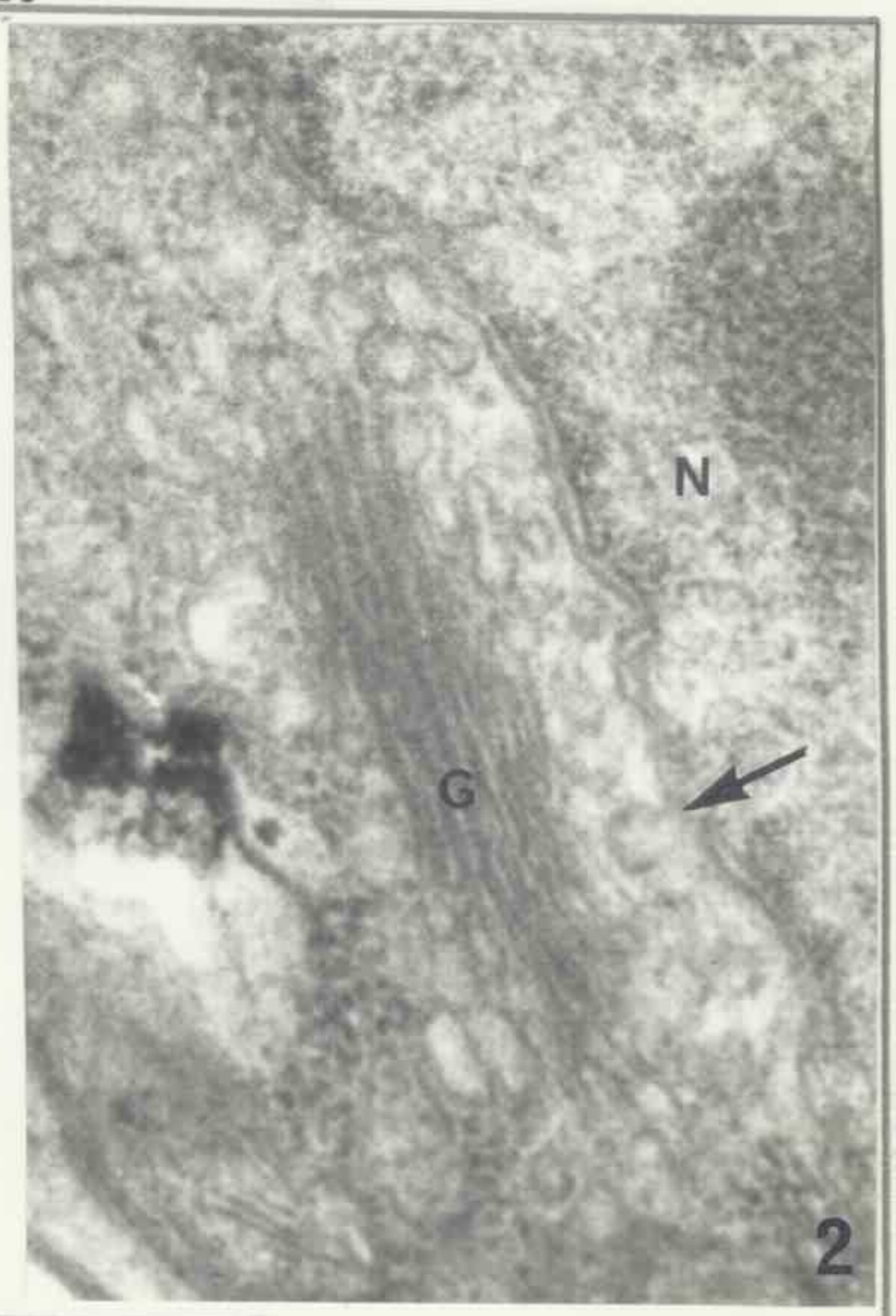


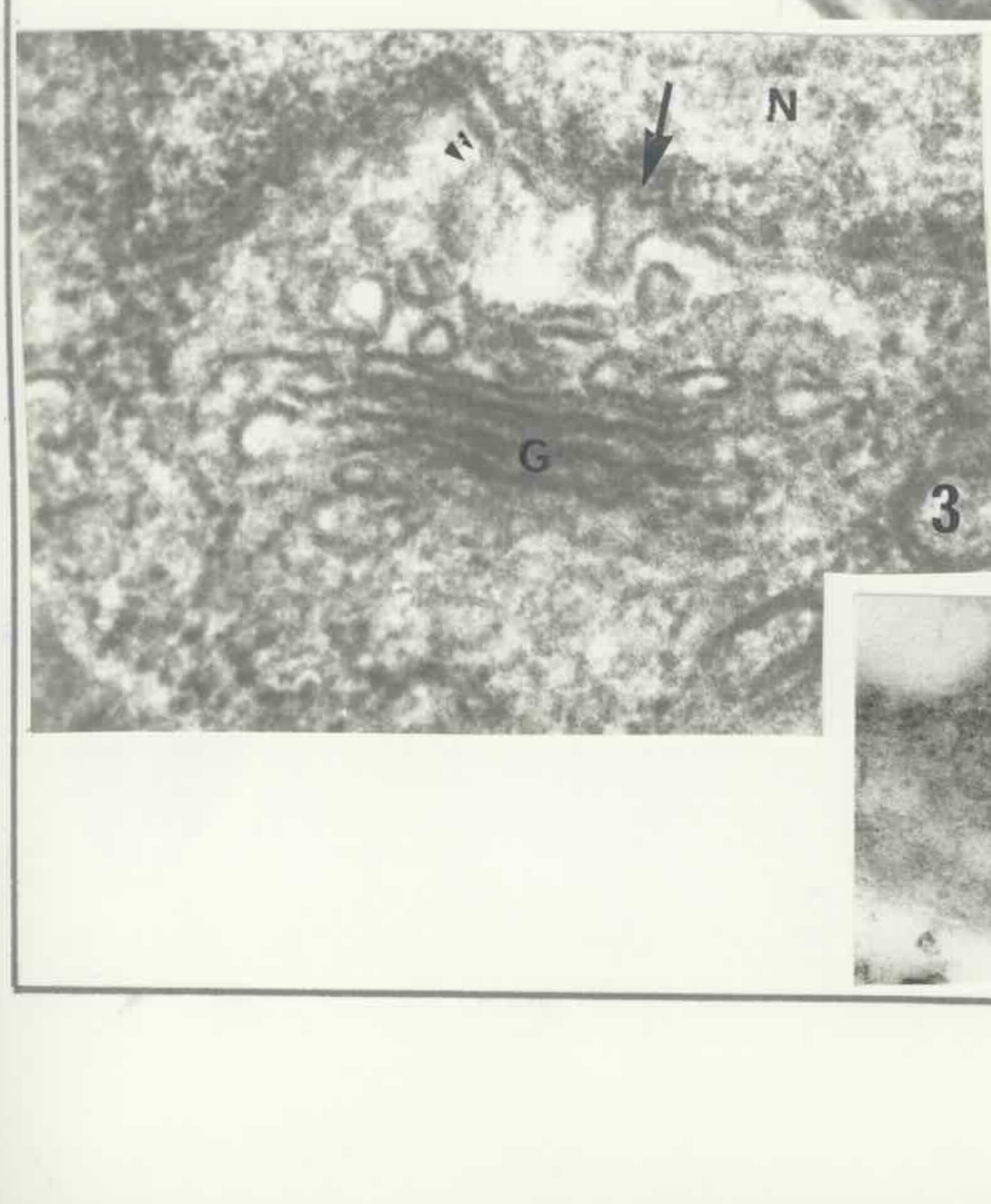


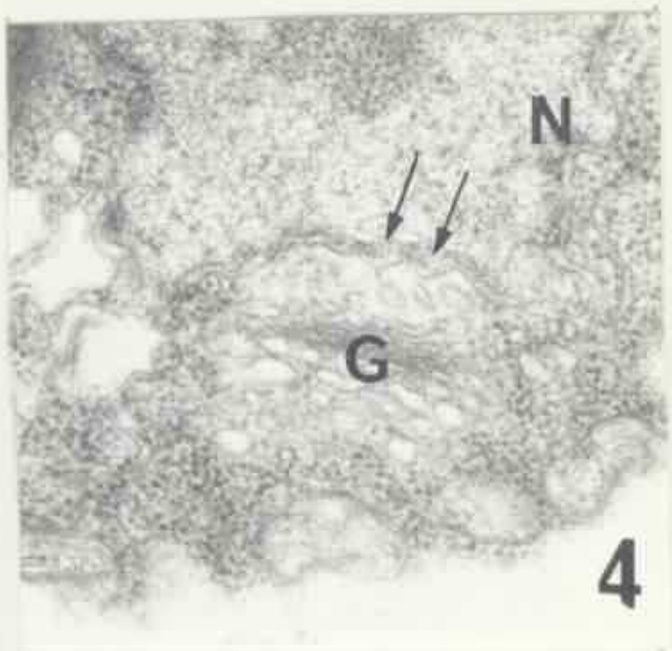


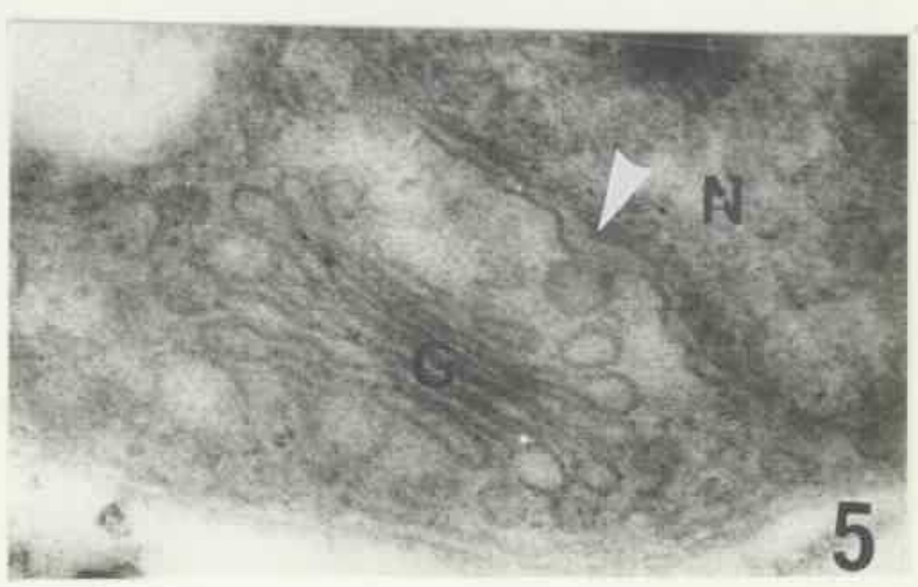
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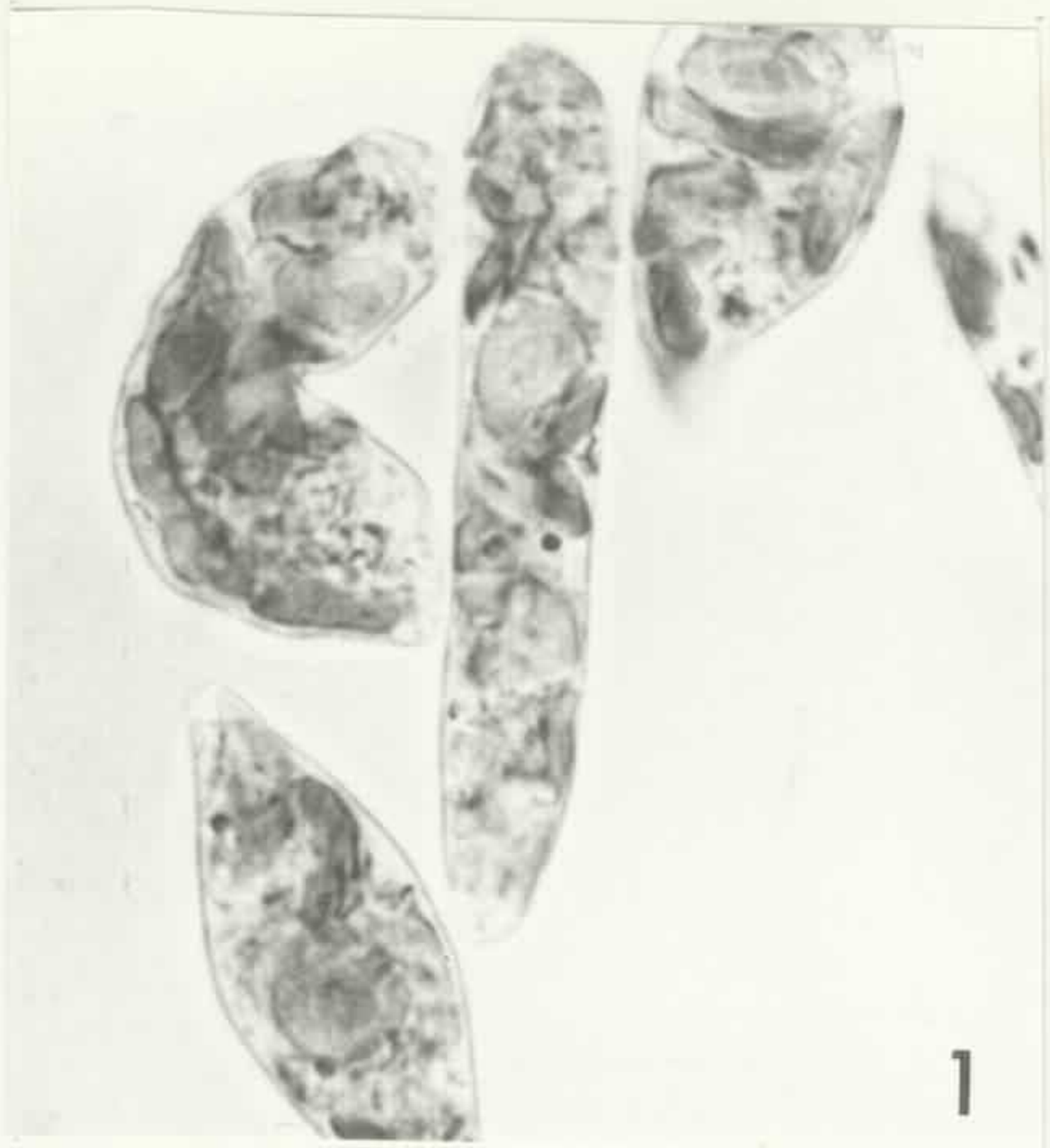








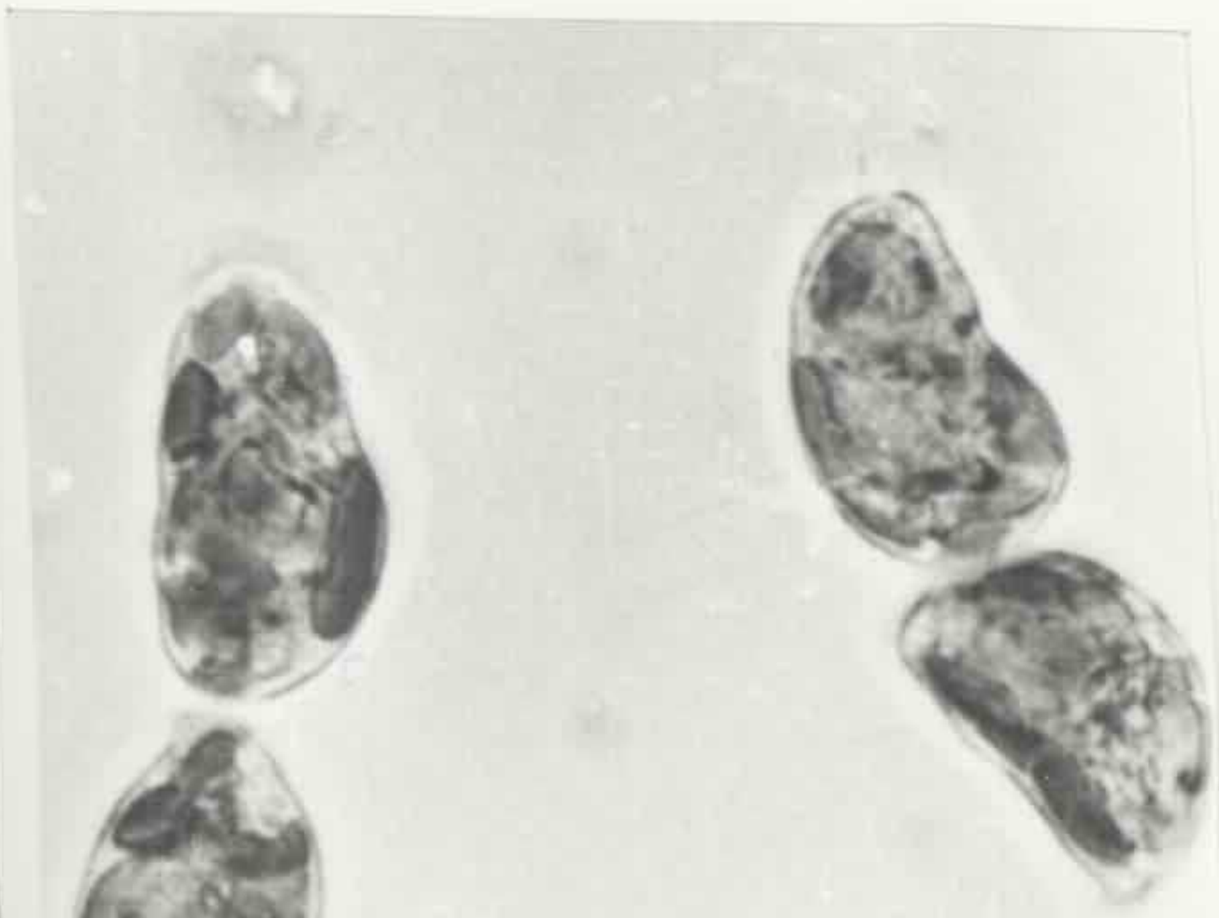




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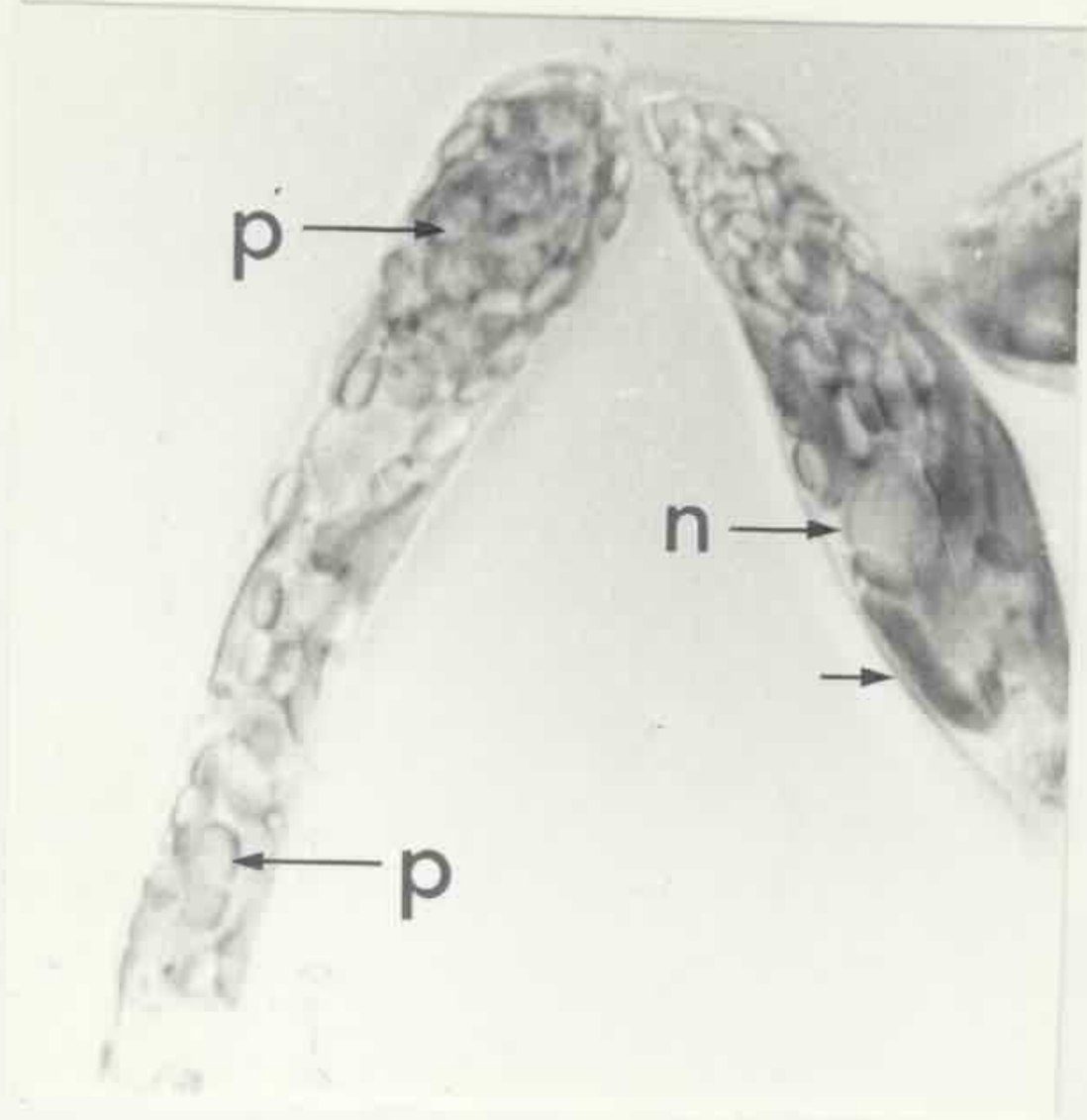


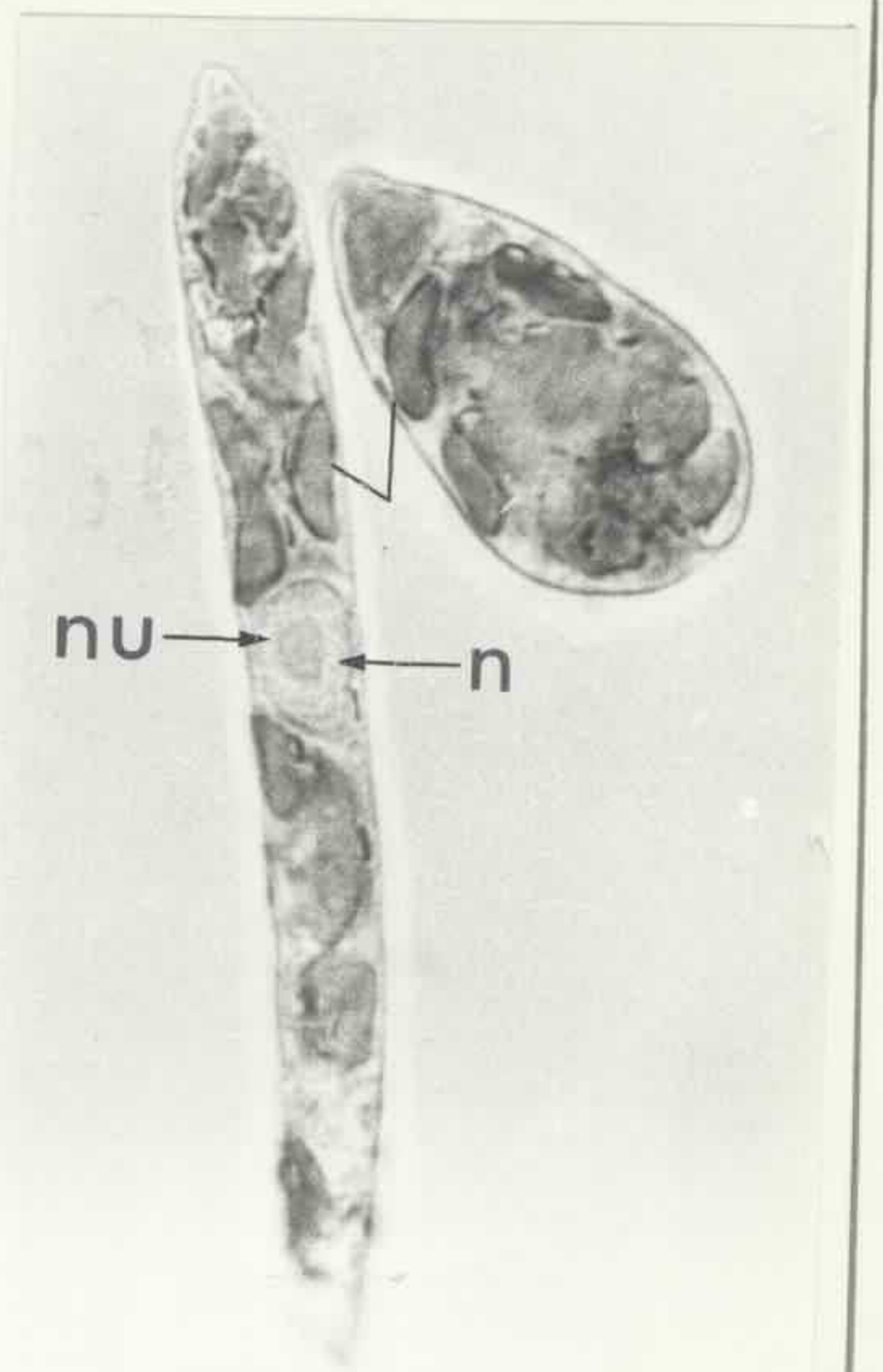
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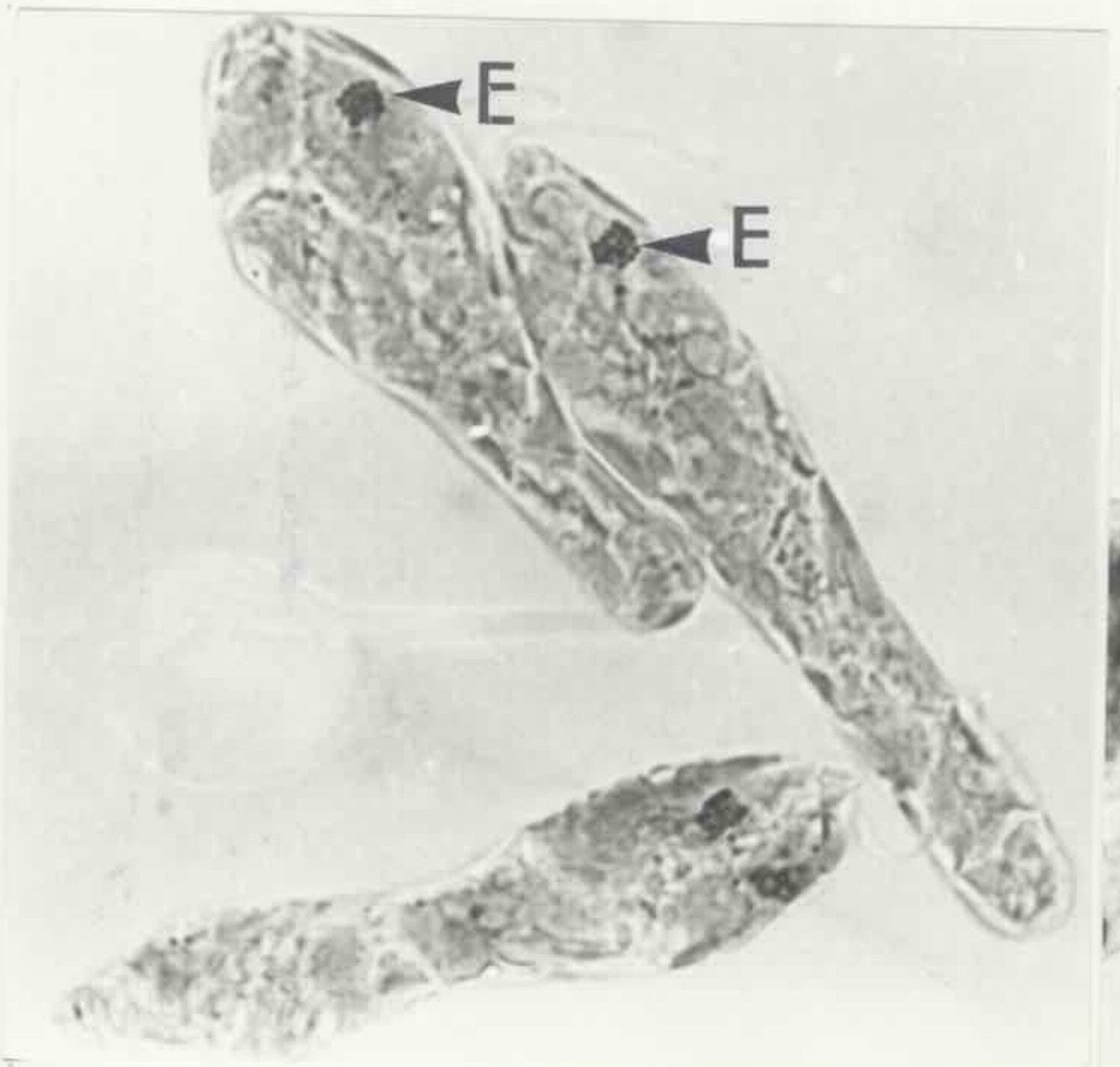


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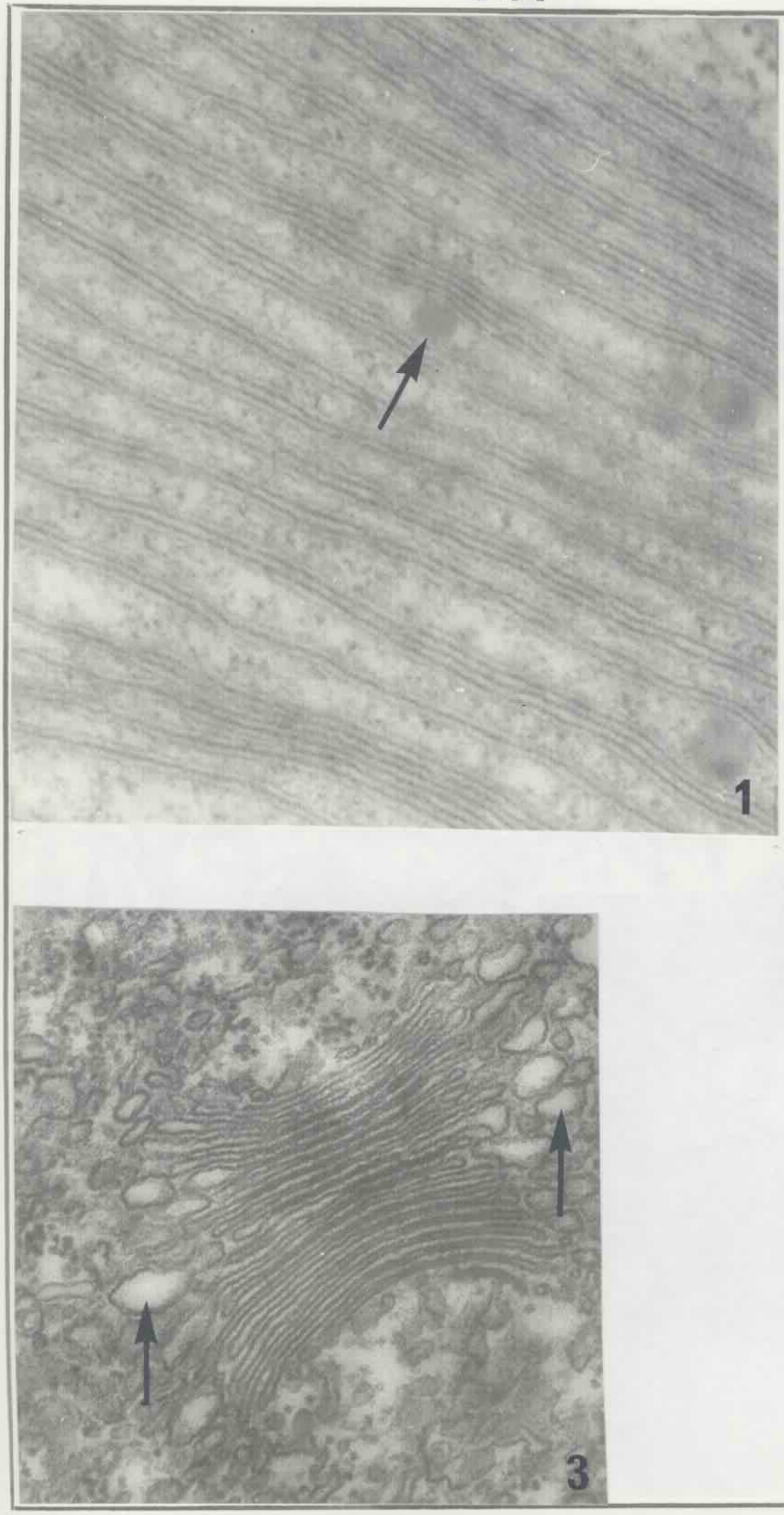




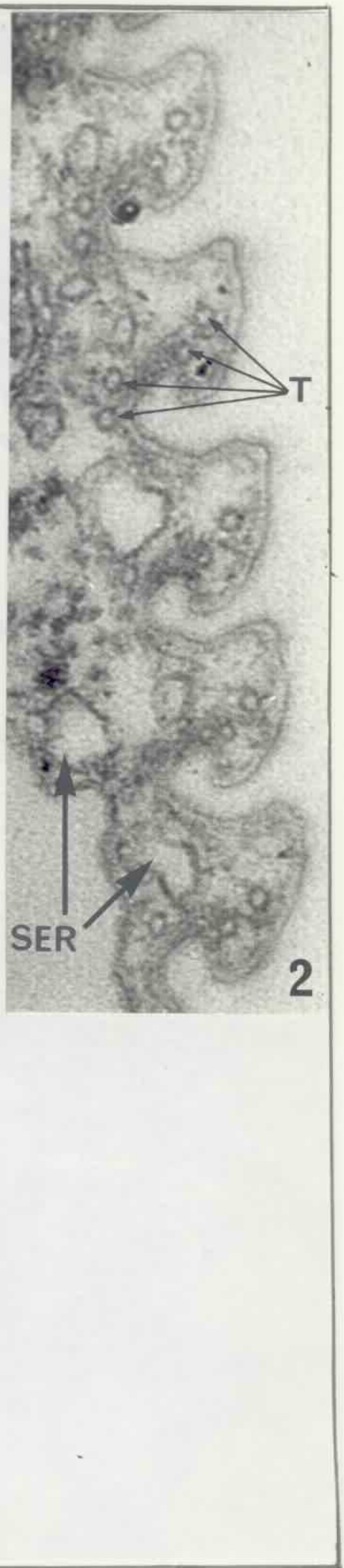


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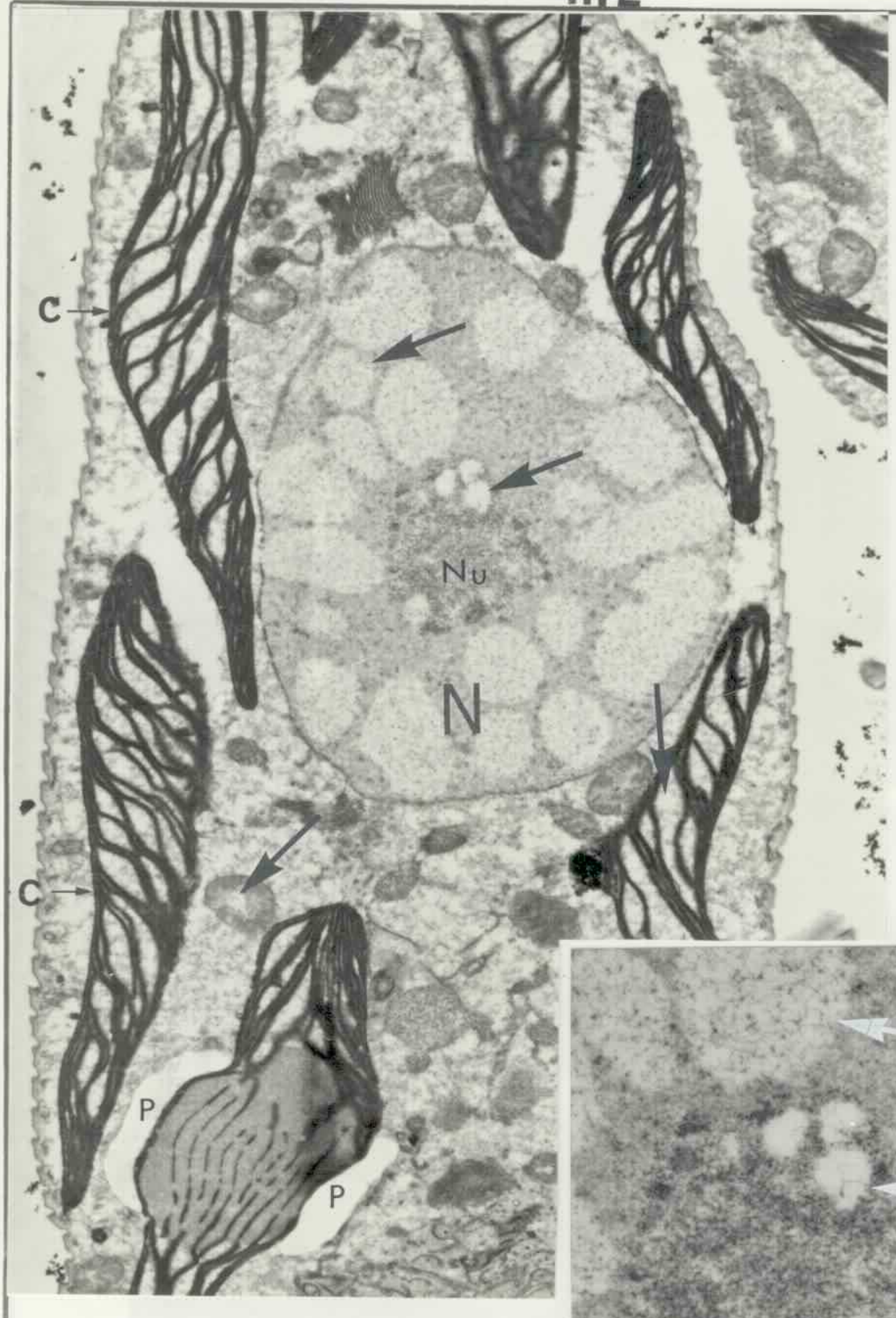
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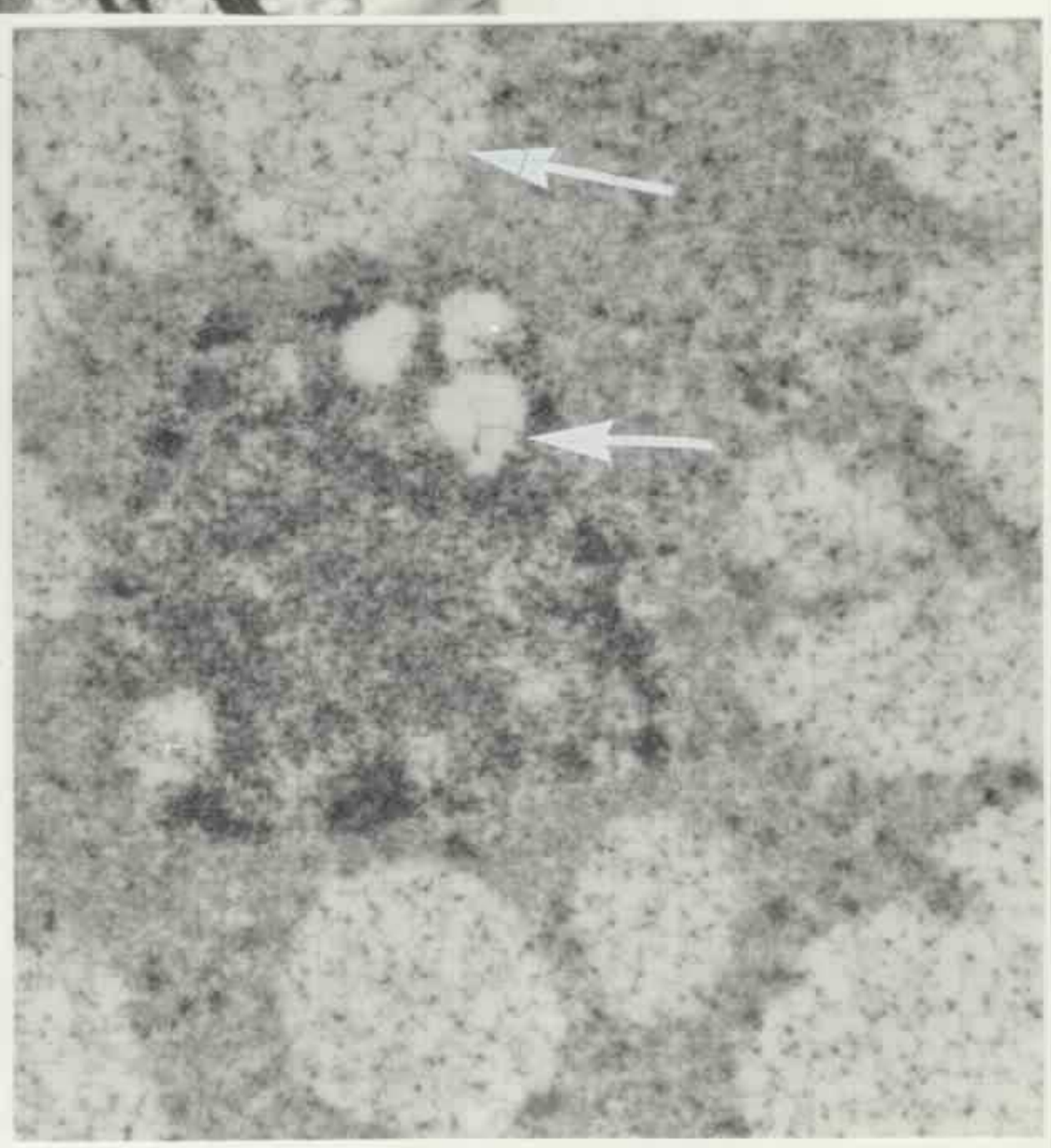


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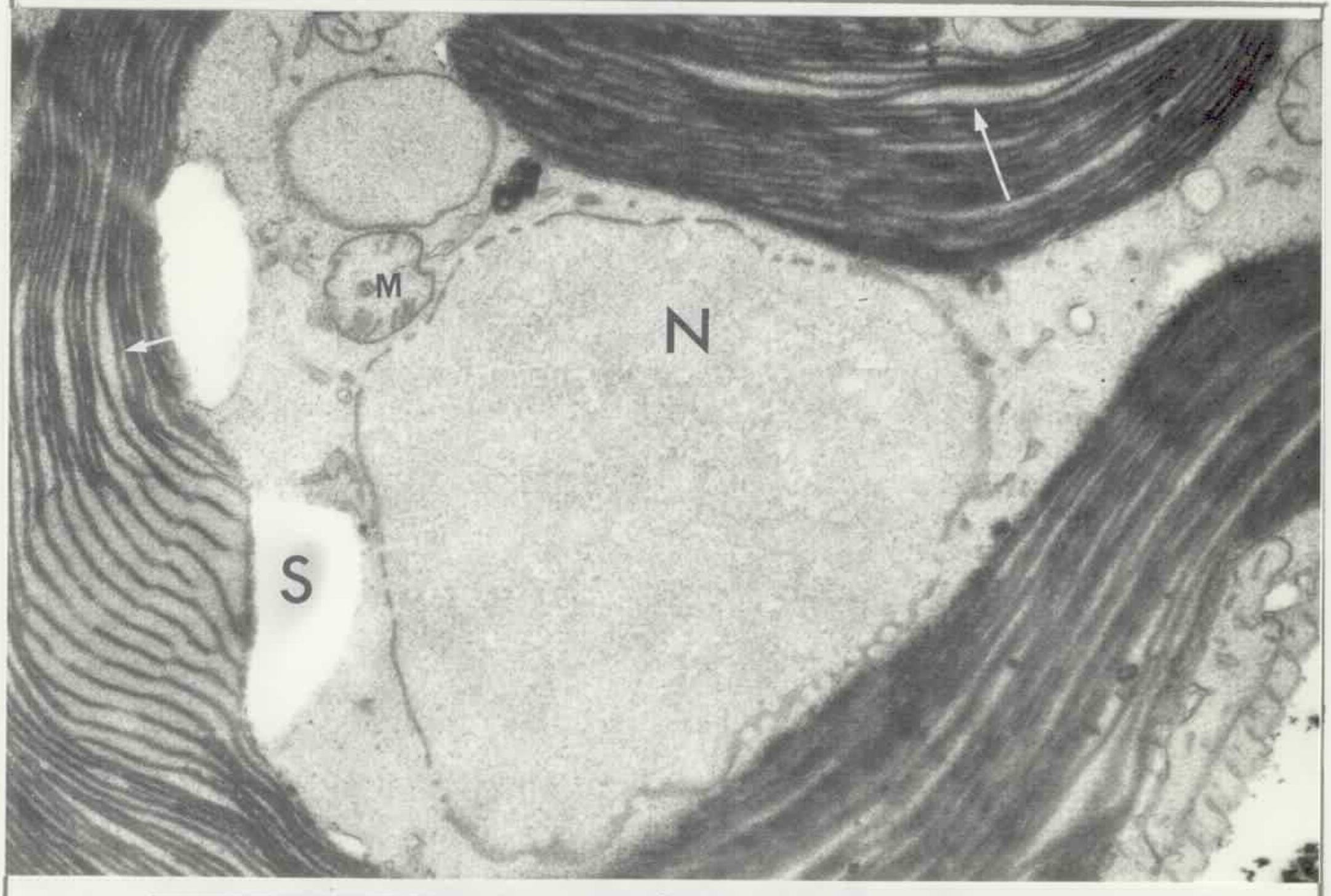
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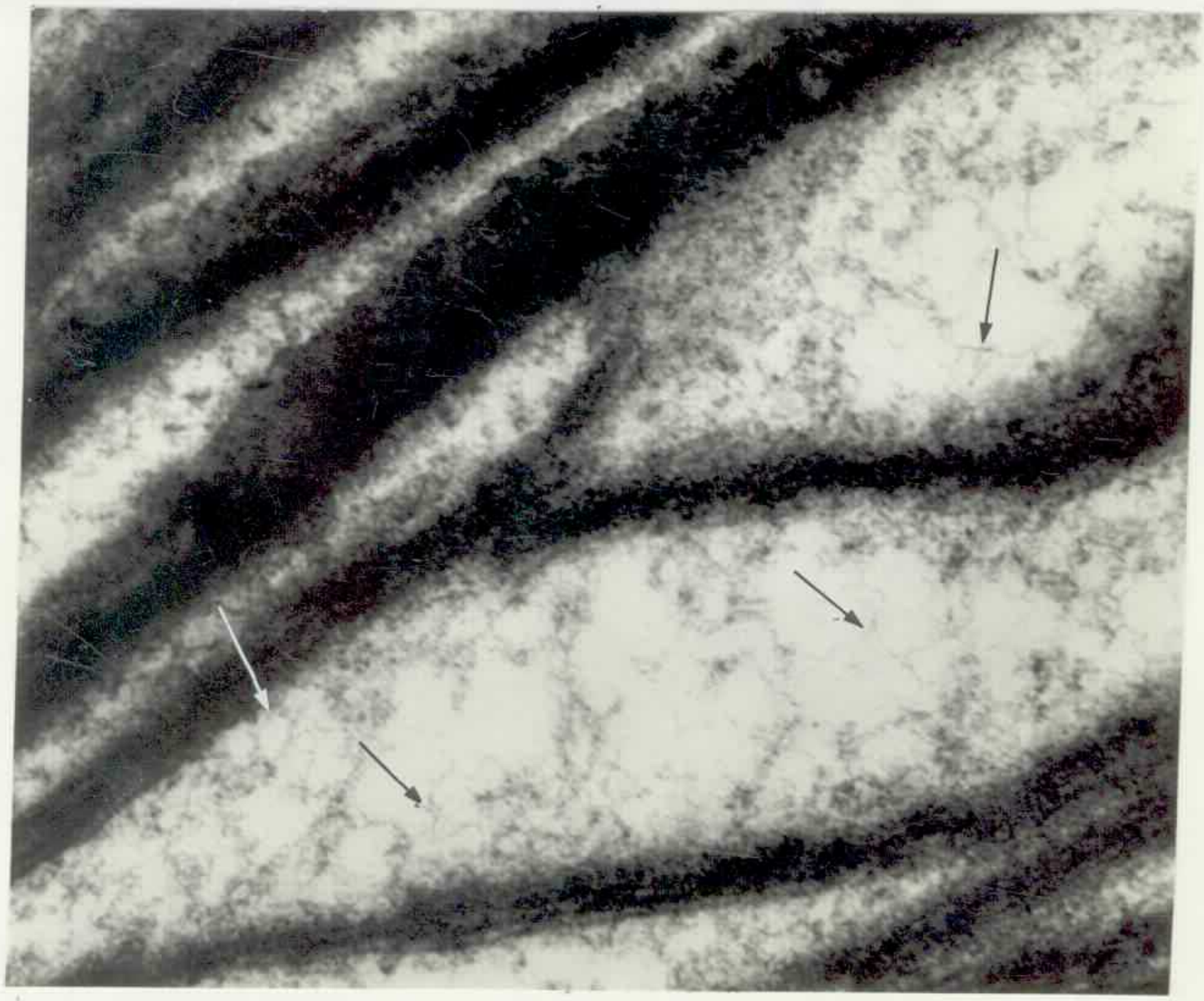
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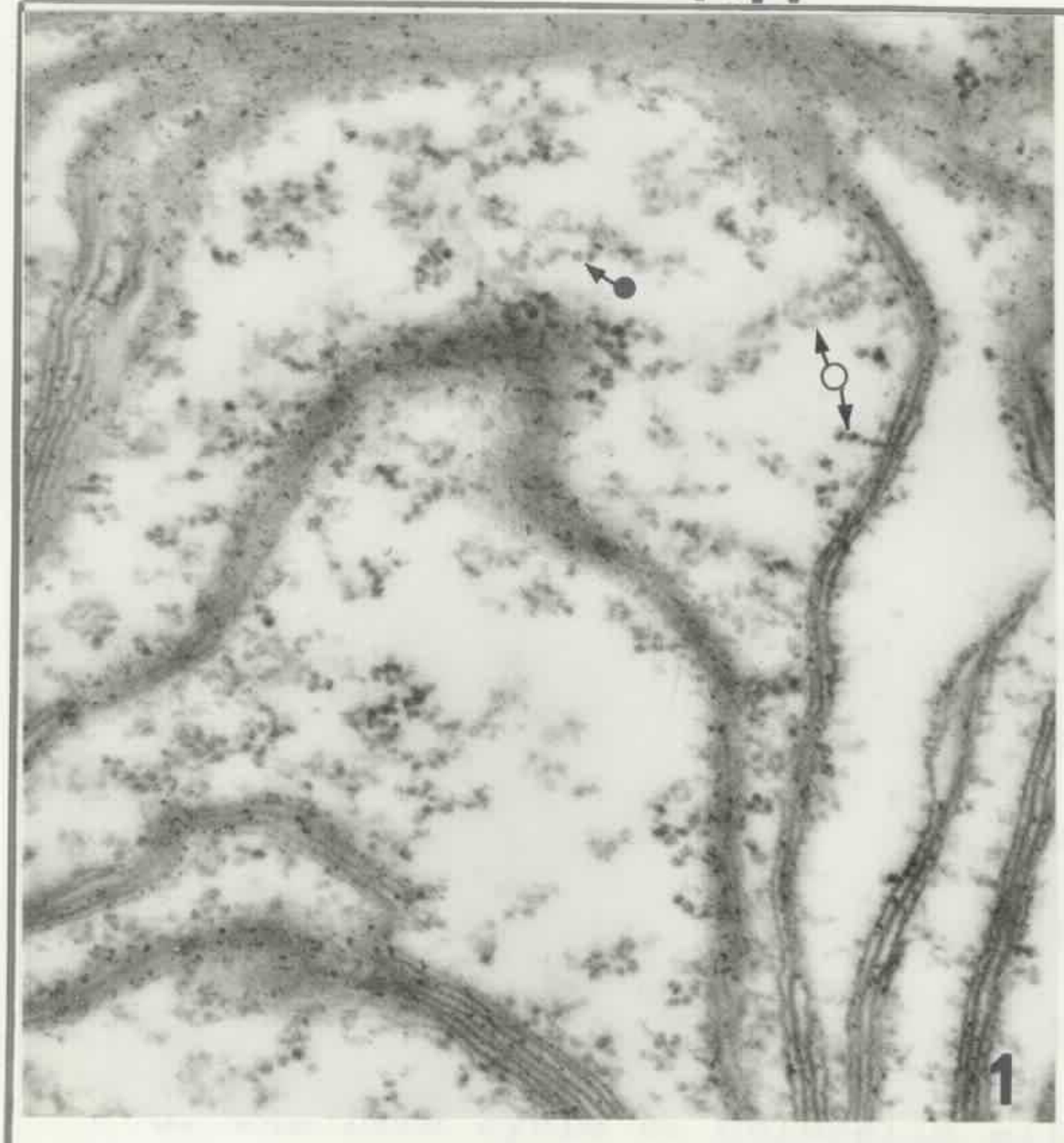


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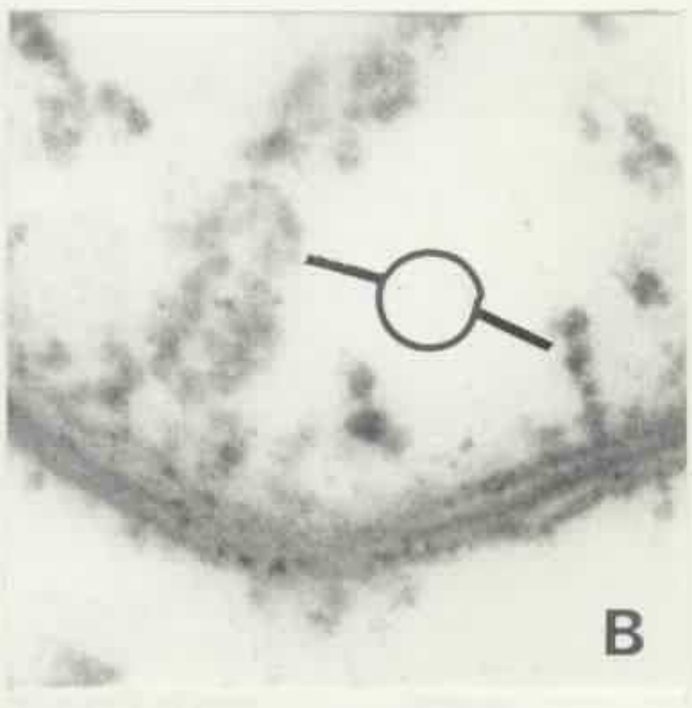


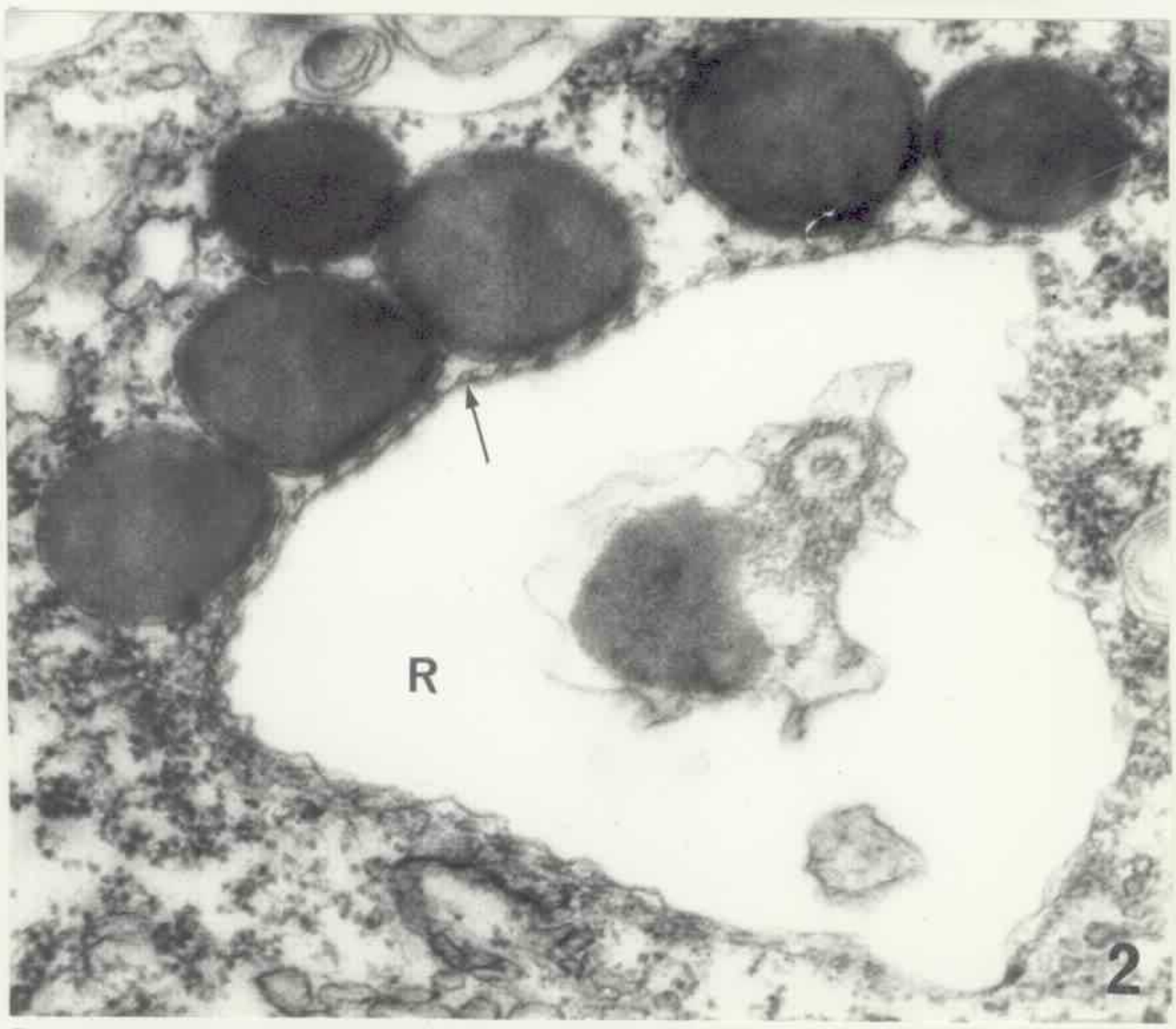


4.14









4.3 Ultrastructural study of cell division in Chlorella NMI

4.3.1 Introduction

There are some apparently conflicting reports on the physiology of Chlorella during cell division. Lorenzon (1958) reported that at a certain state in the life cycle, the nuclei of Chlorella pyrenoidosa became unstainable, while Tamiya et al (1961) showed that they could stain the nuclei with Feulgen stain at all stages of the life cycle of Chlorella ellipsoidea. The limiting factor in cellular division of Chlorella ellipsoidea was reported to be a certain sulphur - containing substance (Hase et al, 1961), whereas in Chlorella prototheccoides, the limiting factor was found to be DNA formation, (Sokawa and Hase, 1967).

Further, Tamiya (1966) indicated that the photoinhibition of cellular division was very strong in some species, but only weak or not apparent in others.

There are also a number of apparently conflicting observations in the reports on ultrastructural studies of cell division in Chlorella, with regards to formation of partition membranes, involvement of Golgi body in cytoplasmic division, time sequence of nuclear and chloroplastic divisions. (Tamiya, 1963; Soeder, 1965; Stahelin, 1966; Bisalputra et al, 1966; Wanka and Mulders, 1967; Guerin-Dumartrait, 1968; Wanka, 1969).

As these apparently confusing observations are probably due to the different species or strains of Chlorella used, and the experimental conditions under which these studies were made, it becomes necessary to study the mode of division of the Chlorella NMI under the conditions to be used in this investigation.

4.3.2 Methods and Materials

A synchronous culture of Chlorella NMI was used. Cells were harvested at intervals, at the end of the dark period, during the light and dark periods. The cells were fixed for electron microscopy, immediately after centrifugation. The fixative were OsO_4 and $KMnO_4$, and fixation procedures 6 and 7 were used (Chapter 3).

In both cases the fixed cells were used for electron microscopy as described in Section 2.4.

4.3.3 Results

Of particular interest in this study were the changes in the Golgi apparatus, and in the processes of cytoplasmic division.

a) Cell at the end of the dark period.

The young cell was nearly filled with a chloroplast containing starch along the periphery of the pyrenoid. Very few lenticular starch grains were observed outside the pyrenoid area. They appeared in the stroma between the bands of lamellae. The nucleus faced the concave side of the chloroplast and was limited by an envelope, consisting of double membranes, with nuclear pores. The cell contained only one Golgi apparatus, which was usually found on one side of the nucleus (Plate 4.1). The elements of the endoplasmic reticulum were sparsely dispersed in the cytoplasm. A continuity existed between the outer nuclear membrane and the membranes of the endoplasmic reticulum (Plate 4.1). A number of small mitochondria, with characteristic cristae, occurred at the periphery of the cell. (Plate 4.2).

A stainable substance was present in the space between the cytoplasmic membrane and the cell wall. This substance was also observed during cell division

(b) Cell during the light period.

The cell increased in size. The chloroplast now contained more starch grains outside the pyrenoid area. The mitochondria moved from the periphery into the inner space of the cell. (Plate 4.3). Towards the end of the light period, a reorientation of organelles occurred within the cell prior to the formation of daughter cells. The mature chloroplast divided by constriction. Division started with an initial constriction around the chloroplast, followed by pinching into two chloroplasts (Plates 4.4 and 4.5). The two parts then moved to the sides of the cell wall with the nucleus between them (Plate 4.9). The Golgi apparatus occupied a perinuclear position (Plate 4.6) and showed a moderate increase in the lateral extent of the cisternae. (Plate 4.7).

No significant differences in morphology of cisternae within the stack were noticed. The Golgi apparatus then divided (dictyokinesis) to give two daughter Golgi complexes. Plate 4.8 suggested that formation of the two Golgi complexes was brought about by division at the middle of the original Golgi apparatus. At this stage, cytoplasmic division was initiated by invagination of the cytoplasmic membrane forming a furrow between the two newly formed Golgi complexes. (Plates 4.9 and 4.10).

c) Cell division

The spherical nucleus elongated and divided, by constriction. During (nuclear) division, the nuclear membrane did not disappear, showing that alga NMI performed nuclear division of the endomitotic type. As the two daughter nuclei moved apart, the division furrow extended down between them. (Plates 4.11 and 4.12). The Golgi complexes moved towards the edges of the furrow and differentiation of cisternal structure was observed. The cisternae on the pole facing the nucleus remained unchanged while the cisternae on the pole facing the furrow widened (Plate 4.11) and exhibited electron transparent interiors. A different class of vesicles, larger than the vesicles generally associated with the unmodified Golgi apparatus, were produced. Those large vesicles, with electron transparent interiors similar to the lumen of the Golgi cisternae, moved from the Golgi apparatus towards the edges of the division furrow.

The division furrow widened as it traversed the cell (Plates 4.12 and 4.13). The substance found between the cytoplasmic membrane and cell wall was present in the furrow. Fairly long profiles of the endoplasmic reticulum became visible. The direction of the division furrow appeared to be correlated with the disposition of the endoplasmic reticulum (Plate 4.12, 4.14 and 4.16). At completion of the first cell division, the Golgi bodies were situated in the

centre of the cell on opposite sides of the furrow (Plate 4.14). Plate 4.15 showed the beginning of division of one of the daughter chloroplasts, and Plates 4.17 and 4.18 clearly demonstrated that chloroplast division occurred before nuclear division, and even before that of the Golgi body. A second cell division at right angles to the first furrow was initiated with division of the Golgi bodies (Plate 4.19). The completion of the second division produced four algal entities (Plate 4.20). When the furrows had divided the original cell, fibres appeared within them and became aggregated in strands (Plate 4.21). Cell wall formation proceeded and four daughter cells were obtained within the original cell wall (Plate 4.22).

4.3.4 Discussion

For simplicity, cells giving four daughter cells have been selected for discussion. The results obtained, however, are also valid for cells which give eight daughter cells.

Chloroplast

Contrary to the observations of Wanka (1968), the present results show clearly that the chloroplast divides before the nucleus and the Golgi apparatus. Division is initiated by a middle constriction of the mature chloroplast (Plates 4.15 and 4.4) and is similar to that in brown and red algae, and in higher plants (von Wettstein; Bouck, Clowes and Juniper). During division in alga NMI, the lamellae of one chloroplast, at the constriction, are in line

with those of the other (Plate 4.5). This suggests that they have been derived from a simple division of the lamellae system. Separation into two daughter chloroplasts is subsequently achieved by the fusion of the inner and outer membranes of the chloroplast envelope.

Mitochondria

Retovsky and Kasterna (1961 b) pointed out that in Chlorella pyrenoidosa and Chlorella vulgaris var viridis the mitochondria form a coherent zone at the periphery of the young cell. As the cell ages, the zone disappears and mitochondria are found well inside the cell. Plate 4..2,3 support and confirm the above statement. Mitochondria contain respiratory enzymes responsible for ATP formation. It is not unreasonable to assume that the distribution of the mitochondria is related to the energy requirements of organelles within the cell. Thus, during growth and cellular division of Chlorella, the mitochondria tend to move closer to the dividing chloroplast.

Endoplasmic reticulum

It is evident that the period of endoplasmic reticulum development starts at a stage prior to cell division and reaches a maximum during the cell division process. This might be expected from the postulated function of the endoplasmic reticulum as an intracellular transport system. It may be involved in the transport of materials to different actively dividing organelles. Further, disposition of the

endoplasmic reticulum seems to correlate with the direction of the division furrow (Plates 4.13, 4.16 and 4.18).

The continuity of the endoplasmic reticulum membranes with the nucleus may imply that genetic information from the nucleus is transported in the endoplasmic reticulum. The presence of endoplasmic reticulum close to and in front of the advancing division furrow suggests a possible nuclear control of cytoplasm division.

Golgi apparatus

One of the earliest organelle changes in the dividing cell is related to the activity of the Golgi apparatus. Growth of the cisternae occurs in a lateral direction, followed by division into two daughter Golgi bodies. (Plate 4.8). The arrangement of these bodies immediately after division is similar to that found by Buvat (1963) in plant cells. This indicates a lateral division of the Golgi apparatus and is different from the type of multiplication suggested by Cronshaw and Wardrof (1964). As cytoplasmic division proceeds, changes in the Golgi apparatus increase. Distention of the cisternae on the pole facing the division furrow occurs. Vesicles with electron transparent interiors similar to the lumen of the Golgi cisternae increase in number and migrate from the Golgi complex towards the edges of the division furrow. These vesicles are usually larger and different from the smaller vesicles which appear in the space between the nucleus and the cisternae.

During these developmental changes which result in an increase in growth and functional activity, new material must be added to the Golgi complex. This material is obtained from the nucleus. Plates 4.7 and 4.8 show blebs from the outer nuclear membrane and vesicles derived from it near the 'formative' faces of the two new Golgi bodies. The swelling of the cisternae facing the furrow may be caused by accumulation of a secretory product. This possibility is supported by observations that similar distention of the Golgi cisternae accompanied by the accumulation of an intracisternal material is encountered in other systems. (Goodman and Porter, 1960, Whaley, 1964).

The involvement of the Golgi apparatus in the deposition of material into the division furrow is suggested by (1) the migration of the larger detached Golgi vesicles containing the product towards the division furrow, (2) their positions at the edges of the furrow, (3) the enlargement of the division furrow as it traverses the cell, and (4) the presence of stainable material in the furrow.

Cytoplasmic division and Cell wall formation

In plant cells with rigid cellulosic walls, the essential feature of the cytoplasmic division is the presence of a cell plate formed by the fusion of vesicles in the equatorial plane of the dividing cell. (Mollenhauer and Whaley, 1963; Frey-Wyssling *et al*,1964). The origin of these vesicles is not clear, but the most widely accepted hypothesis is that the vesicles are derived from the Golgi

apparatus (Pickett - Heaps and Northcote, 1966).

Stahelin, (1966); and Bisalputra et al, (1966), reported that during cytoplasmic division in Chlorella vulgaris, a partition membrane appeared between the new Golgi bodies derived from the division of the original single Golgi apparatus. They suggested that the partition membrane originated from the fusion of small vesicles produced by the adjacent Golgi bodies between the two daughter nuclei. Bisalputra et al (1966) also showed that invaginations of the ectoplast occurred, and complete division of the cell into two parts was brought about by the meeting of the invaginations with the partition membranes. In studies with Chlorella pyrenoidosa, using the freeze-etching method, Guerin-Dumartrait (1968) also pointed out that after division of chloroplast and nucleus, partition membranes appeared between the daughter nuclei. On the other hand, Wanka (1968) reported that in the Chlorella strain 211 - 8b, the Golgi bodies were located close to the nucleus but opposite to the side of the developing cell - plate. He indicated that small droplets or vesicles in the equatorial plane are formed by vacuolization of the cytoplasm. These vesicles coalesced giving rise to the cell plate. Furthermore, in Chlorella strain 211 - 8b, cytoplasmic division was mostly observed at the tetranucleate stage (Wanka and Mulders, 1967; Wanka 1968).

Our observations on cytokinesis in Chlorella NMI do not agree with the concept that division originates from vesicles aggregating and fusing in the equatorial plane of the dividing cell. However, vesicles produced by the Golgi complex are involved in cell wall formation. Under the growth conditions of the present study, cytoplasmic division occurs at the binucleate stage, and contrary to the observations of Soeder (1965); Wanka and Mulders (1967) and Wanka (1968) the second nuclear division starts after completion of the first (Plates 4.17 and 4.18) cytoplasmic division. Furthermore, it appears that initiation of cytoplasmic division occurs before nuclear division (Plates 4.9 and 4.10).

In Chlorella NMI, no partition membranes are detected. A furrow originates from the invagination of the plasma membrane in the area between the two Golgi bodies (Plate 4.9). From the micrographs showing its progress across the cell, it is evident that we are concerned with a true division furrow. The Golgi bodies, resulting from the division of the original Golgi body, start to move as soon as the division furrow is initiated. The Golgi bodies move along the edges of the advancing division furrow and stop in the middle of the cell, where their further division initiate the formation of division furrows at right angles to the initial one.

As mentioned previously the spatial distribution of the Golgi bodies in the middle of the dividing cell (Plate 4.20), the increased distention of the Golgi cisternae, and the close proximity of the Golgi vesicles to the edges of the furrow strongly indicate that the Golgi bodies are involved in the transport of material across the plasma membrane into the division furrow. Cell wall formation is initiated after cytoplasmic division of the mother cell is completed. The material in the furrow is first organised into fibres, which become aggregated into strands (Plate 4.21) and finally cell walls are produced.

Observations of the division process of Chlorella NMI clearly indicate that autospore production occurs by means of successive bipartitions, which involve a nuclear division followed by a division of the protoplast into two equal parts. However, it is apparent that another type of cell division, progressive cleavage, exists in Chlorella sp. (Wanka, 1968). In this type of division, several nuclear divisions occur, resulting in a multinucleate cell, followed later by divisions of the protoplast, to produce a number of units, each possessing a single nucleus.

It appears, therefore, that both types of cleavage are encountered in Chlorella sp. These two types of cleavage have also been reported in Chlorococcum sp (Deason, 1965).

4.4 Summary

1. Autotrophic cultures of Chlorella NMI are synchronised by alternating light and dark periods of 16:8 hours. The cell divides to give eight autospores.

2. Double fixation procedures (Glutaraldehyde-Osmium, and Glutaraldehyde-Pottasium permanganate) are used to study the ultrastructure of Chlorella NMI and Euglena.

3. The chloroplasts of Chlorella NMI are positively stained by the Feulgen-fluorescent dye. Under the electron microscope, DNA fibrils in the chloroplast are represented as linear, single-loop, and double-loop forms. The DNA molecules are distributed throughout the chloroplast, and also accumulate in areas of low electron density between the lamellae and around the pyrenoids.

4. Associations are shown between DNA fibrils and the lamellar membranes, and between DNA fibrils and ribosomes. Polysomes are also present at these sites.

5. The outer layer of the nuclear envelope is involved in the production of membranous material (initially as blebs and subsequently as detached vesicles) for the morphogenesis of the Golgi body.

6. Mitochondria of Chlorella NMI contain DNA fibrils and ribosomes.

7. DNA fibrils, ribosomes and polysomes associated with lamellar membranes are demonstrated in chloroplasts of Euglena.

8. The course of cell division in Chlorella NMI is described on the basis of electron-micrographs. There is an organised movement of organelles prior to cell division.

9. Cytoplasmic division is initiated after that of the Golgi body and apparently before nuclear division. The chloroplast divides before the nucleus.

10. Division of the algal cell into two parts (successive bipartition) is brought about by a true division furrow, originating from the invagination of the plasma membrane at a point between the two Golgi bodies. The second cytoplasmic division begins after completion of the first.

11. The Golgi bodies are located close to the division furrows, and contribute to the deposition of material into the furrows for the formation of cell walls of the daughter cells.

Plate 4

Key to labelling

N	-	nucleus
CW	-	cell wall
ER	-	endoplasmic reticulum
G	-	golgi body
S	-	starch
L	-	chloroplast lamella
M	-	mitochondria
Py	-	pyrenoid
C	-	chloroplast
Ve	-	vesicle

Plate 4.1 — Section of Chlorella NMI cell. Note continuity between nuclear envelope and ER membranes (arrow). Nucleus has clear regions containing fine beaded fibrils and darker regions with fine granular appearance.
Glut - $KMnO_4$. x 53,000

Plate 4.2 — Section of Chlorella NMI showing a number of small mitochondria located along the periphery of the cell.
Glut - $KMnO_4$. x 24,000

Plate 4.3 — Section of Chlorella NMI showing the new position of mitochondria, deep inside the cell and close to the chloroplast.
Glut - $KMnO_4$. x 24,000

Plate 4.4 — Section of Chlorella NMI cell showing a chloroplast dividing by constriction at the middle.
Glut - $KMnO_4$. x 24,750

Plate 4.5 — Section of Chlorella NMI cell. The nucleus has moved to a position between the dividing chloroplast. Glut - $KMnO_4$. x 24,000

Plate 4.6 — Section shows perinuclear Golgi body. Note number of vesicles between nucleus and forming face of Golgi body. Glut - $KMnO_4$. x 66,300

Plate 4.7 — Section showing lateral increase of Golgi cisternae. Arrow indicates formation of a bleb by the nuclear envelope. Double arrows indicate vesicles liberated from nuclear envelope. Glut - $KMnO_4$. x 52,500

Plate 4.8 — Section shows dictyokinesis. Position of the two Golgi bodies suggests a lateral division of the Golgi body. Arrows indicate blebs and vesicles from the outer nuclear membrane adjacent to the forming face of the Golgi body. Glut - $KMnO_4$. x 52,500

Plate 4.9 — Section of a dividing Chlorella NMI cell. F indicates the division furrow formed by invagination of the plasma membrane between the daughter Golgi bodies. Arrow indicates stainable material between plasma membrane and cell wall. Glut - $KMnO_4$. x 38,000

Plate 4.10 — Section of dividing Chlorella NMI cell. Note the Golgi vesicles (VE) close to the division furrow (F). Glut - $KMnO_4$. x 22,500

Plate 4.11 — Section showing nucleus has divided and the division furrow has pyrenoids between the two nuclei. The shape of the two daughter nuclei (arrows) suggests nuclear division has occurred by constriction. Note position of ER near advancing division furrows.
Glut $KMnO_4$. x 26,000

Plate 4.12 — Section shows a more advanced stage of cell division.
Glut - $KMnO_4$. x 53,000

Plate 4.13 — Cytoplasmic division of the cell is nearly complete. Note positions of the Golgi bodies on opposite side of the division furrow (F).
Glut - $KMnO_4$. x 39,600

Plate 4.14 — Division into two daughter cells is completed. Golgi bodies now occupy position at the middle of the cell. More ER present.
Glut - $KMnO_4$. x 53,000

Plate 4.15 — Section shows start of chloroplast division in the two new formed daughter cells.
Glut - $KMnO_4$. x 28,100

Plate 4.16 — Note proliferation of ER near the division furrow (F).
Glut - $KMnO_4$. x 24,000

Plate 4.17 — Section shows divided chloroplasts at the two-cell stage.
Glut - $KMnO_4$. x 24,000

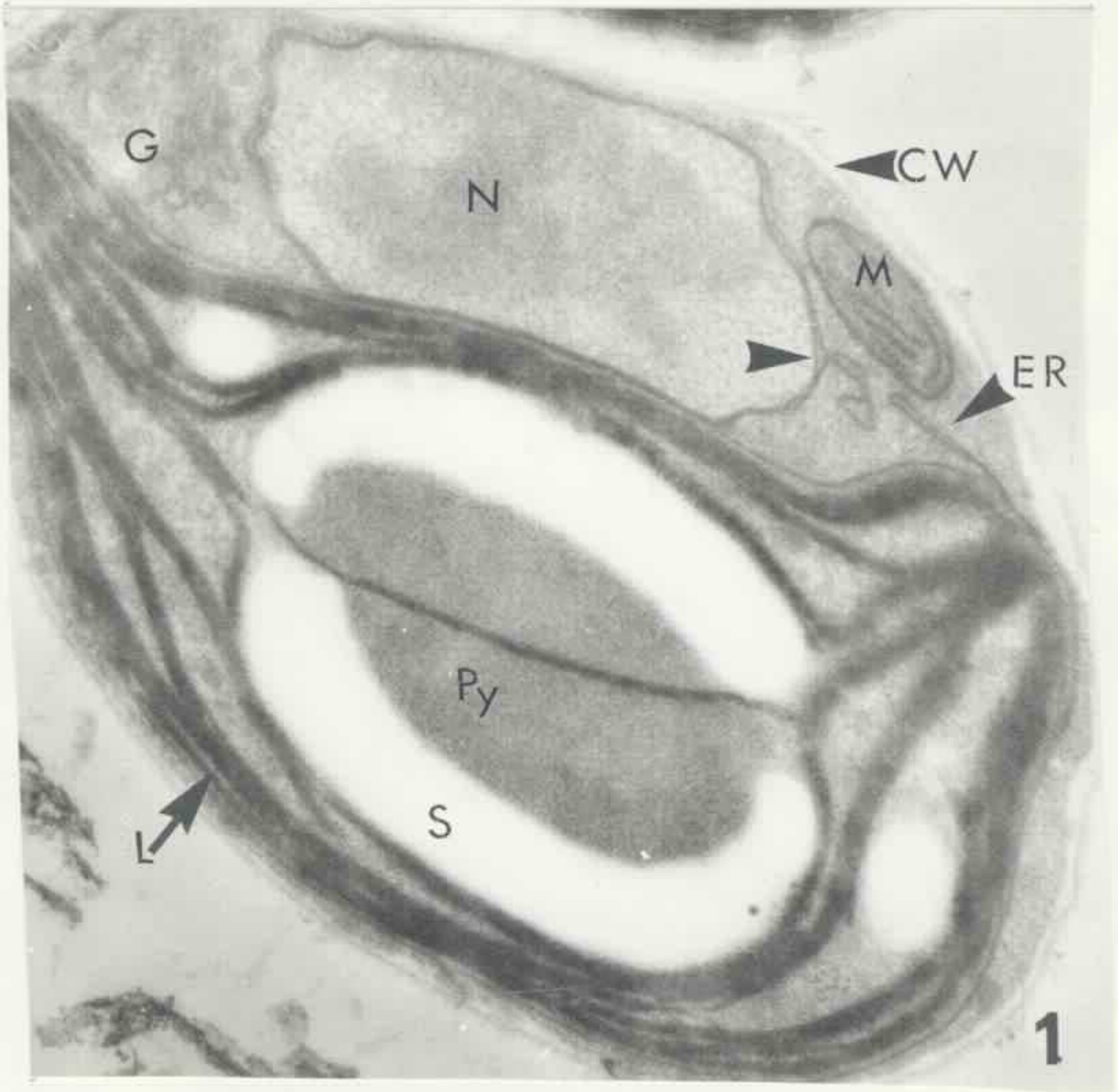
Plate 4.18 — ER near division furrow (F). Section shows that chloroplast division is complete before nuclear division starts.
Glut - $KMnO_4$. x 24,000

Plate 4.19 — Section shows divided Golgi bodies, at the centre of the cell.
Glut - $MNnO_4$. x 26,000

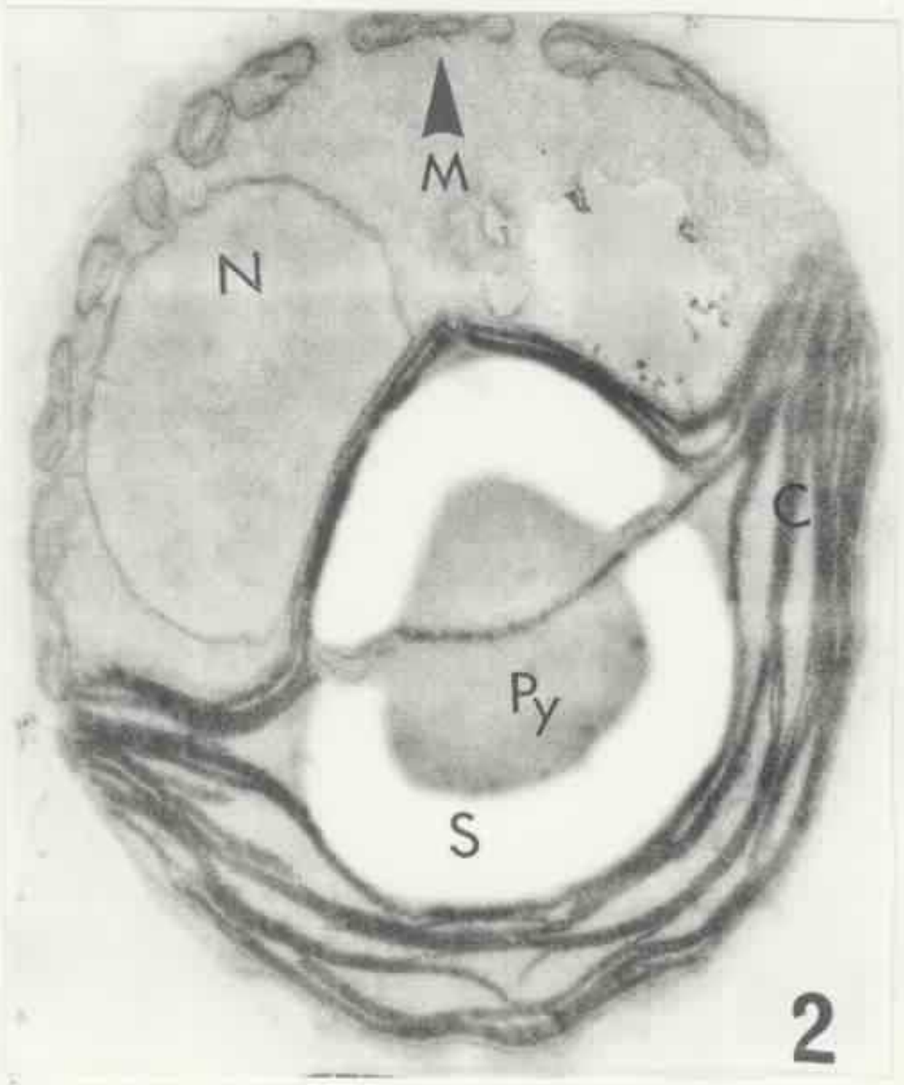
Plate 4.20 — Section shows four-cell stage. Note position of Golgi bodies in the centre of cell, and large vesicles close to the edge of the furrows.
Glut - $KMnO_4$. x 24,000

Plate 4.21 — Stainable material in the division furrows is organised into fibres (arrows).
Glut - $KMnO_4$. x 43,000

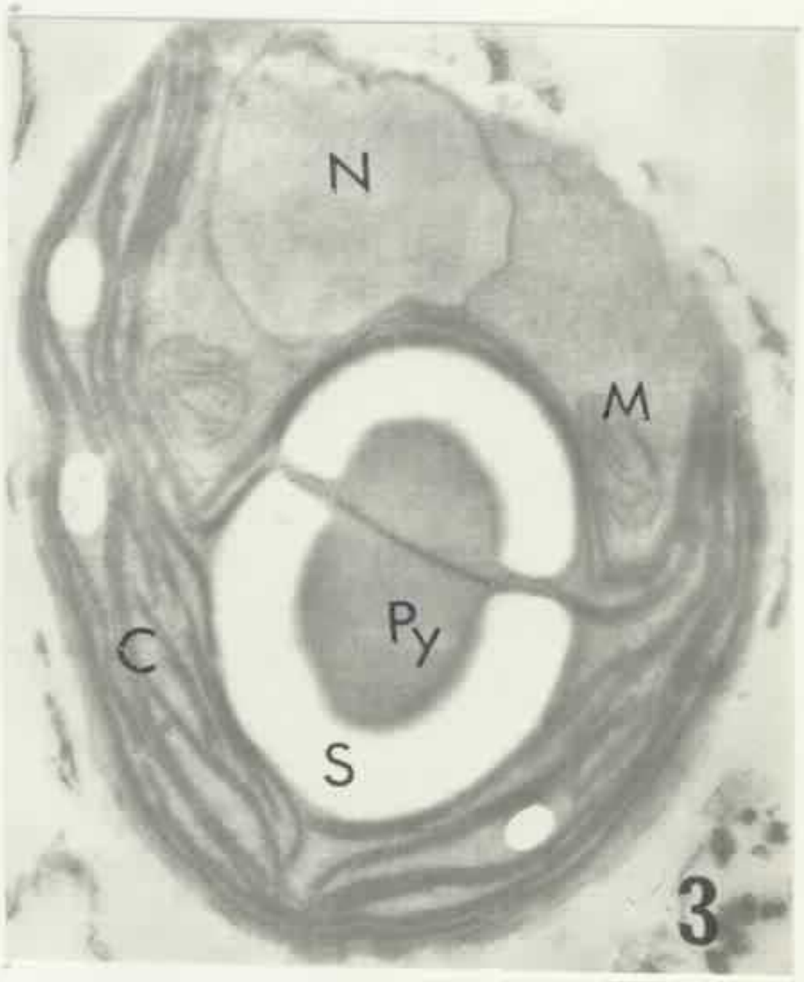
Plate 4.22 — Section shows completed division into four daughter cells. Note cell wall of mother cell is still present.
Glut - $KMnO_4$. x 36,000



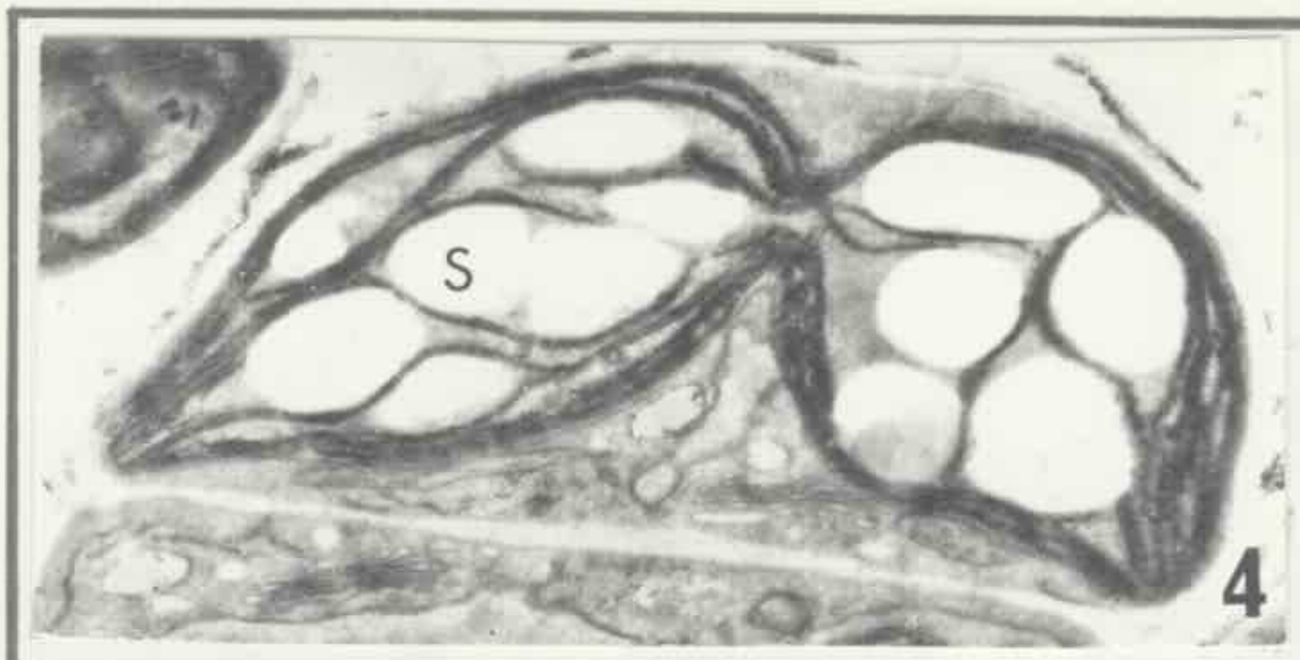
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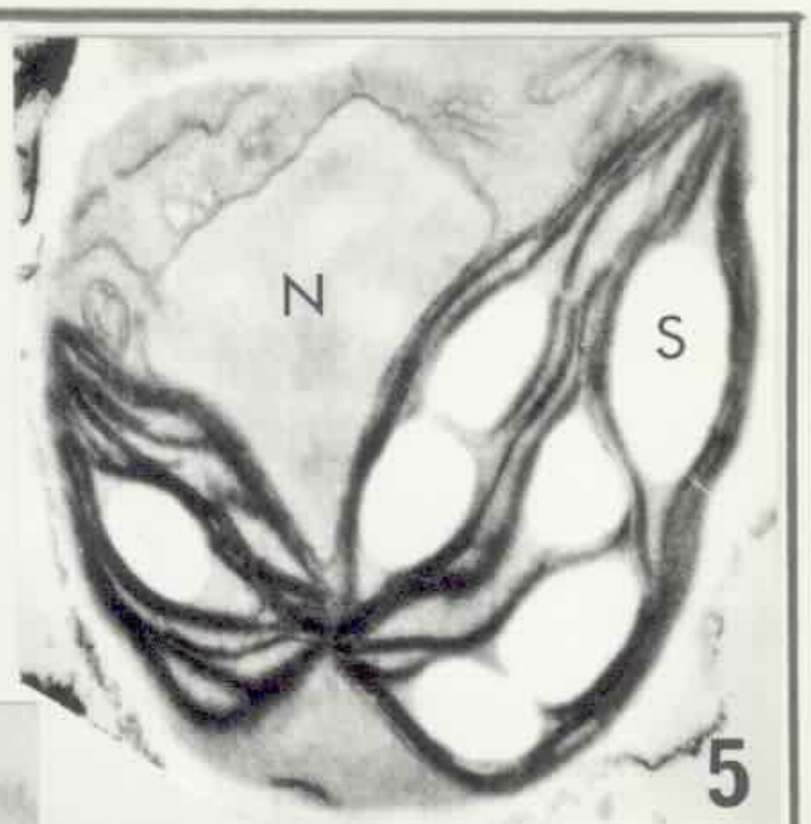


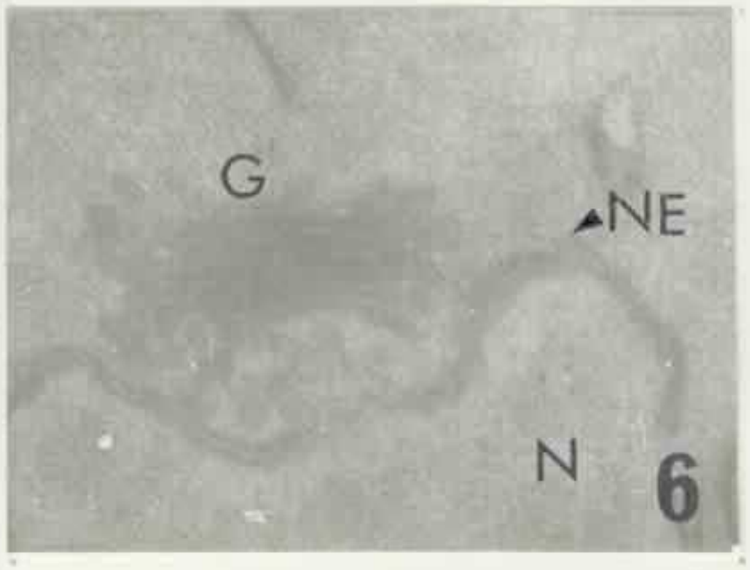
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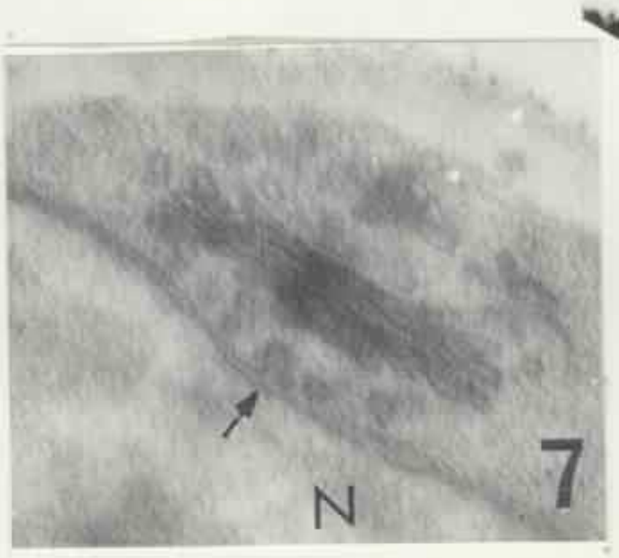


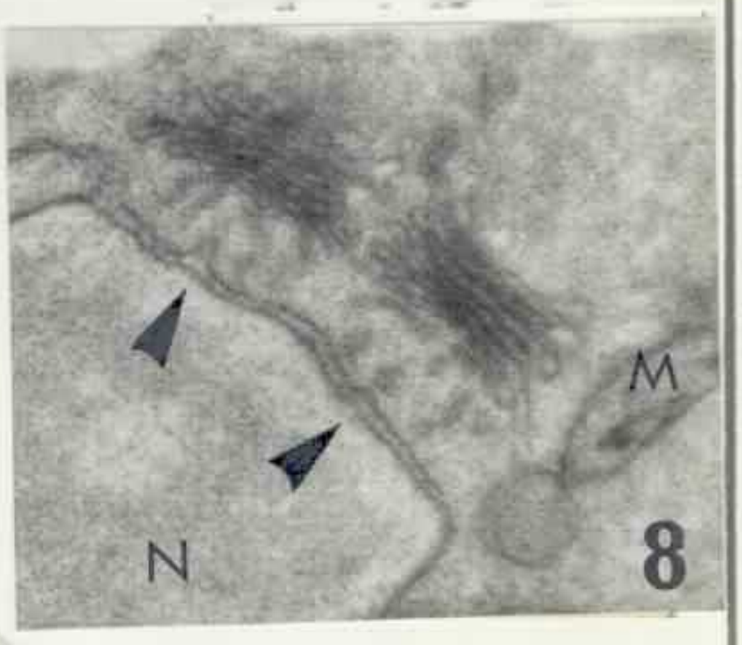
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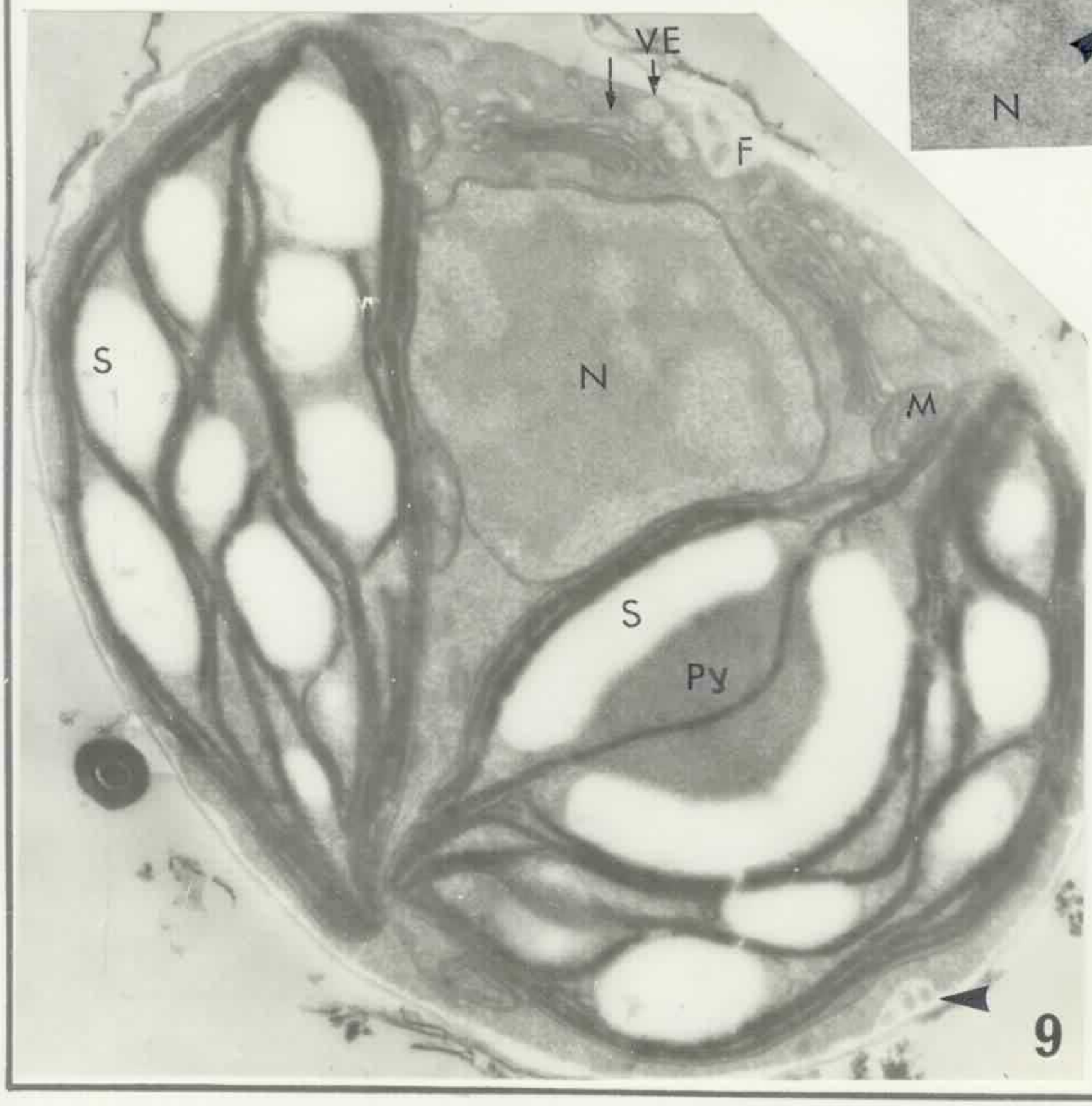

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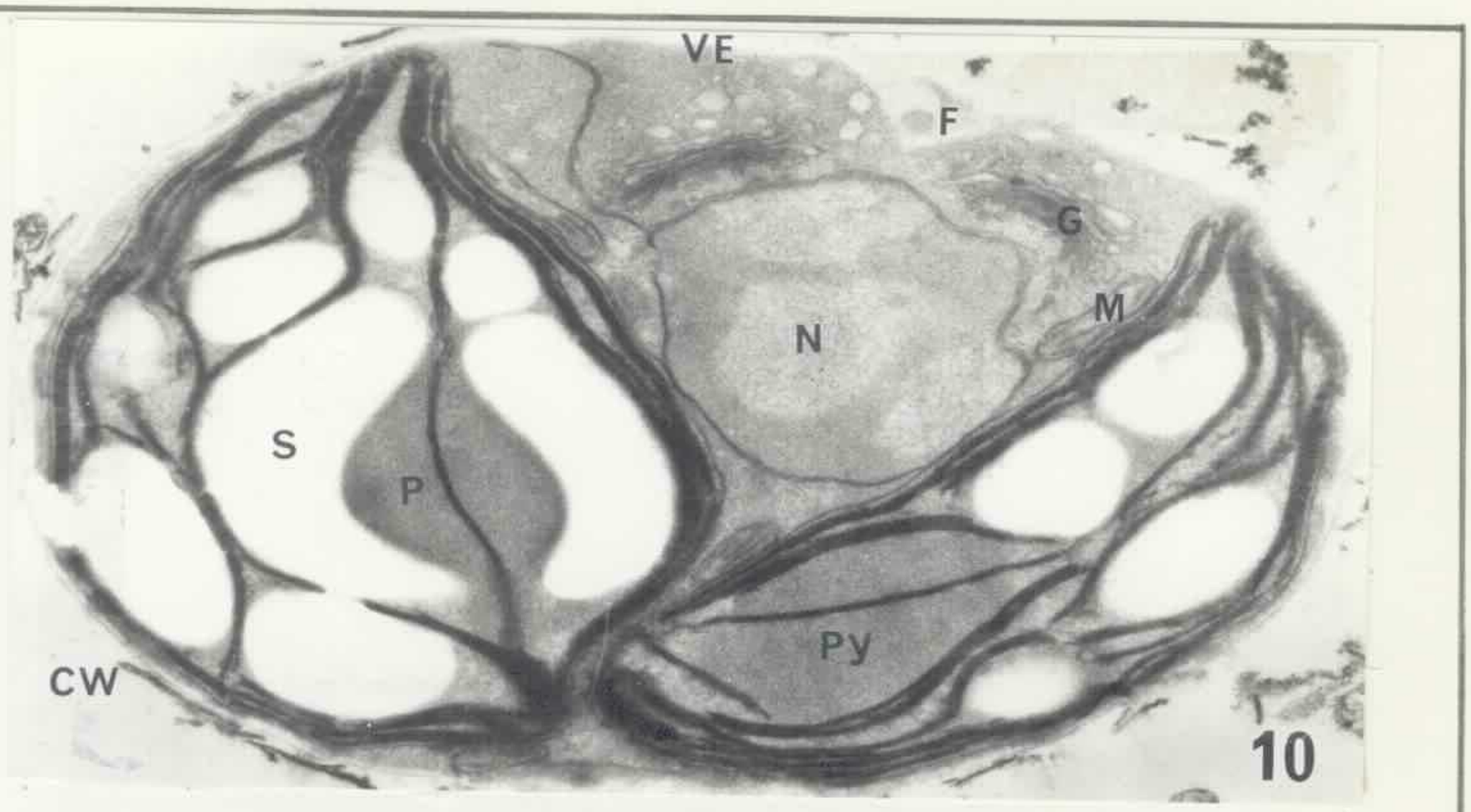

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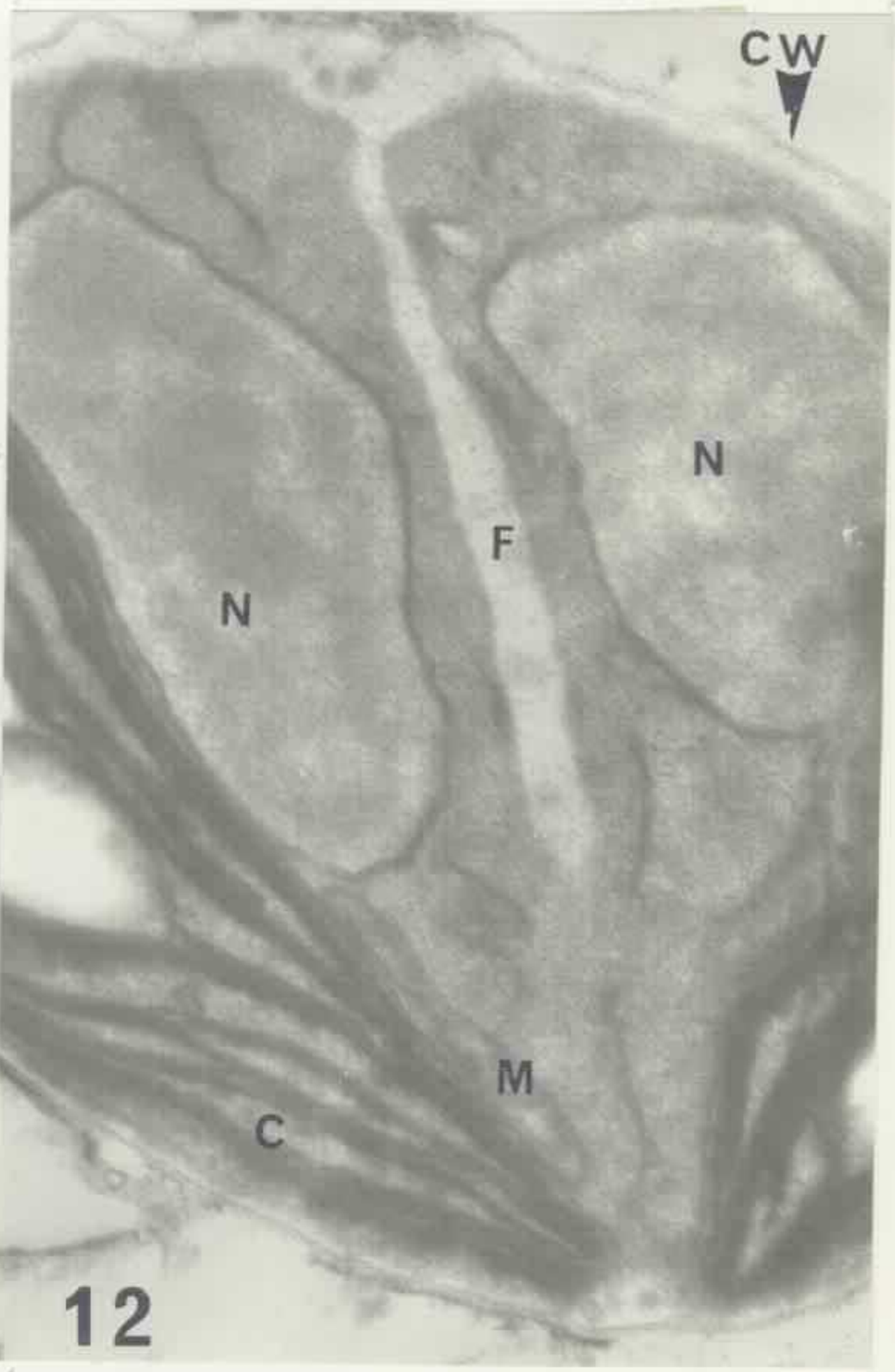

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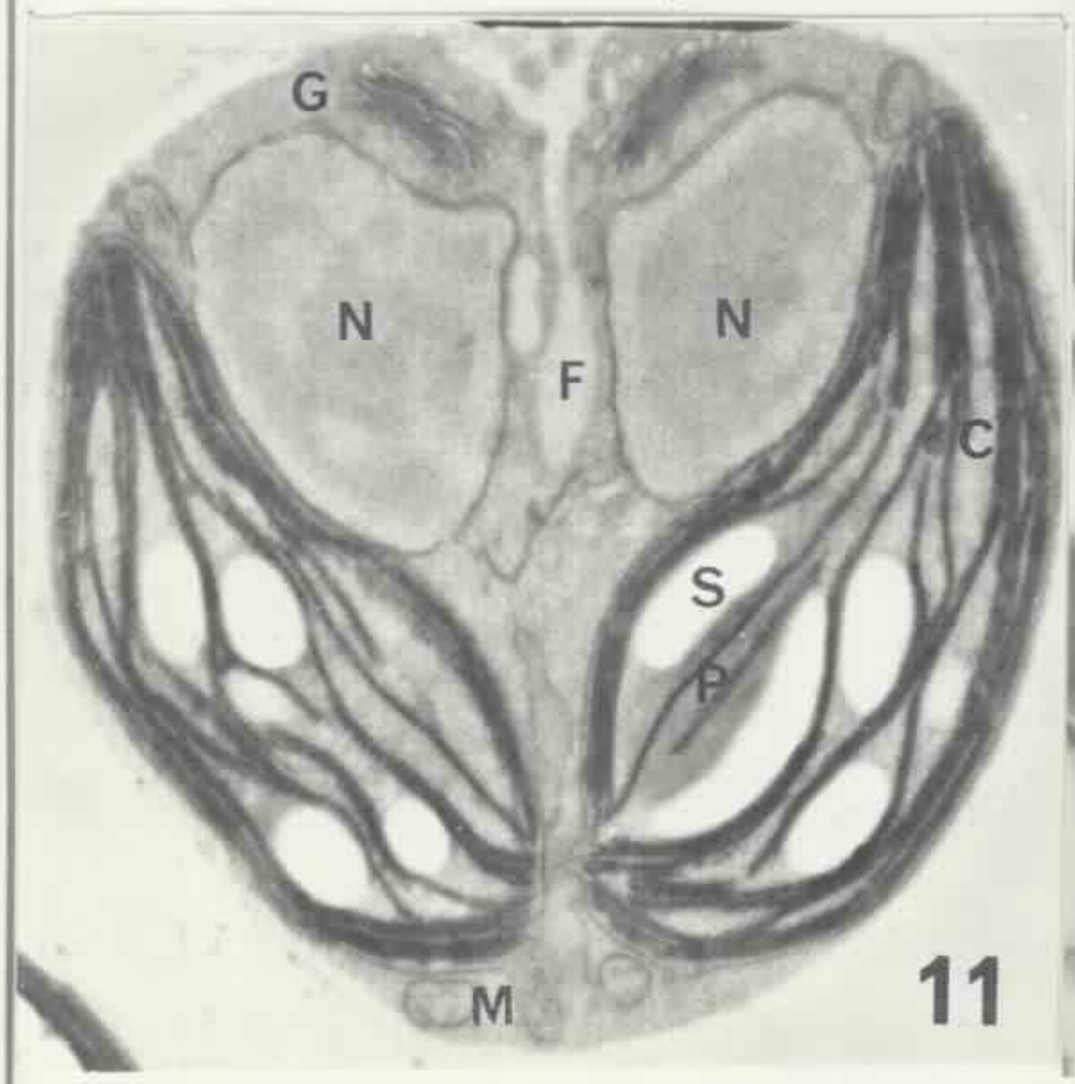

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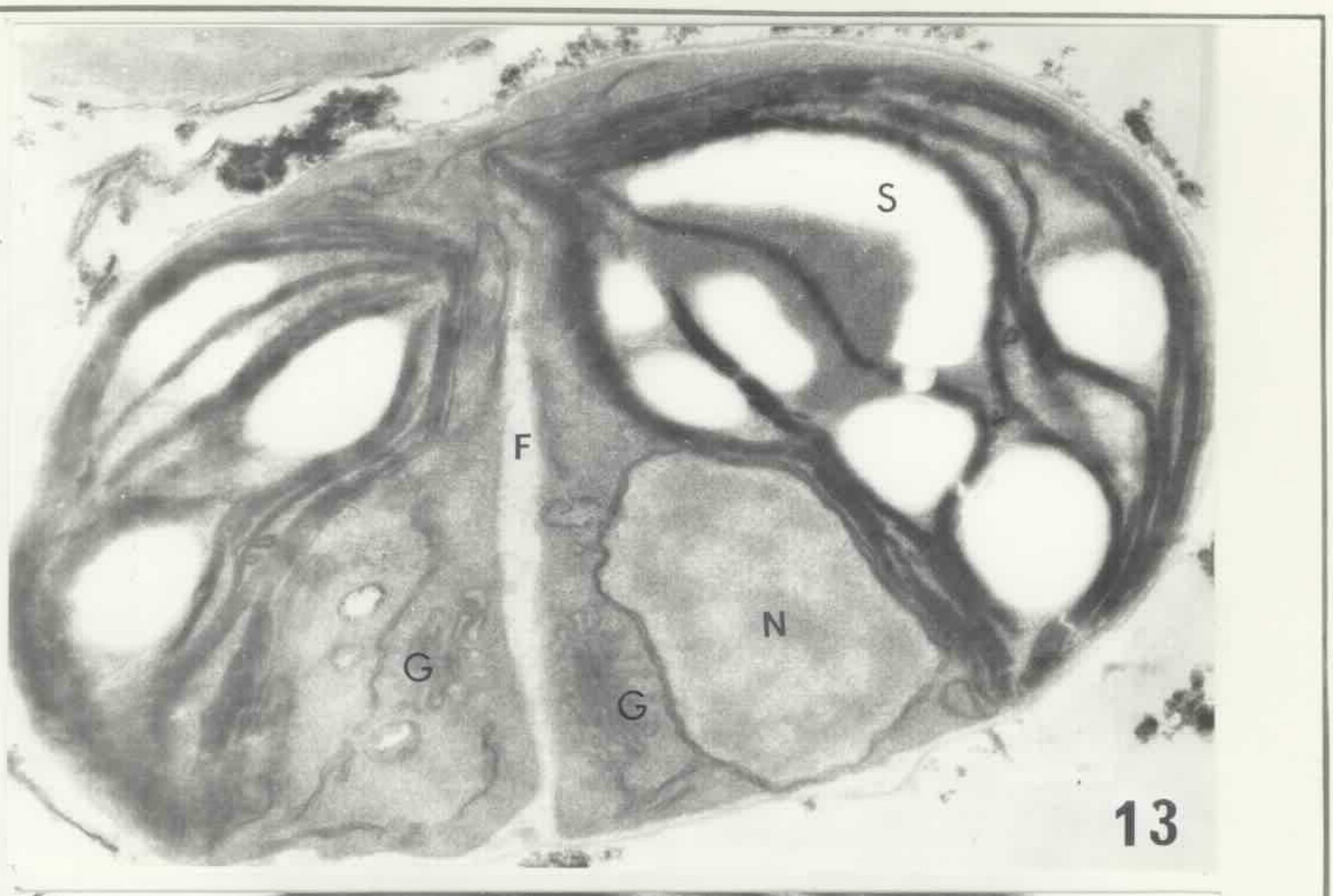
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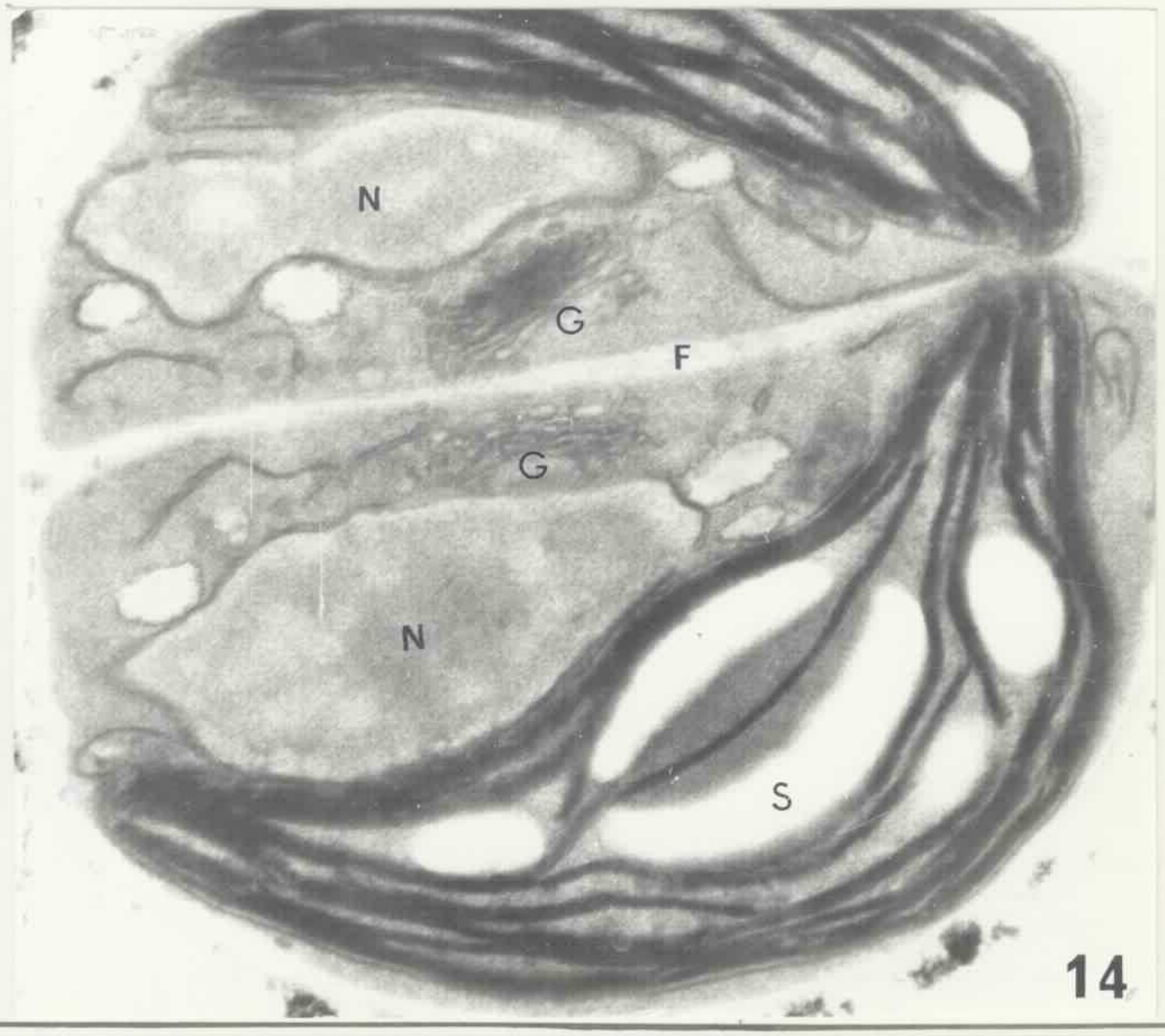
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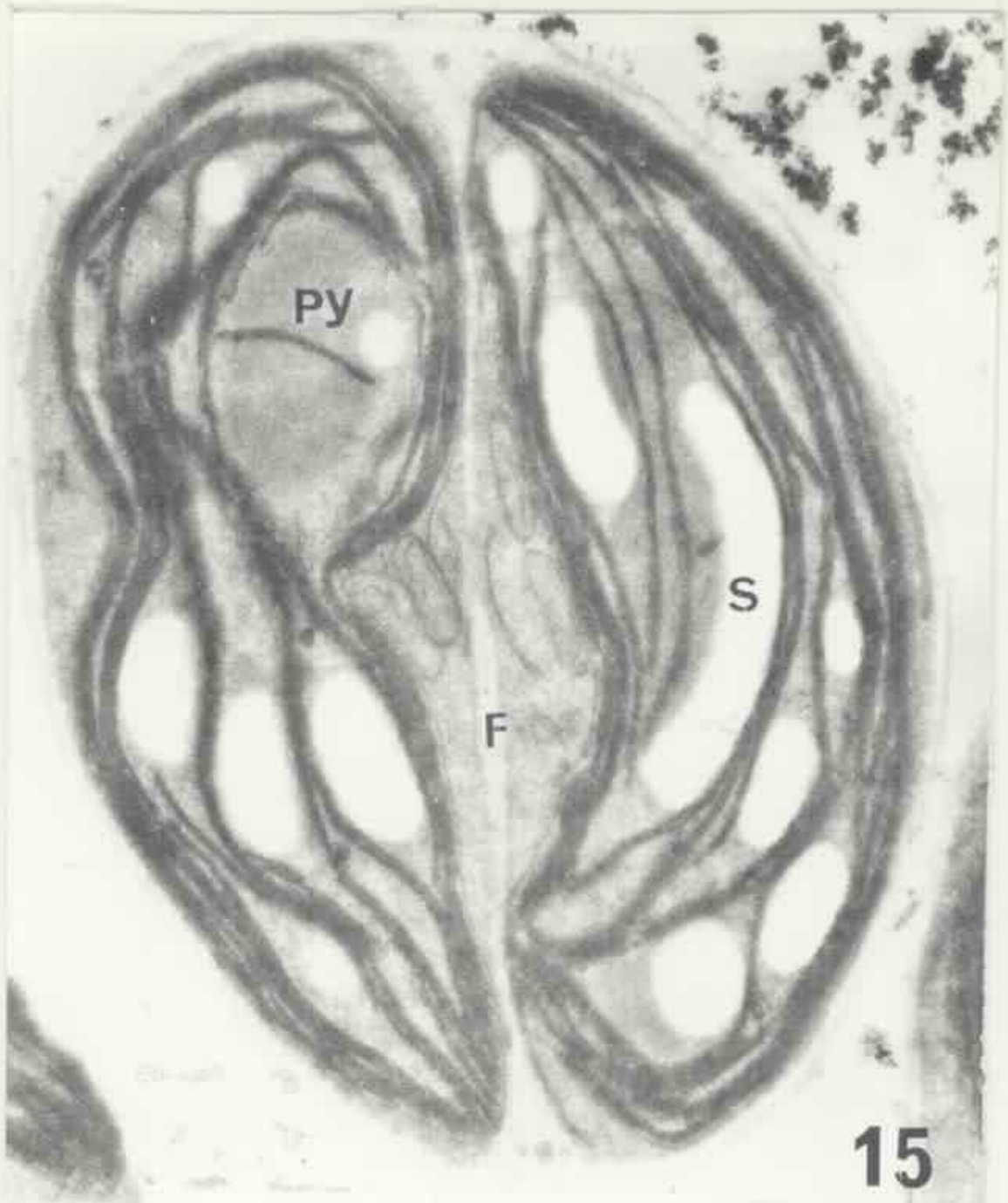
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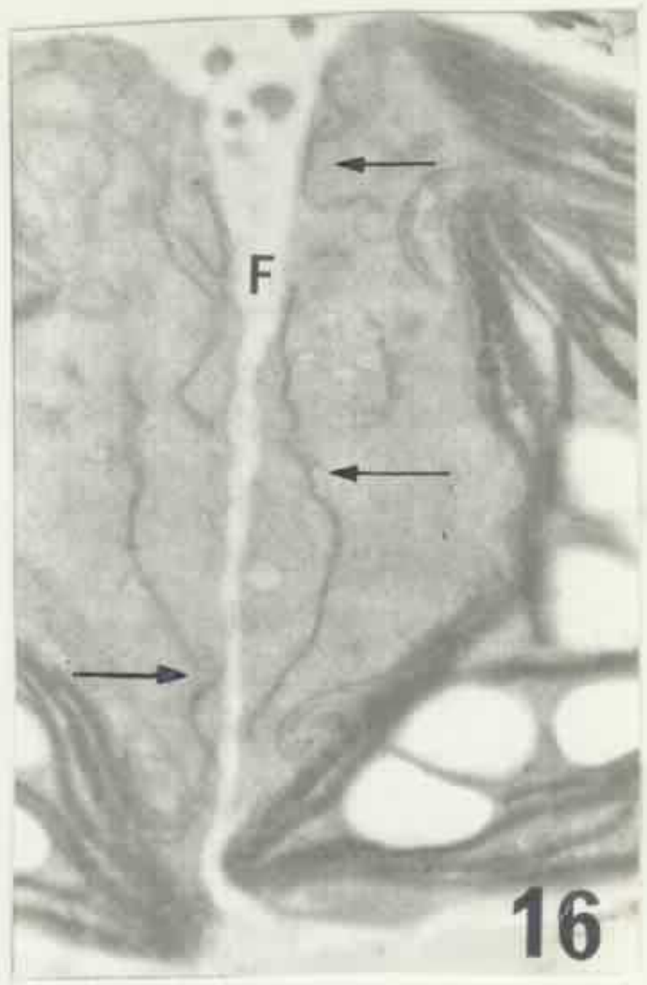
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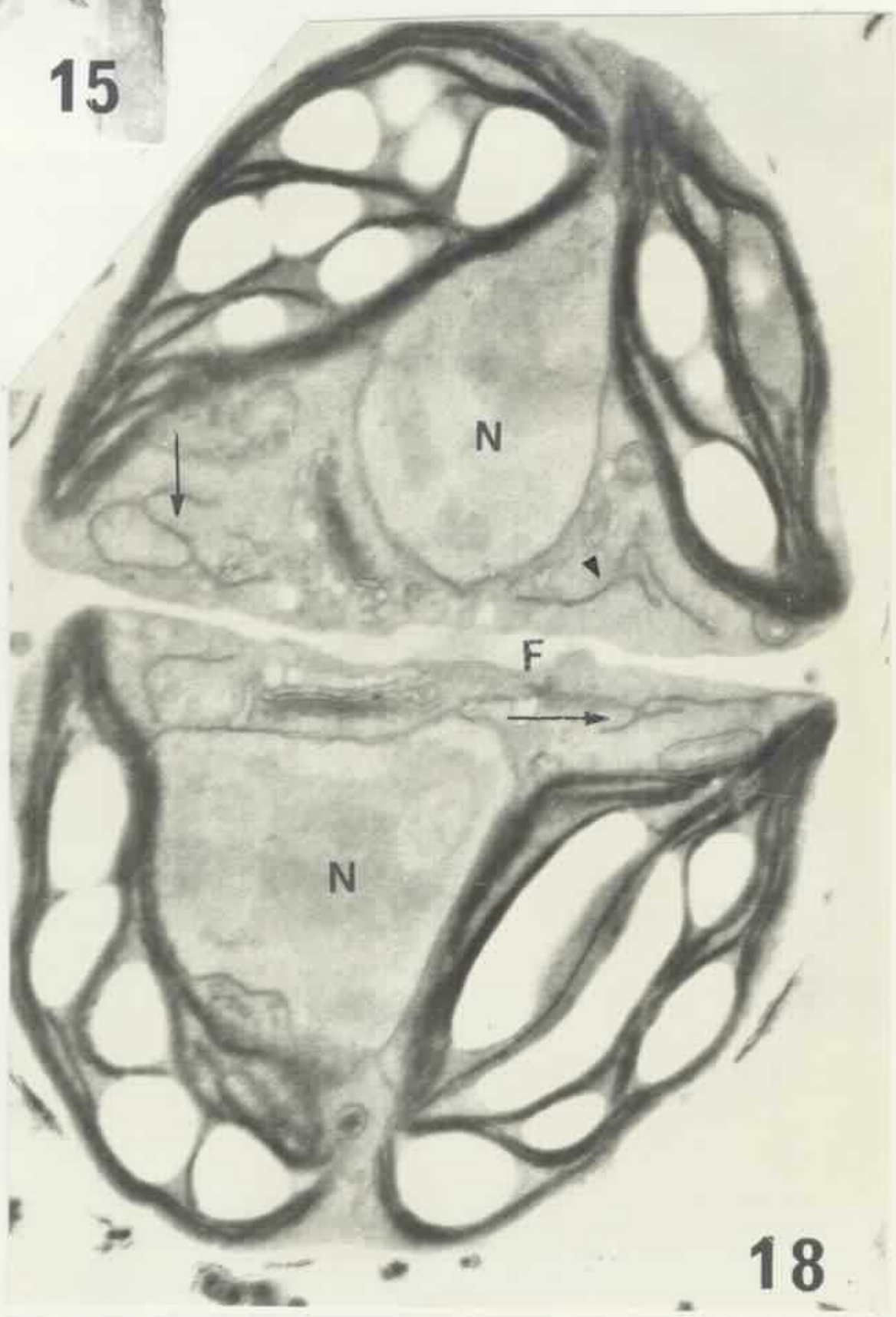
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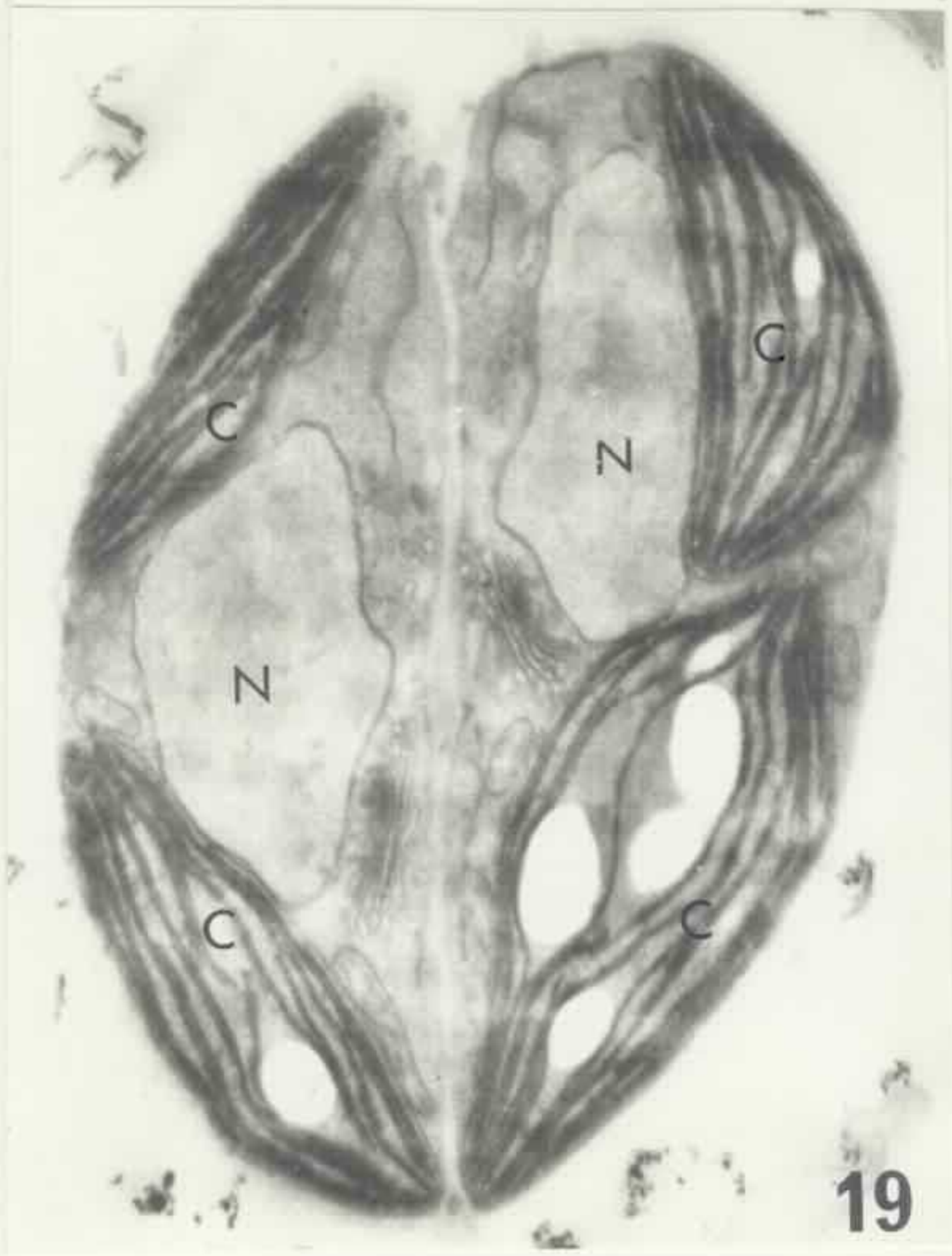
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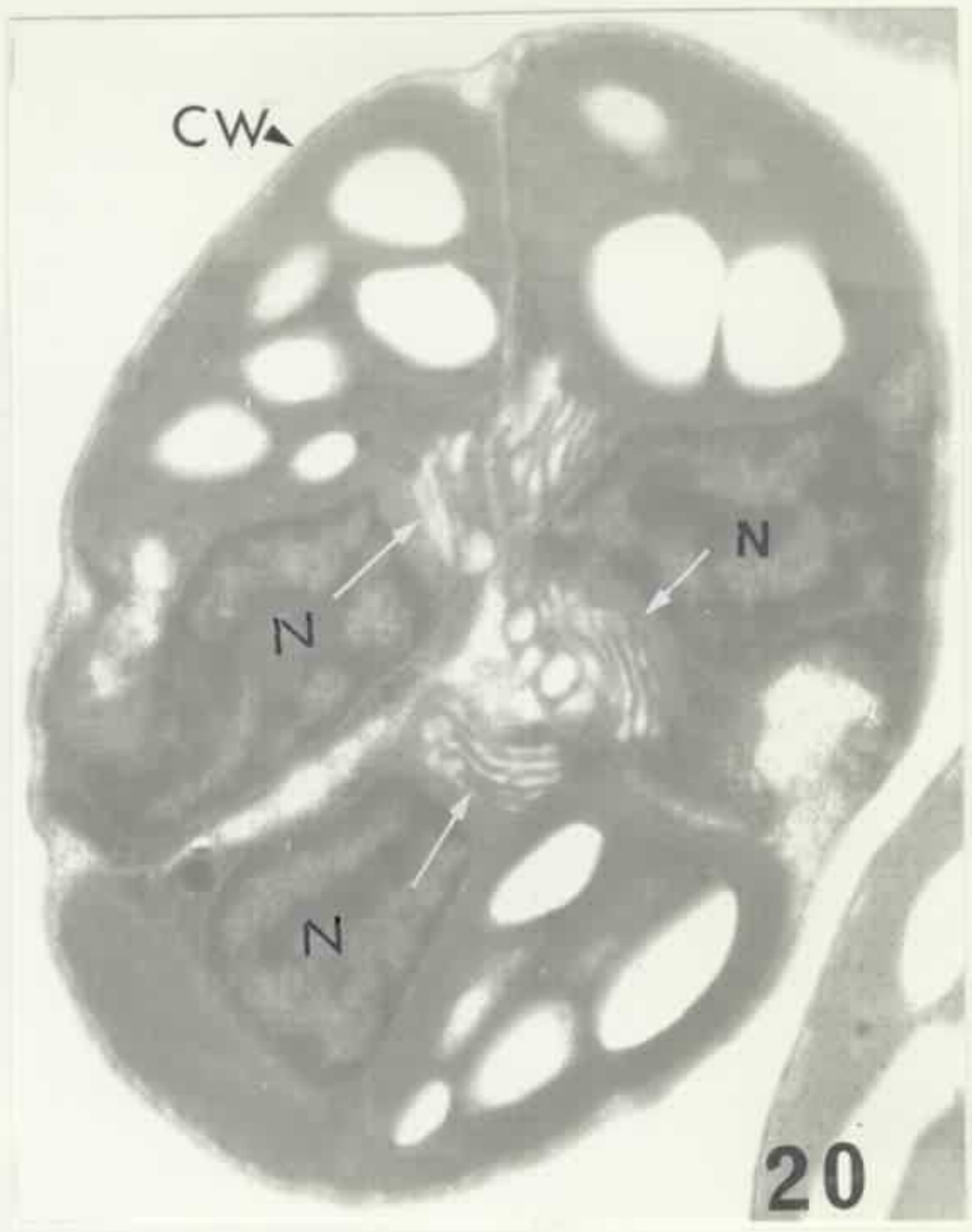
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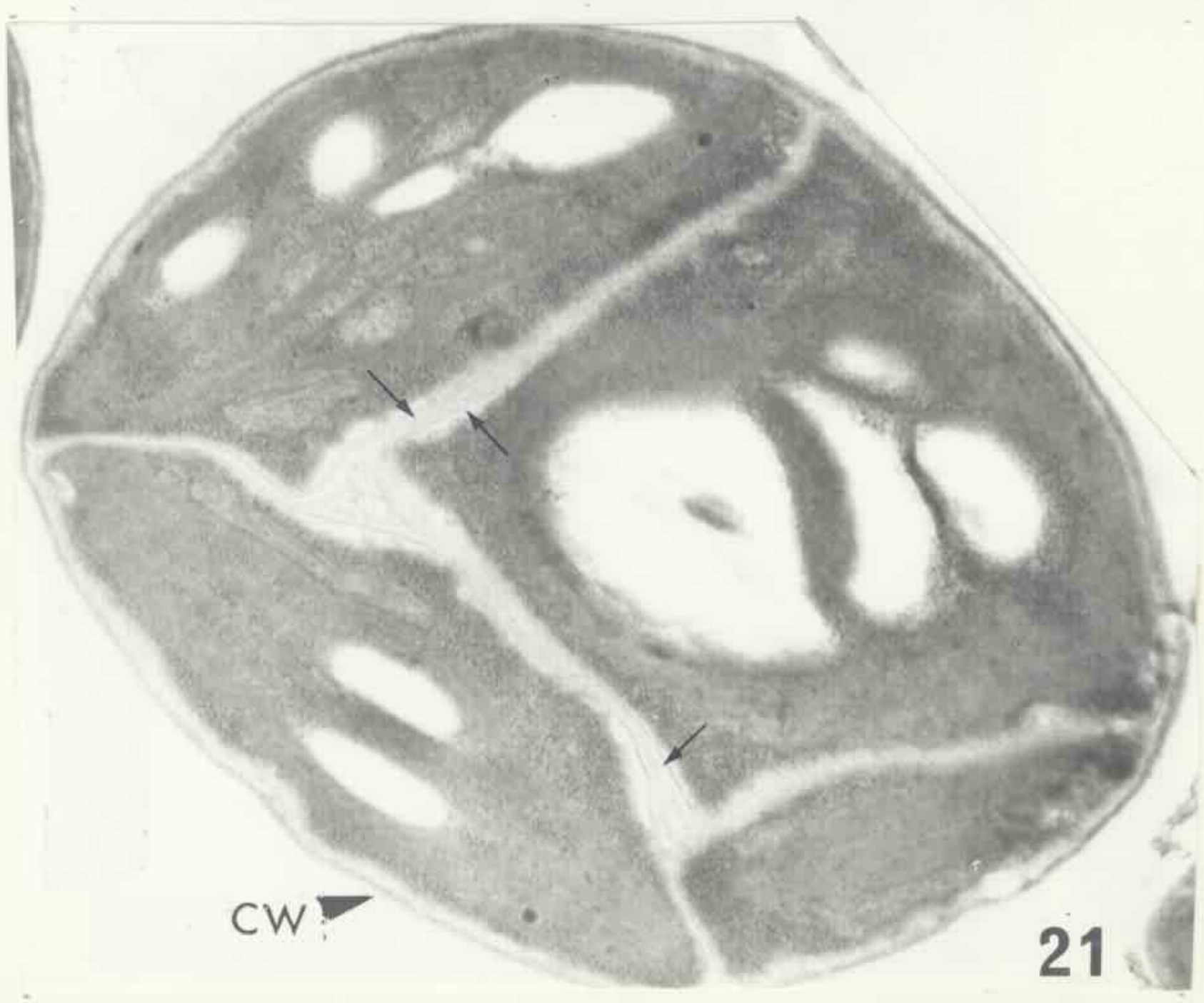
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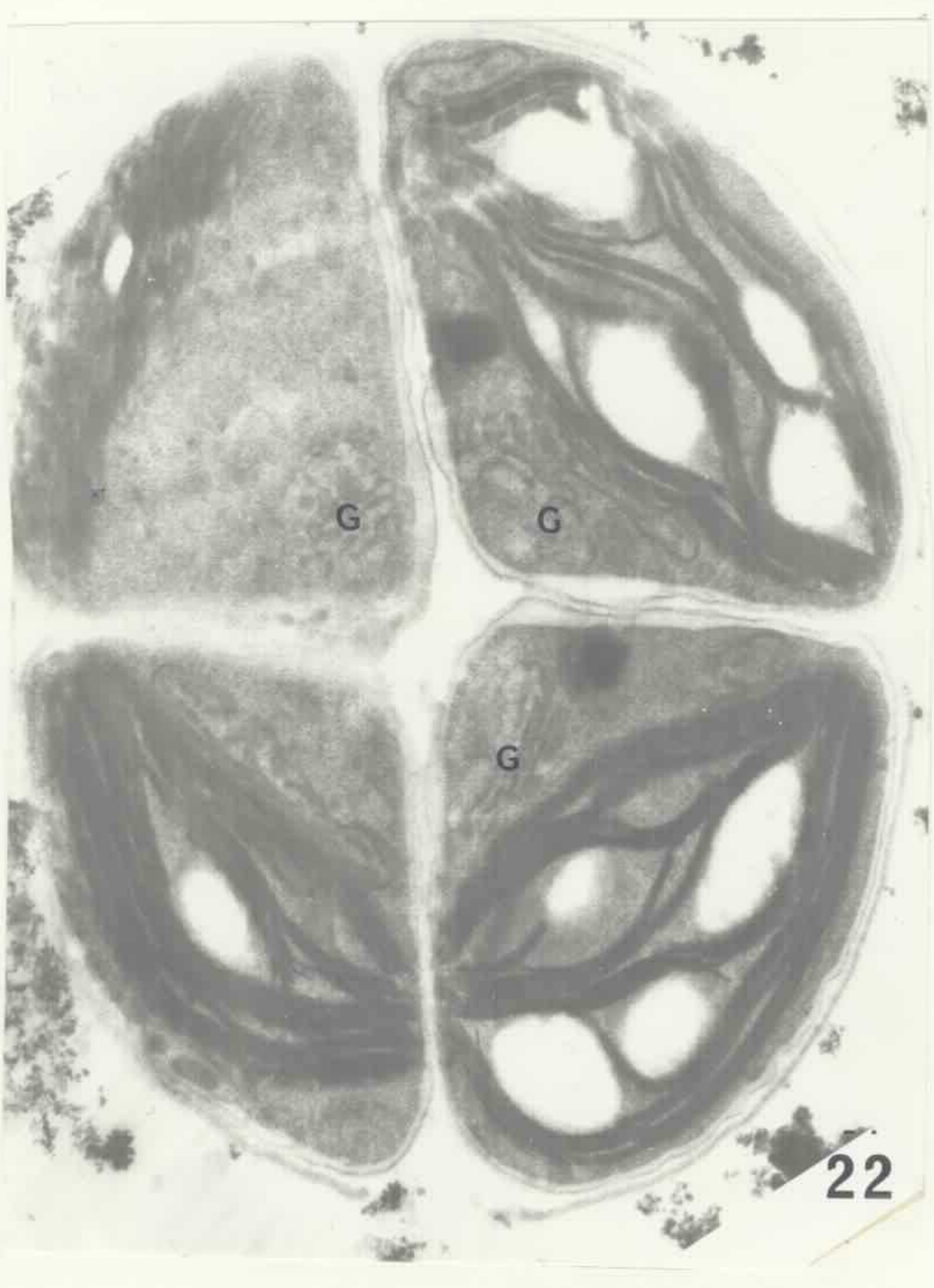
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CHAPTER 5

EFFECTS OF PHENYLUREA HERBICIDE (DIURON) ON PHOTO AUTOTROPHICALLY GROWN CHLORELLA NMI

5.1 Inhibition of growth in relation to concentration of diuron, and density of inoculum.

5.1.1 INTRODUCTION

To ascertain the effectiveness of diuron as an inhibitor of growth of alga NMI, the influence of this compound on cell number and chlorophyll content was studied.

5.1.2 MATERIALS AND METHODS

The following concentrations of diuron, 0, 0.02, 0.05, 0.1, 0.2 and 0.5 ppm were added to equal numbers of NMI cells (6.5 x 10 cell/ml) in sterile nutrient medium in duplicate culture vessels. These were incubated with shaking at 28 $\pm$ 1^{o}C in a water-bath, and illuminated with two 150 watts incandescent lamps. A stream of air, enriched to 5% CO_2 was continuously passed through the cultures during the growth period.

After 46 hours, cell number was determined as described in Section 2.3. The algal pigments were extracted with 80% acetone. Chlorophyll concentration was then determined as in Section 2.2.

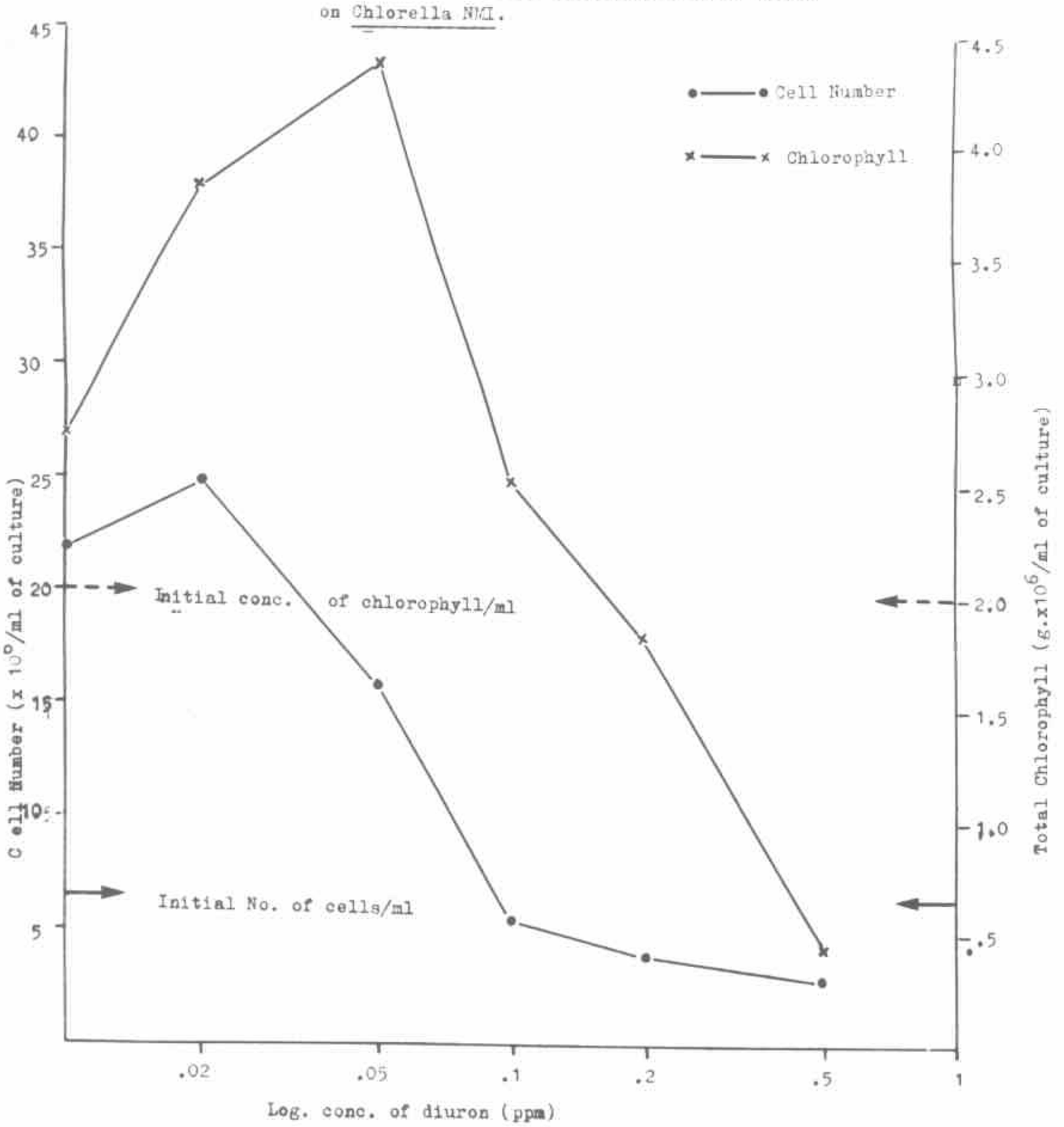
5.1.3 RESULTS

Fig. 1 showed the effects of the different concentrations of diuron on cell number and photosynthetic pigments of Chlorella NMI after 46 hours incubation. At 0.02 ppm, cell division was stimulated. A marked inhibition in the rate of cell division occurred at 0.05 ppm diuron, and the rate of inhibition increased with higher concentrations of the herbicide. Pigment synthesis was stimulated at the lower concentrations of the herbicide (0.02-0.05 ppm). The curves showed that rate of cell division was more sensitive to diuron than pigment production. A concentration of 0.069 ppm of diuron which reduced cell number by 50% still caused stimulation of pigment formation.

5.1.4 DISCUSSION

50% inhibition of growth, expressed as cell number, was obtained at 0.069 ppm diuron /(Fig.1). At the lowest concentration (0.02 ppm), the herbicide stimulated cell division. Photosynthetic pigments were less sensitive to the herbicide. Concentrations of 0.02-0.05 ppm stimulated production of the pigments as compared to that of the untreated control. 0.27 ppm of diuron were needed to cause 50% inhibition of pigment production. Matsuka and Hase (1966) had shown that light-enhanced chlorophyll

FIG. 1 Effects of different concentrations of diuron on Chlorella NMI.



formation in the glucose-bleached Chlorella cells was only slightly suppressed when the photosynthetic CO_2 fixation was blocked by monuron. They suggested a non-photosynthetic nature of light action in chlorophyll formation. Sokawa and Hase (1967a) however, found that although lack of CO_2 did not appreciably affect chlorophyll in the early phase of greening, a certain amount of suppression occurred in later phases. The results shown in Fig. 2 agreed with the above observations as conc. of 0.03-0.05 ppm had been shown to suppress CO_2 fixation in chloroplast and algae (Bishop, 1958; Jagendorf and Marguilies, 1960; Schiff et al, 1967).

It was also found earlier (Chapter 3, Part I) that stimulation of growth of Chlorella NMI occurred at very low concentrations of other phenylurea herbicides. This observation might be described as an example of the common phenomenon that certain compounds are inhibitory at high concentrations, but stimulatory at very low concentrations. Stimulation of growth by herbicides have been often reported (Ashman, 1964; Atkins, 1965; Swietochowski et al. 1964). Phenylureas had been found to show cytokinin activities (Bruce and Zwar, 1965). During the process of greening of glucose bleached Chlorella cells, under photosynthetic conditions, formation of chlorophyll proceeded in parallel with that of protein (Sokawa and Hase, 1967). Stern et al, (1964 a,b)

had reported that rate of photosynthesis during chloroplast development paralleled the formation of pigments and lamellae. It is, therefore, reasonable to assume that the increase in the amount of photosynthetic pigments at low concentrations of herbicide implied an increase in protein synthesis in the alga. This assumption was strongly supported by the facts that cytokinins stimulated synthesis of protein and nucleic acids in leaves (Osborne, 1962; Nichiporovich et al, 1967) and stimulated growth and cell division in red, brown and green algae (Isawaki, 1965; Pederson, 1968; Spencer, 1968; Jennings, 1969; Guerin-Du Martrait, 1966).

In studies of inhibition of O_2 evolution by diuron in chloroplast suspension, Izawa and Good (1965) observed that higher amounts of diuron were needed to produce the same degree of inhibition when the density of the chloroplast suspension was increased. It has been shown earlier (Chapter 4, Part I) that, with the same concentration of herbicide, inhibition of growth of Chlorella NMI was much lower with heavier algal inoculum. This reduction in inhibition with increasing inoculum density could be explained by the need for a critical amount of diuron in the chloroplast for inhibition to occur. Izawa and Good (1965); Monaco and Moreland (1966)

showed that diuron acted at a photosynthetic reaction centre which comprised a unit of several hundred chlorophyll molecules. This finding was supported by the work of Stanger and Appleby (1972) who also concluded that when sufficient diuron molecules were not available to inhibit all reaction centres, the unaffected photosynthetic units could continue to function. As the chlorophyll:diuron ratio increased from 200:1 to 300:1 the inhibitory effect decreased and at the 800:1 ratio, no toxic effect was observed (Stanger and Appleby, 1972). Thus, by increasing the cell number in the inoculum, the chlorophyll:diuron ratio was increased, resulting in reduction of sensitivity to diuron.

Van Rensen (1971) found stimulation of O_2-evolution by very low concentrations of diuron in Scenedesmus. This increase of O_2-evolution could be explained by the stimulation of chlorophyll pigment production, resulting in an increase in intensity of photosynthesis and not necessarily involving the redox balance as suggested by Van Rensen (1971). However, it is possible that the small amount of diuron, producing a very high chlorophyll:diuron ratio, could at the same time render the redox balance more favourable for O_2-evolution (Van Rensen, 1971).

5.2 Inhibition and photosynthetic pigments

5.2.1 Introduction

In view of the difference in sensitivity of cell division and pigment production to diuron, it was of interest to study the effects on the pigments by a concentration of diuron which caused complete inhibition of cell division.

5.2.2 Materials and Methods

Chlorella NMI cells were harvested at the end of the dark period from a synchronous culture. The cells were washed once with distilled water before use in the experiment.

Duplicate cultures of the cells were set up with 1 ppm of diuron and without diuron. The experimental vessels were incubated in a shaker water-bath at $28^{o} \pm 1^{o}C$ and illuminated with two 150 watts incandescent lamps. Air enriched to 5% CO_2 was continuously passed through the cell cultures.

Effects of diuron on the Chlorella cells were examined by cell count, pigment measurement and electron microscopy at intervals during the experimental period.

5.2.3 Results

At 36 hours cell count showed that diuron (1 ppm) completely inhibited cell division of the Chlorella cells, while the untreated culture had a 7-fold increase in cell number (Table 4). Total chlorophyll content increased with time in the untreated culture, but decreased in the treated culture. After incubation for 90 hours, inhibition of chlorophyll production was relieved in the treated culture. The percentage of chlorophyll content showed an increase as compared to that after 36 hours' incubation. For a more detailed study of the effects of 1 ppm diuron on photosynthetic pigments, concentrations of chlorophyll a (Chl.a), chlorophyll b (Chl.b) and carotenoids were determined and expressed as percentages of the pigment contents of the inoculum (Table 5). Chlorophyll production was more sensitive to diuron than carotenoids. The herbicide had a greater effect on Chl.b formation than on Chl.a (Table 5), resulting in an increase in Chl.a to Chl.b ratio (Table 4). After 90 hours, however, diuron toxicity was relieved and the Chl.a to Chl.b ratio was found to be identical to the untreated culture.

Table 4 Effects of diuron on cell number and photosynthetic pigments of chlorella (NMI)

Diuron conc. in ppm	Time in hours	No. of cells/µl culture (x 10^6)	Chlorophyll a:b ratio
0	0	2.2	1.95
	12	3.0	1.58
	36	15.1	1.66
	90	40.0	1.66
1	0	2.2	1.95
	12	2.4	2.38
	36	2.3	3.35
	90	2.0	1.91

Table 5 Effects of diuron on cell number and photosynthetic pigments of chlorella NMI (Results are expressed as percentages of measurements at start of experiment)

Diuron conc. ppm.	Time in hours	Cell No. %	Total chlor. %	Total 'a' %	Total 'b' %	Total conc. %
1	0	100	100	100	100	100
	12	108	87	93	76	103
	36	107	71	82.7	48	100
	90	91	88	86	88	92

Electron microscopy

Plate 5.1 shows Chlorella NMI cells grown photoautotrophically for 12 hours, and Plate 5.2 represents Chlorella NMI cells treated for 12 hours with 1 ppm. diuron. In comparing these electron micrographs of $KMnO_4$ fixed cells, it was observed that diuron treated cells contained no starch, and had large mitochondria, with numerous crístae, lying close to the chloroplasts. The single chloroplasts of the untreated cells were cup-shaped, with the nuclei generally facing the concave side; the chloroplasts of the treated cells, however, had lost the cup-shaped appearance and tended to assume a spherical shape (Plate 5.2). A large vacuole was sometimes present in the treated cell.

Plate 5.3 illustrates a diuron-treated cell fixed by the glutaraldehyde-osmium procedure. The absence of starch and slight rounding off of the chloroplasts were evident. The number of thylakoids making up the grana-like regions in the treated cell chloroplast was greatly reduced (Plate 5.4). The majority of such areas contained only two thylakoids, and very few had larger numbers, which was in direct contrast to the grana-like regions in the chloroplast

of the untreated cell. Ribosomes and DNA fibrils could be seen in the chloroplast, and there was a high concentration of ribosomes in the cytoplasm.

Plate 5.5 represents a Chlorella cell after 36 hours incubation. The lamellae organisation was severely disrupted and the lamellae have broken down into smaller units. However, DNA fibrils could still be seen in the stroma. The mitochondrion had enlarged considerably, and the cristae were short and swollen and reduced in number. Both ribosomes and DNA fibrils could be distinguished in the organelles.

5.2.4.

DISCUSSION

Of interest in the present study is the observed increase in chlorophyll a:b ratio during treatment with 1 ppm diuron. This increase may be due to either a change in the concentration of chlorophyll a or chlorophyll b.

In the experiment, it is found that total chlorophyll content is decreased, but concentration of chlorophyll b is reduced by 24% after 12 hours and 52% after 36 hours as compared to only 7% and 17.5% for chlorophyll a. These results indicate that the increase in chlorophyll a:b ratio in the diuron-treated cell is due to a reduction in chlorophyll b. This is in line with the finding that a higher chlorophyll a:b ratio is often due to reduction or absence of chlorophyll b in mutant plants Hordeum vulgare (Goodchild et al, 1966), Arabidopsis thaliana (Hirono and Redei, 1963), and Chlorella pyrenoidosa (Allen, 1958).

The concept of photosynthesis consisting of two light reactions, System I and System II, is now widely accepted. Recent work on chloroplast fragmentation has shown that chlorophyll a and chlorophyll b are associated with both systems. Fraction one, with a

chlorophyll a:b ratio of 2, is primarily responsible for system II, and fraction two, with chlorophyll a:b ratio of 6, is responsible for system I (Boardman and Anderson, 1964). In view of the accumulating evidence that chlorophyll b is formed from chlorophyll a, (Shylk, 1964; Bogorad, 1966; Jones, 1968; Ellsworth et al, 1968, 1969), it can be speculated that the photosynthetic system contains a form of chlorophyll a that can be converted to chlorophyll b. The assumption of two forms of chlorophyll a in the photosynthetic system is supported by the findings of Duysens (1963). He showed that the fluorescence of chlorophyll a is excited more effectively by the light absorbed by pigment system II, than that absorbed by pigment system I. Furthermore, the presence of higher concentrations of chlorophyll b in System II is consistent with the findings of Losada et al (1961) and Allen (1958). Losada et al (1961) showed that some assistance of light energy is required in the splitting of water, and it involves accessory pigments, such as chlorophyll b. Allen (1958) suggested that chlorophyll b might be necessary for effective use of light energy, and that it might stabilize the photo-synthetic apparatus.

The increase in chlorophyll a:b ratio observed in diuron-treated cells may be the result of a direct action on System II, by inhibiting the conversion of chlorophyll a to chlorophyll b. The change in chlorophyll ratio interferes with the activity of System II, and thus the use of light energy by the cells is less efficient, resulting in inhibition of photosynthesis.

Since chlorophyll b may be important in the stacking of the thylakoids in the photosynthetic apparatus (Allen, 1958; Goodchild et al, 1966; Highkin et al, 1969), then diuron would interfere with the formation of grana-like areas in Chlorella NMI chloroplast. This is confirmed by the electron microscope studies (Plate 5.4) Smith (1954) has observed that chlorophyll b may not be necessary for oxygen evolution. As photolysis of water, however, is considered to take place in the grana of chloroplasts (Wettstein, 1958), it is reasonable to assume that an increase in chlorophyll a:b ratio which impairs grana formation in the chloroplast may have some effect on oxygen evolution. Homann and Schmid (1967), Woo et al (1971), and Bishop et al (1971) have reported that stacking in the lamellae of the chloroplasts of higher plants is necessary for the normal activity of Photosystem II.

5.3 Summary

1. Action of different concentrations of diuron show that cell division is more sensitive to the herbicide than chlorophyll production.

2. Very low concentrations of diuron (.02 to .05 ppm) stimulate chlorophyll production.

3. An analysis of data on content of chlorophylls a and b of cells treated with 1 ppm diuron indicates a definite differential inhibitory action of diuron on the synthesis of chlorophyll b. Chlorophyll a to b ratio is increased.

4. After 12 hours, mitochondria have migrated from the periphery to lie close to the chloroplasts. The latter become spherical in shape, have no starch grains, few stacks of thylakoids, and the grana-like regions contain fewer thylakoids.

5. After 36 hours, the lamellae system is severely disorganised. The lamellae are broken in several places into shorter units. The chloroplasts contain ribosomes and DNA fibrils. Mitochondria are large, with fewer and shorter cristae.

Plate 5

Plate 5.1 — Untreated *Chlorella* NMI cells after 12 hours. The chloroplast (c) is cup-shaped and contains large starch grains (c). Nucleus (N) faces concave side of chloroplast. Note mitochondria (arrows) at periphery of cell.

Glut - $KMnO_4$. x 10,000

Plates 5.2, 5.2.A — *Chlorella* NMI cells treated with 1 ppm diuron for 12 hours. The chloroplast (c) tends to be spherical in shape. Note absence of starch, and large mitochondria, with numerous cristae, close to the chloroplast.

Glut - $KMnO_4$

5.2 x 20,000
5.2.A x 30,000

Plate 5.3 — *Chlorella* NMI cells treated with 1 ppm diuron for 12 hours. Note absence of starch. Chloroplast (c) has lost its cup-shaped appearance.

Glut - OsO_4. x 20,000

Plate 5.4 — *Chlorella* NMI cell treated with 1 ppm diuron for 12 hours. Fewer grana-like structures. Majority of lamellae are made up of 2 thylakoids. Note large mitochondria (M) near chloroplast.

Glut - OsO_4. x 66,300

Plate 5.5 — *Chlorella* NMI cell treated with 1 ppm diuron for 36 hours.



1



2

2A

5



5





CHAPTER 6

EFFECTS OF DIURON ON PHOTOAUTOTROPHICALLY GROWN EUGLENA CELLS

6.1 Introduction

Because of the widespread use of Euglena sp. in a variety of biological studies and particularly in investigations controlling the loss and development of chloroplasts, Euglena appeared to be an ideal choice to study the effects of diuron on photosynthetic cells.

It is expected that a study of changes in the ultrastructure of herbicide-treated Euglena cells would shed some additional light on the action of diuron.

6.2 Preliminary investigation of inhibition of growth of Euglena by diuron

Euglena cultures were grown photo-autotrophically with three different concentrations of diuron (1, 2 and 5 ppm) and as an untreated control. The culture vessels were incubated, with mechanical shaking, in a water-bath at 26 ± 1°C, and illuminated with two 150-watt incandescent lamps. Growth was estimated by turbidimetry measurements and expressed as percent of the control. Readings were taken daily.

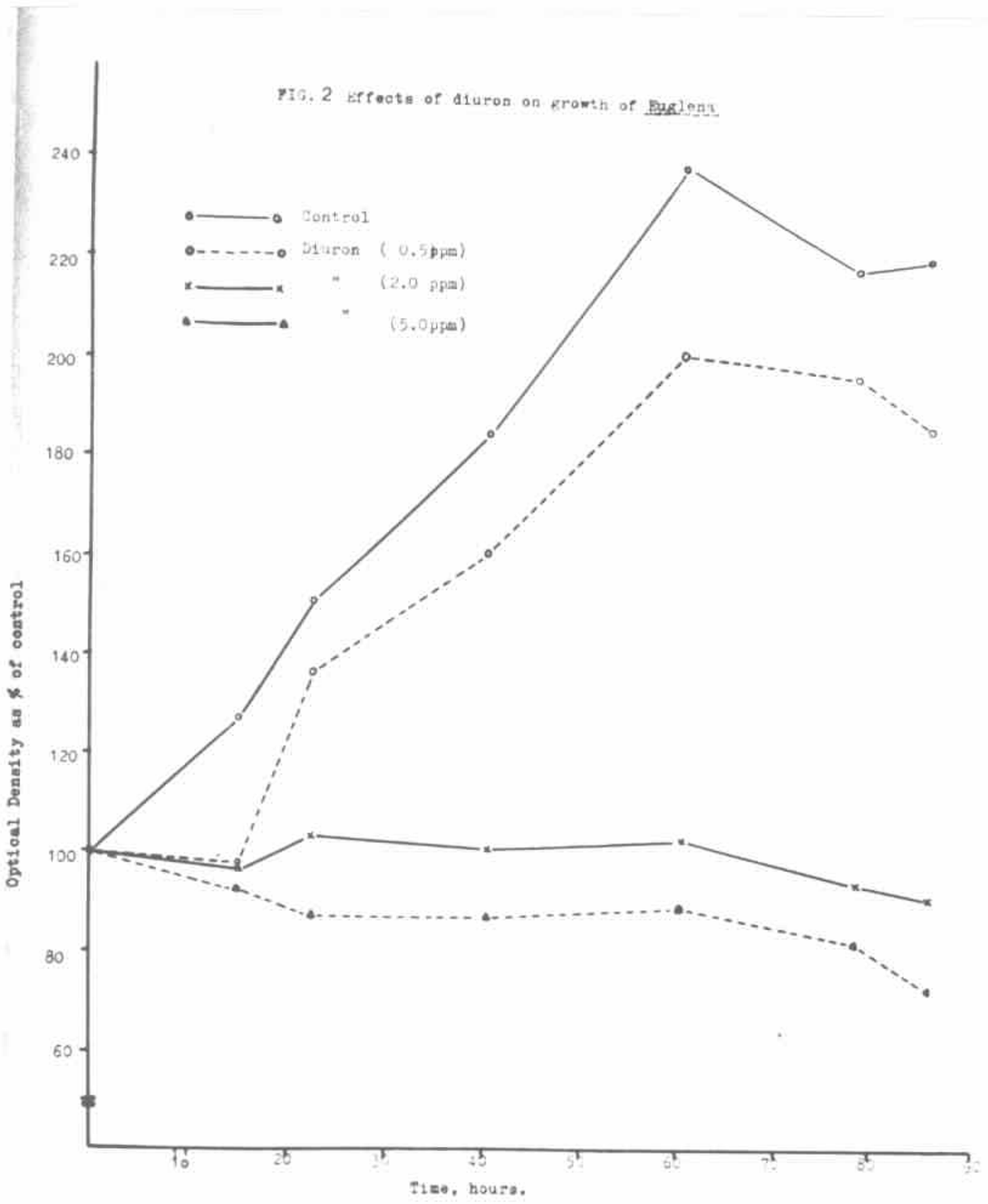
Fig. 1 shows the effects of the three concentrations of diuron on photoautotrophic growth of Euglena.

Growth was only slightly inhibited at 0.5 ppm of diuron. At 2 and 5 ppm diuron, there was a complete inhibition of growth.

6.3 Effects on cell division, pigment synthesis and fine structure of Euglena in relation to the concentration of diuron

A series of cultures of Euglena were set up with diuron at the following concentrations - 0, 0.05, .01, .05, 0.1, 0.2, 0.5, 1.0, 3.0, 5.0 and 10.0 ppm. The cells were incubated at 26 ± 1°C with shaking, and illuminated at 400 ft. candles with fluorescent 'White-warm' lamps.

FIG. 2 Effects of diuron on growth of Euglena



After 48 hours growth, cell number was counted and the algal pigments were extracted with 80% acetone and chlorophyll concentration determined as described in Section 2.2.

Light microscopy

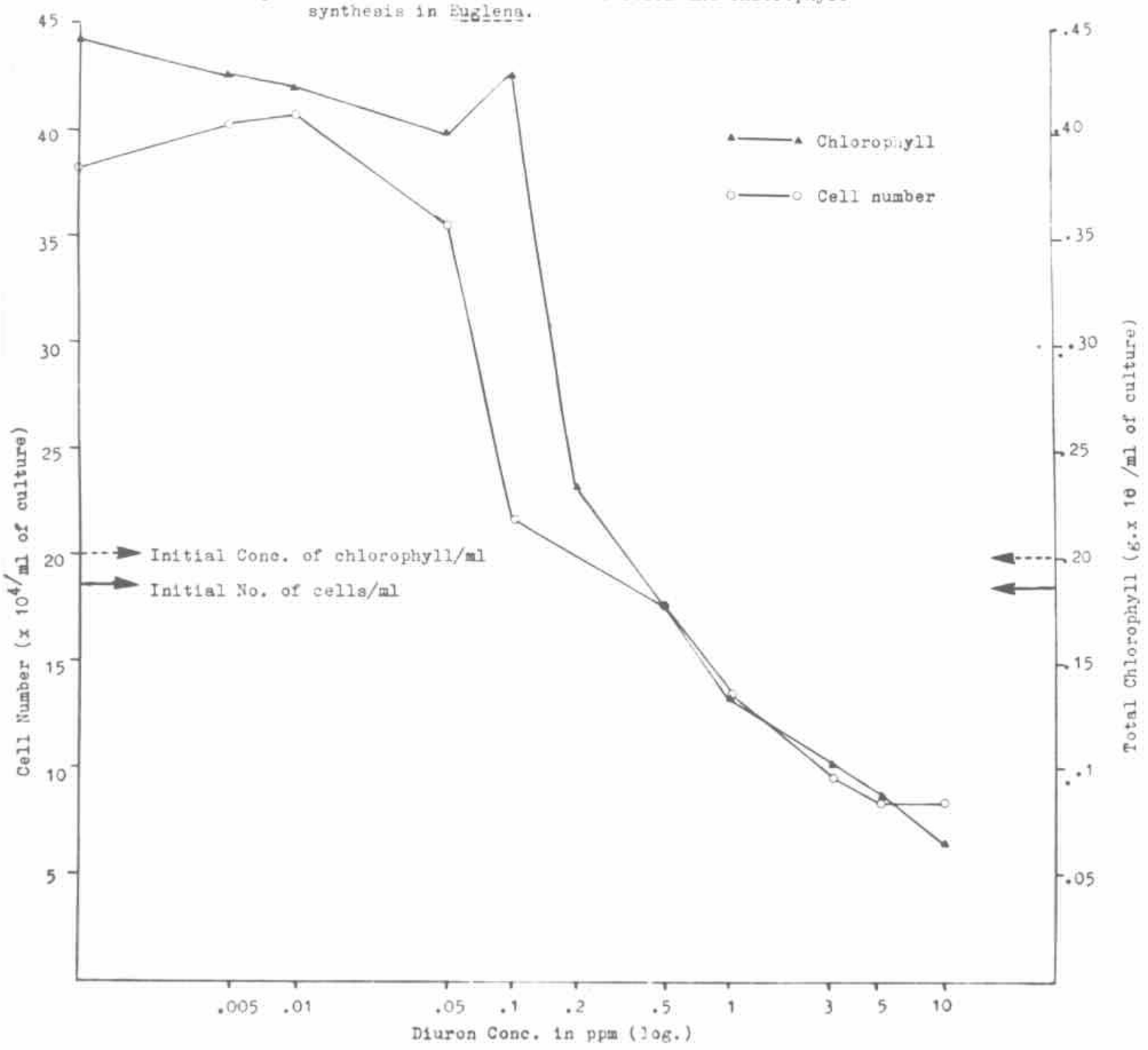
Photomicrography of the algal cultures after 48 hour growth was taken using a Leitz Ortholux microscope, equipped with a camera, a 100 x oil immersion objective and a 10 x ocular lens. The cell culture was recorded on Ilford Pan F or Kodak Pan Plus-x film.

Electron microscopy

Samples were taken from the treated and untreated cultures after 48 hours, and fixed with the double fixation procedure (Glutaraldehyde-Osmium) as described in Section 4.2.2.

Fig. 3 shows that the untreated Euglena cells had doubled in number after 48 hours incubation (from 19.0 x 10^4 to 38.3 x 10^4 cells/ml). During that period chlorophyll concentration had also doubled. The very low concentrations (.005-.01 ppm diuron) did not affect cell division nor chlorophyll synthesis. At a concentration of 0.05 ppm, the herbicide caused a decrease in cell number, but not in the total amount

FIG. 3 Effects of diuron on cell division and chlorophyll synthesis in Euglena.



of chlorophyll. Increase of diuron concentration to 0.1 ppm still did not affect total chlorophyll contents, although cell division was almost completely inhibited. Above 0.2 ppm, the herbicide inhibited both cell division and chlorophyll synthesis. At these concentrations, there was a decrease in cell number and pigment content with increasing amounts of the herbicide.

In the present study, chlorophyll contents of the cells, reduction or increase in chlorophyll "a" and chlorophyll "b" in treated cells were expressed as percentage of these pigments in the untreated cells after 48 hour incubation (Table 6). The results showed that at the lowest concentration which did not inhibit cell division, inhibition or stimulation of the two chlorophyll pigments was not observed. At 0.01 and 0.05 ppm diuron, however, there was a reduction in chlorophyll "b" and a stimulation in chlorophyll "a" production. At 0.1 ppm, the chlorophyll content per cell was nearly double that of the untreated cell, and although chlorophyll "a" content was nearly doubled, there was comparatively a low increase in chlorophyll "b" (Table 7). This is indicated by the high chl. "a" to chl. "b" ratio (Table 6). At 0.2 ppm, diuron caused marked reductions in chlorophyll "a" and "b"

Table 6 Effects of diuron on production of chlorophyll pigments in Euglena grown autotrophically in light for 48 hours

Diuron conc. (ppm)	Chl.a, g/ml of culture ($\times 10^{-5}$)	Chl. b, g/ml of culture ($\times 10^{-5}$)	Chl.a / Chl.b
0	3.44	0.99	3.7
.005	3.43	0.82	4.2
.01	3.36	0.84	4.6
.05	3.26	0.71	4.6
.10	3.63	0.63	5.8
.20	1.92	0.39	4.9
.50	1.42	0.35	4.0
1.0	1.06	0.27	3.9
3.0	0.83	0.20	4.1
5.0	0.71	0.18	3.9
10.0	0.49	0.15	3.3

Table 7 Effect of diuron on contents of chlorophylls a and b of Euglena grown in light for 48 hours. (Chlorophyll content is expressed as percentage of that of control)

Diuron conc. in ppm.	Chl.a/cell % of control	Chl.b/cell % of control	Total chl/cell % of control
0	100	100	100
.005	99	84	95.5
.01	105	80	99
.05	106	80	100
.10	192	116	175
.20	112	80	104.5
.50	113	96	109
1.0	92	84	90
3.0	95	80	92
5.0	92	76	82
10.0	61	64	53

but the chl. "a" to chl. "b" ratio was still high compared to that of the untreated cells. At higher concentrations (0.5 to 1.0 ppm), the herbicide was highly toxic to the cells and both pigments were inhibited at nearly the same rate, as shown by the decrease in pigment ratio (Table 6).

Light Microscopy

Untreated cells were full of paramylon bodies after 48 hour growth in light (Plate 6.1). When exposed to 0.1 ppm level of diuron, paramylon bodies could not be distinguished, and the chloroplasts were big and highly distorted (Plates 6.2 and 6.3). At 0.5 ppm of diuron, some giant and small chloroplasts were found within the cell (Plate 6.4). At higher concentrations, reddish brown material was visible within the cell. Only three to four chloroplasts remained, and these were small and dense (Plates 6. 5, 6 and 7).

Electron microscopy

Plate 6.8 shows part of a Euglena cell from the culture used as inoculum. The cell contained a number of elongated chloroplasts, with small paramylon bodies adjacent to the pyrenoids. The chloroplast had the typical features of a normal mature plastid (Section 4.3.2). Plate 6.9 represents an untreated cell, grown photo-

autotrophically for 48 hours. The only difference from cells of the inoculum was the large number of paramylon bodies in the cytoplasm.

After 48 hours in diuron at 0.05 ppm, the cells contained chloroplasts similar to those in the untreated cell. There was, however, a small number of large chloroplasts (Plate 6.10). Paramylon bodies, although present, were fewer in number.

Concentrations of 0.1 and 0.2 ppm, of diuron caused certain structural changes within the algal cell. Only two to three chloroplasts were visible in the sections (Plate 6.11). The chloroplasts were odd shaped and very large. The lamellae were arranged in an irregular manner. They were not packed together as in the chloroplasts of the untreated cells, but were rather curved at different angles leaving larger spaces of the stroma (Plate 6.12). Another conspicuous change was the almost complete absence of paramylon bodies. Pyrenoid areas were visible, with very small paramylon bodies (Plate 6.12). Other changes were also observed in the cytoplasm of these treated cells. Small vacuoles, occasionally found in control cells, could be easily observed in the cytoplasm. Sometimes a few of the vacuoles contained electron dense material.

These membrane bound vacuoles were comparable to the cytolysomes or autophagic vacuoles described in mammalian cells (De Duve & Wattiaux, 1966).

Cells treated with 0.5 ppm diuron contained a few large odd shaped chloroplasts (Plate 6.14). The chloroplast of Euglena contained only one pyrenoid (Section 4.1). This agreed with the studies of Gibbs (1960) on Euglena pyrenoids. Therefore, the presence of more than one pyrenoid in the large chloroplast and the shape of the latter (Plate 6.15) indicated that the large chloroplast could be formed from the fusion of neighbouring chloroplasts as shown clearly in Plate 6.13. Such an occurrence would be in accord with the reduction in number of chloroplasts per cell. The empty vacuoles were larger and more numerous. The cytolosome vacuoles contained lipid like bodies, electron dense material (Plate 6.14) and sometimes vesicles and multivesicular bodies. The mitochondria were thin, long and irregular in shape (Plate 6.14). At 1 ppm diuron, electron dense bodies, resembling the granules of the eye-spot could be seen scattered within the cytoplasm (Plate 6.16).

At 2 ppm diuron the cell section showed two to three small chloroplasts (Fig. 6.17). More and larger cytolysomes were found in the cytoplasm

(Plates 6. 19, 20). Vesicles bounded by a unit membrane, containing fat bodies, myelin figures and unit membranes, (Plate 6.18) similar to those identified as phospholipid vesicles by Leedale (1965a) were also present. With increasing diuron concentrations, clearing of the cytoplasmic contents became more pronounced, probably due to autolysis in the cytolysomes. Large bodies composed of concentric lamellae and large lipid bodies appeared in the sections (Plate 6.17). These bodies were thought to be degenerated or improperly formed chloroplasts. Comparable structures have been found by Siegesmund et al.(1962) in starved cells of Euglena. Thin, bizarre shaped mitochondria were distinguishable (Plate 6.17). At 5 ppm and 10 ppm diuron small and broken chloroplasts were present in the sections (Plates 6.21 and 6.22). At the highest concentration used (10 ppm), the cytoplasmic contents had been almost used up and part of a cytolysome vacuole could still be observed (Plate 6.22). It was interesting to note that although at the high diuron concentrations the nuclear content appeared different from that of normal cells, the nuclear envelope and pellicle were apparently not adversely affected. At 10 ppm, when most of the

contents of the cell appeared completely disrupted, the pellicle still retained its typical sculptured organisation and the nucleus its unbroken nuclear envelope, (Plate 6.22).

6.4 DISCUSSION

It is clear that diuron is an effective inhibitor of growth of Euglena grown autotrophically in the light (Fig.2). The addition of 0.5 ppm diuron causes a short lag period, but it seems to have little effect on the pattern of Euglena growth, although it slightly inhibits the optimal level of growth achieved in the control culture. Schiff et al (1967) have shown that 10 μM (2.33 ppm) of diuron completely inhibited carbon dioxide fixation in dark grown resting cells of Euglena gracilis var bacillaris, incubated in the light, but had little effect on chlorophyll formation. At a concentration of 0.1 μM (0.023 ppm) diuron, CO_2 fixation was inhibited about 95 per cent. It is possible that 0.5 ppm used in the present study is sufficient to inhibit photosynthetic CO_2 fixation, but is inadequate to produce a marked difference in optical density from that of the control. However, the failure to detect a difference between normal and treated cells may be due to the fact that, at the chosen wavelength (662 nm) for turbidity measurements, a decrease in cell number may be compensated by an increase in pigment content per ml. of culture.

In the second experiment (6.3) the effects of different concentrations have been investigated separately on cell numbers and pigment synthesis. Response of chlorella NMI to the lower concentrations of diuron shows that cell division is more sensitive to the herbicide than chlorophyll synthesis (Fig.3). A concentration of 0.05 ppm of diuron which does not affect chlorophyll synthesis, decreases the rate of cell division. It must be noted, however, that at that concentration, storage material, in the form of paramylon bodies, is considerably depleted (Plate 6.10), with regard to the untreated culture (Plate 6.9). This is in agreement with the biochemical finding that diuron blocks carbon dioxide fixation, hence carbohydrate production. However, the presence of a few paramylon bodies (Plate 6.10) suggests that photosynthesis is not completely inhibited at that concentration of diuron.

The increase of diuron to 0.1 ppm results in complete inhibition of carbon dioxide fixation, as indicated by the absence of paramylon bodies in the cell (Plate 11). The differential effect of the herbicide on cell division and pigment production is more pronounced. Cell division is completely inhibited, but total pigment synthesis is unaffected, a doubling of pigment concentration per cell was observed (Table 3).

This may imply a certain independence of chlorophyll production from carbon dioxide fixation for a supply of precursors and energy source. Although a doubling of pigment concentration per cell is observed (Table 7), more chlorophyll 'a' is produced with relation to chlorophyll 'b', and this is reflected in the very high increase in chlorophyll a:b ratio (Table 6). These results support the finding (Section 5.2.3) that diuron preferentially inhibits the formation of chlorophyll b in the photosynthetic system. As discussed earlier (Section 5.2.4) this shift in chlorophyll a:b ratio may impair theproper functioning of the photosynthetic unit, and cause a breakdown of the lamellae organisation.

It is interesting to find that under the influence of diuron, Euglena behaves like some animal cells in a pathological situation. At concentrations of diuron which inhibit carbohydrate synthesis, the reserve material (paramylum) is rapidly metabolised and used by the cell. As outside carbon becomes unavailable, the Euglena cell makes use of the non-essential components of its cytoplasm to maintain its vital basic metabolic processes. It appears that this is brought about by isolation of parts of the cytoplasm within membrane-bound vacuoles, cytolysomes. Similar

responses have been observed in a number of tissues under a variety of physiological and pathological conditions (Ashford and Porter, 1962; Blum and Buetow, 1963; De Duve, 1963; Novikoff and Essner, 1962; Malkoff and Buetow, 1964). Apparently digestive enzymes appear within these structures, and breakdown of the contents by hydrolysis occurs (Brandes, et al, 1964; Novikoff and Essner, 1962; Erickson and Trump, 1964).

In the present study, the formation of cytolysomes can be interpreted as a mechanism for survival of the Euglena cell by utilisation of the breakdown products of cytoplasmic contents. As the contents of cytolysomes undergo progressive degeneration, small vesicles, multi-vesicular bodies, unit membranes and myelin figures are formed. At high concentrations, the toxic effects of diuron are more severe, and the Euglena cell responds by the formation of more and larger cytolysomes. Degeneration of cytoplasmic contents is rapid, resulting in death of the cell.

6.5 Summary

1. Cell division of Euglena is more sensitive than chlorophyll production to diuron. Low concentrations of the herbicide (.01 to .1 ppm) do not affect chlorophyll production, but show a differential inhibitory action on the biosynthesis of chlorophyll 'b', causing an increase in the chloroplast a to b ratio.

2. Light microscopy shows that, at low concentrations of diuron, giant chloroplasts are formed. Higher concentrations produce a reduction in number and size of the chloroplasts.

3. In electron microscopy, the first morphologic change is the disappearance of paramylum, and fusion of neighbouring chloroplasts into odd-shaped giant chloroplasts.

4. At higher concentrations, areas of cytoplasm are segregated by limiting membranes to form cyto-lysomes. The chloroplasts are reduced in size, the lamella organisation is disrupted, and finally the chloroplasts break down.

5. Nuclear changes are minimal and the pellicle still retains its sculptured organisation at the highest concentration used (10 ppm).

Plate 6

Plate 6.1 — Untreated Euglena grown photoautotrophically for 48 hours. Note number of paramylon bodies (arrow) within the cell.

Plates 6.2 and 6.3 — Cell treated with 0.1 ppm. diuron for for 48 hours. Arrows indicate giant chloroplasts. Note their distorted shapes.

Plate 6.4 — Cell treated with 0.5 ppm. diuron for 48 hours. Arrow indicates giant chloroplast.

Plate 6.5 — Cell treated with 2 ppm. diuron for 48 hours. Note round chloroplasts and their granular appearance.

Plate 6.6 — Cell treated with 5 ppm. diuron for 48 hours. Chloroplasts are small and dense (arrows). Note presence of nucleus with nucleolus.

Plate 6.7 — Cell treated with 10 ppm. diuron for 48 hours. Chloroplasts are very small and dense (arrows).

Key to labelling

C - Chloroplast
M - Mitochondria
G - Golgi body
Py - Pyrenoid
P - paramylon body
R - Reservoir
CV - Contractile vacuole
N - Nucleus
V - Vacuole
E - Eyespot
Ly - lysosome - like vacuole

Plate 6.8 Section through part of Euglena cell from inoculum. The chloroplast contains 12-16 well oriented lamellae. Pyrenoid (Py) with associated paramylon bodies (P) is indicated. Cytoplasm contains mitochondria (M). Glut - O_sO_4. x 12,000.

Plate 6.9 Section of Euglena grown autotrophically in light for 48 hours. Note large number of paramylon bodies (P) in cytoplasm. Glut - O_sO_4. x 12,000.

Plate 6.10 Section through part of cell treated with 0.05 ppm. diuron for 48 hours. A giant and a normal chloroplast (C) are seen. Note a great reduction in number of paramylon bodies as compared to that in the control cell. (Plate 6.9). Glut - O_sO_4. x 6,000

Plate 6.11 Micrograph shows three cells from culture treated with 0.1 ppm. diuron. Note presence of both normal and large, distorted chloroplasts (C). Vacuolisation (V) of cytoplasm is apparent, and small vacuoles (Ly) containing electron dense material can be seen. Glut. - O_sO_4. x 5,250

Plate 6.12 Section through part of cell treated with 0.1 ppm. to illustrate effect of the herbicide on chloroplast. Note disruption of orientation of lamellae, and large stroma spaces. Concentration of chloroplast ribosomes (Cr) is high. Arrows indicate small lysosome-like vacuoles. Glut.-O_sO_4. x 12,000.

Plate 6.13 Section through part of a cell treated with 0.5 ppm. diuron. Arrow indicates point of fusion of two chloroplasts. Glut. - O_sO_4. x 15,000.

Plate 6.14 Micrograph shows a number of cells from culture treated with 0.5 ppm. diuron. Note greater vacuolisation of cytoplasm, large cytolysome-like vacuoles (Ly), giant chloroplasts (C). Nucleus is not affected.
Glut.- O_sO_4. x 3,000

Plate 6.15 Cell treated with 0.5 ppm. diuron. Micrograph shows giant chloroplast with several pyrenoids (Py).
Glut.-O_sO_4. x 15,000.

Plate 6.16 Cell treated with 1 ppm diuron. Chloroplasts are small and lamellae are not well-defined. Structures with large lipid globules and very fine membranes are apparent, and may represent degenerated chloroplasts (arrow). Note the presence of small osmiophilic bodies, resembling eye-spot granules, scattered within the cytoplasm.

Glut- O_sO_4. x 12,720

Plate 6.17 Cell treated with 2 ppm diuron. Chloroplast with densely packed lamellae (C). Structures with large lipid globules and fine membranes are seen (large arrow). A number of small multivesicular bodies are also present (small arrows).

Glut- O_sO_4. x 12,000

Plates 6.18, 6.19, 6.20 Sections of cell treated with 2 ppm diuron. Cytolysomes containing myelin figures (6.18), fat body and concentric membranes (6.19) and multivesicular bodies (6.20).

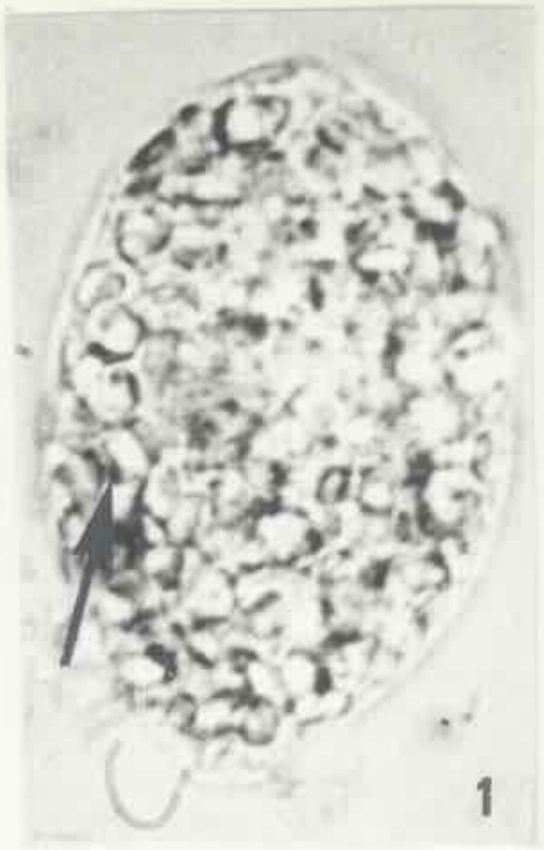
Glut- O_sO_4. x 52,500

Plate 6.21 Cell treated with 5 ppm diuron. Only a few cytolysomes are present. The empty spaces (v) may represent true vacuoles and cytolysomes in which the visible content has been completely digested. Few small mitochondria are seen. Chloroplasts are in degenerating stages.

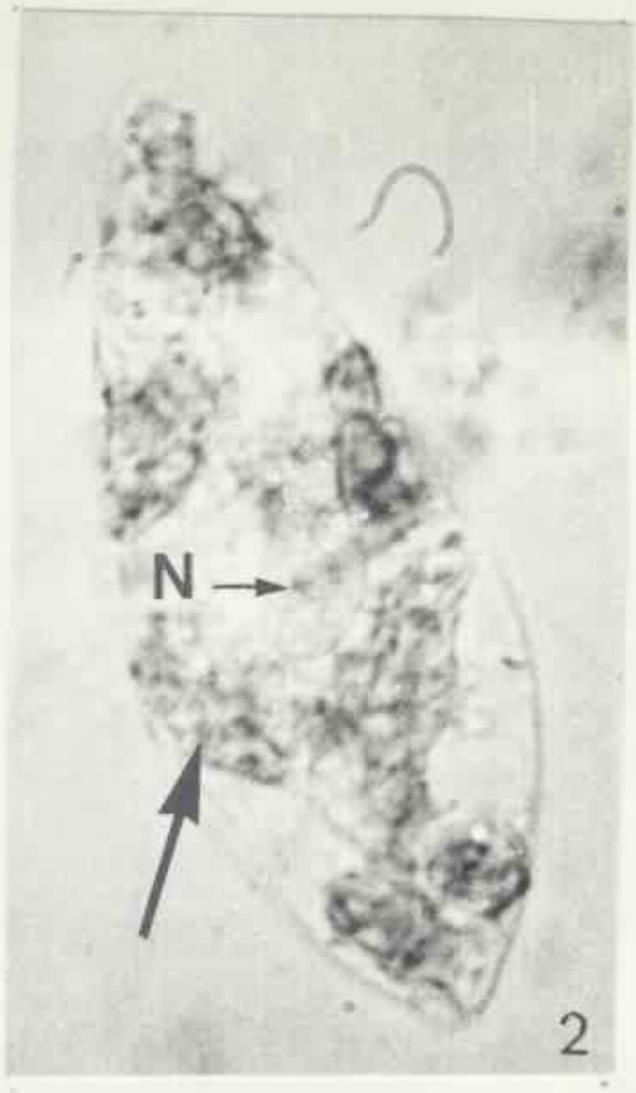
Glut- O_sO_4. x 7,650

Plate 6.22 Cell treated with 10 ppm diuron. Advanced stage of degeneration. Most of the structures within the cytoplasm have disappeared. Vestiges of mitochondria (arrow) can be seen.

Glut- O_sO_4. x 15,000



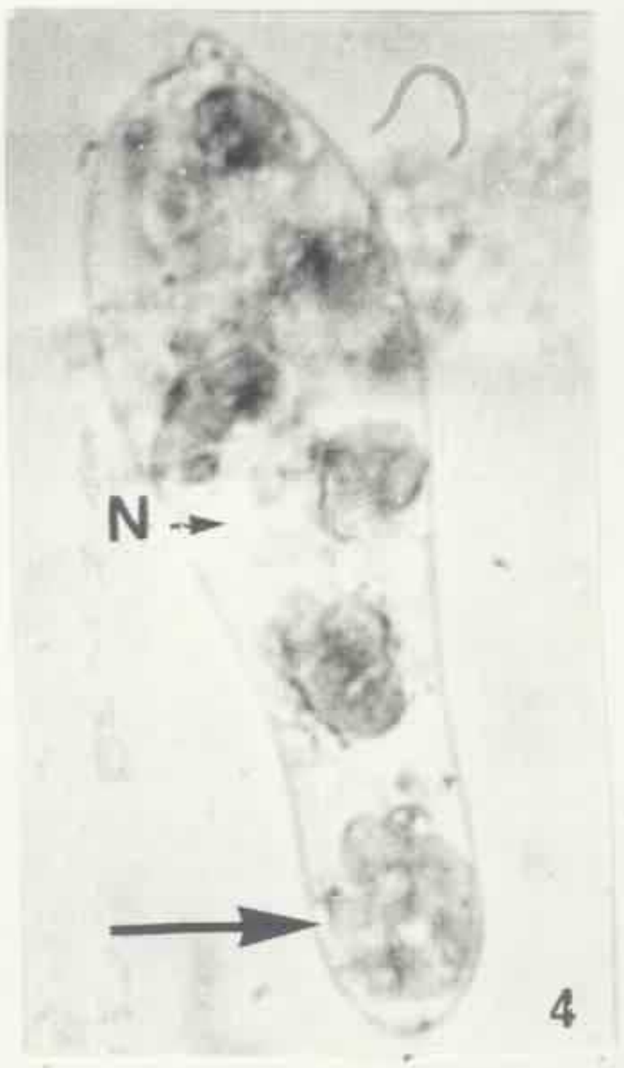
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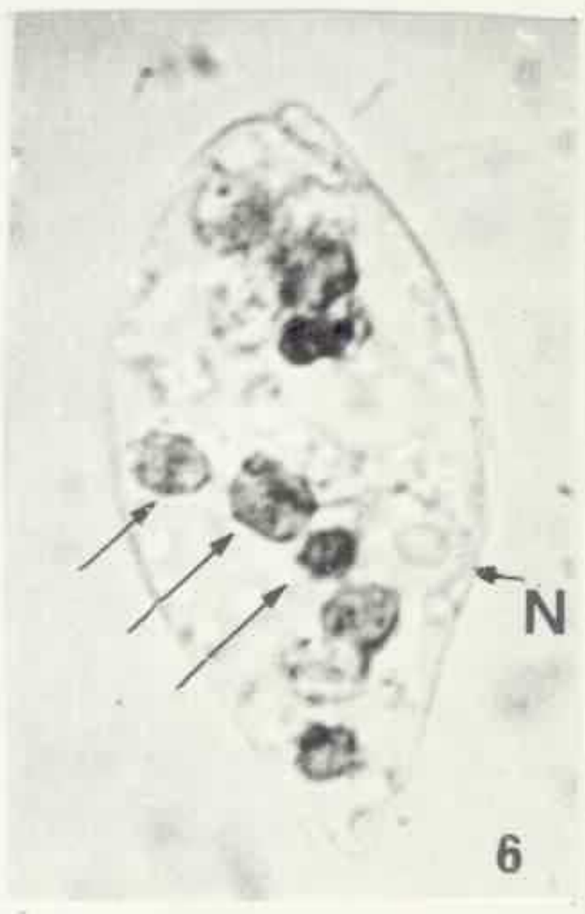
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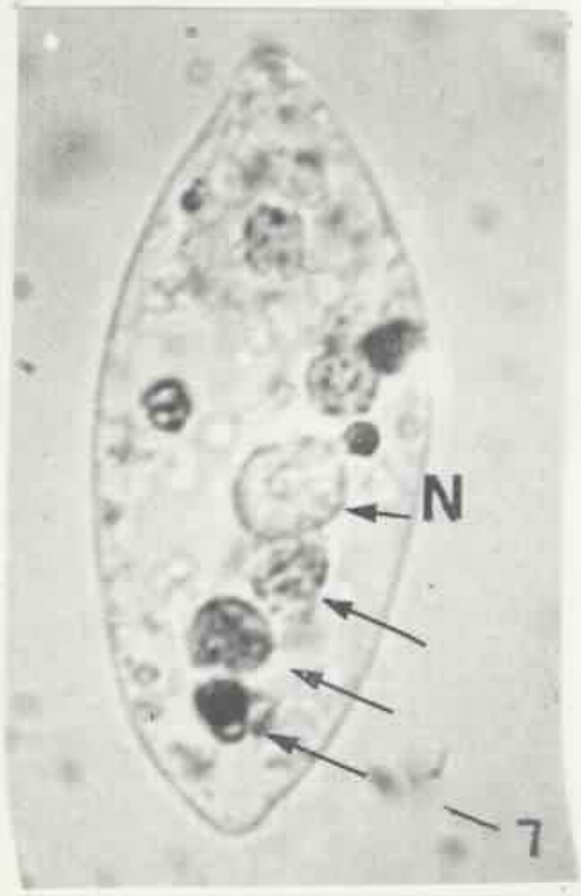
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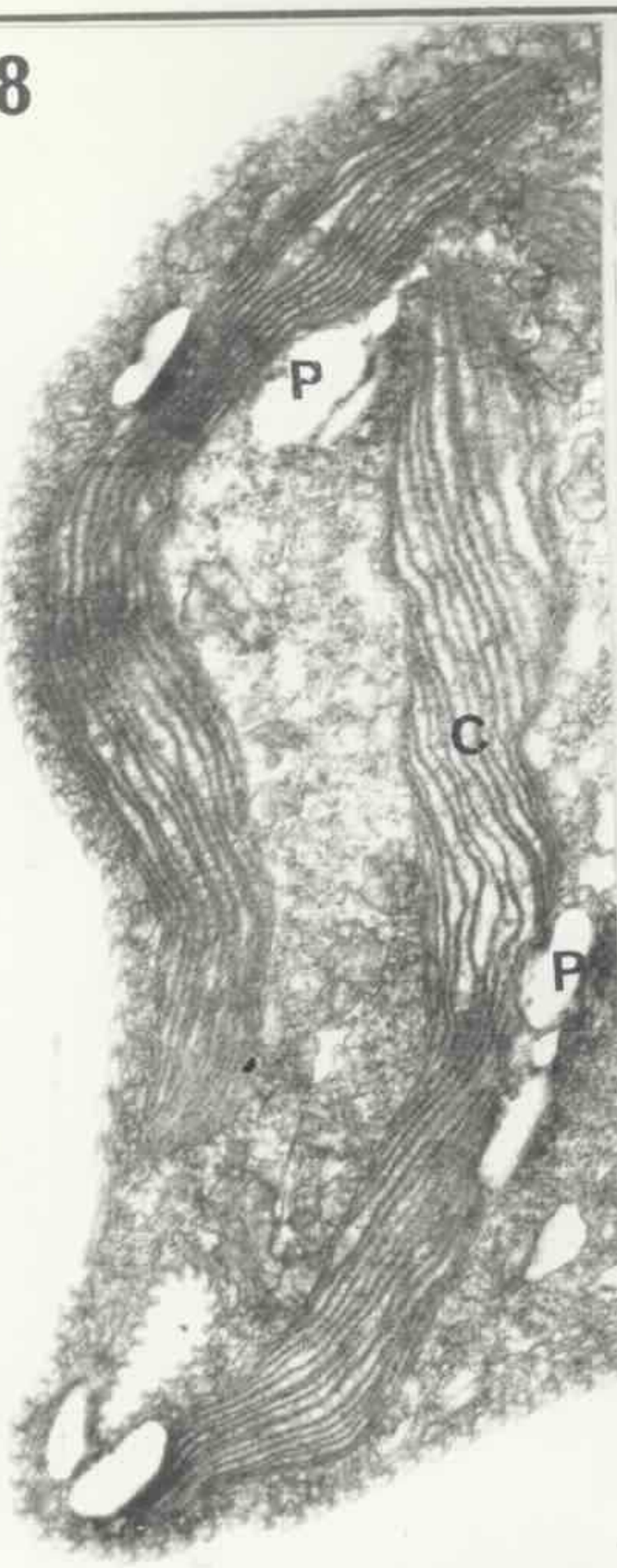


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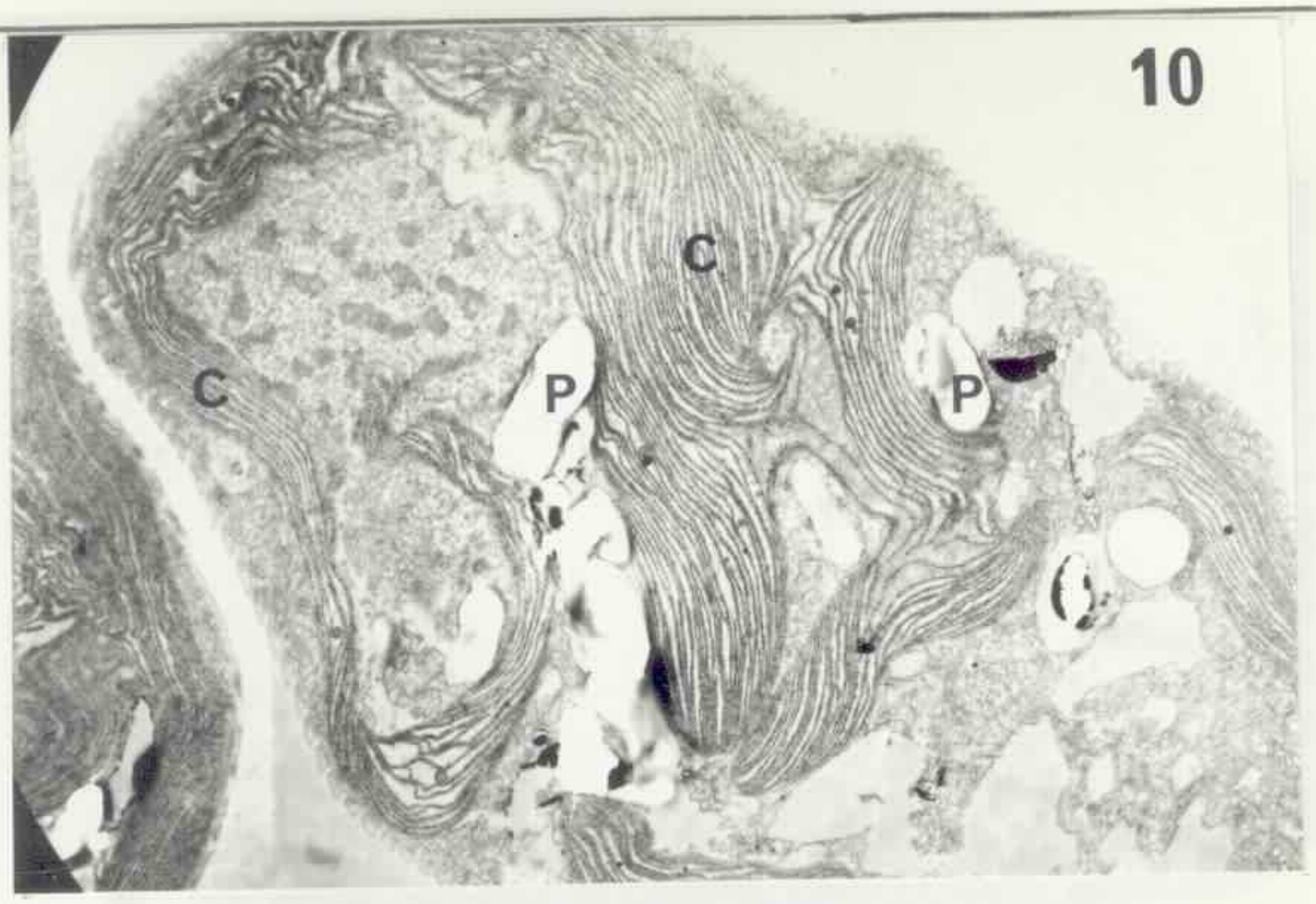
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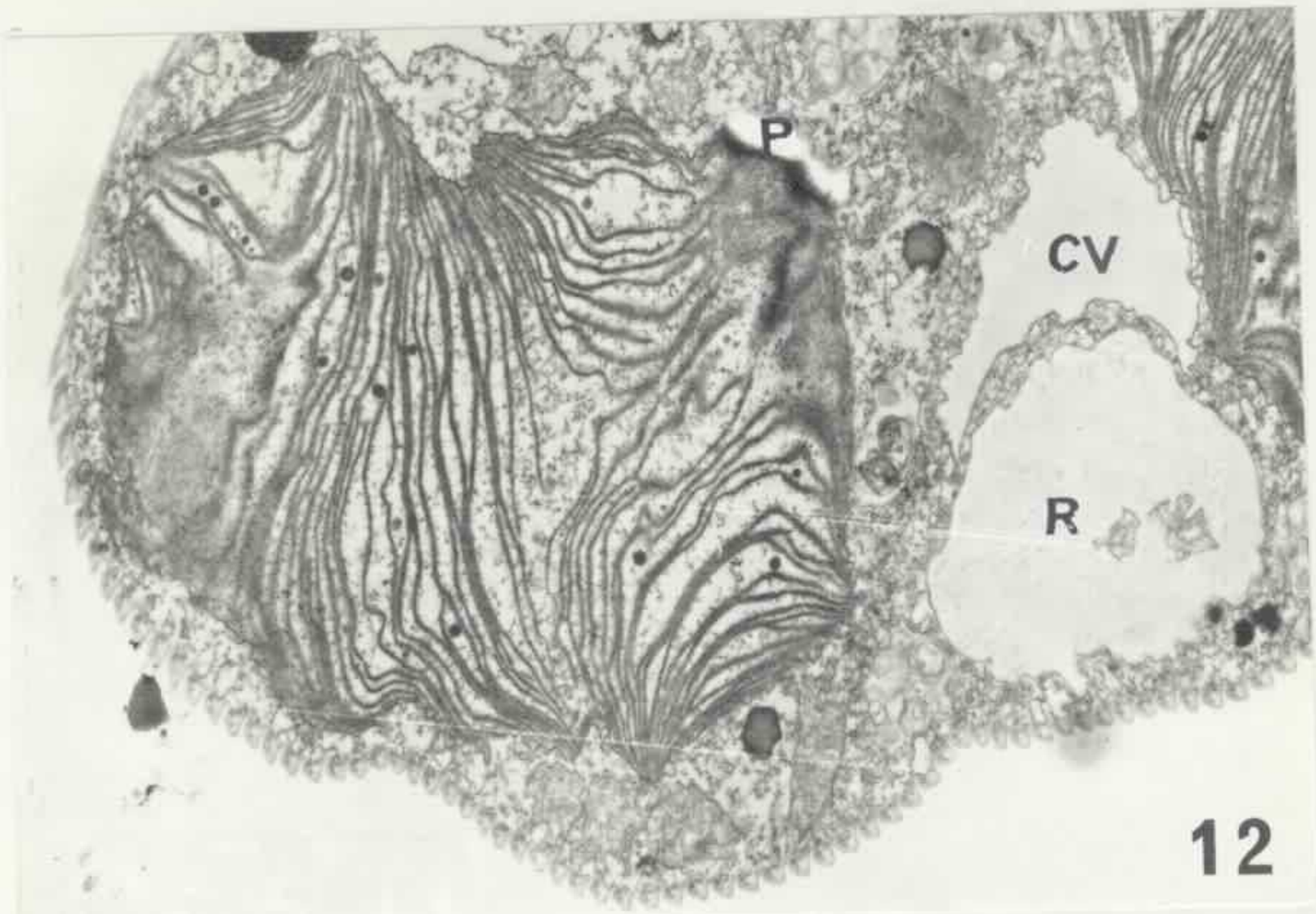
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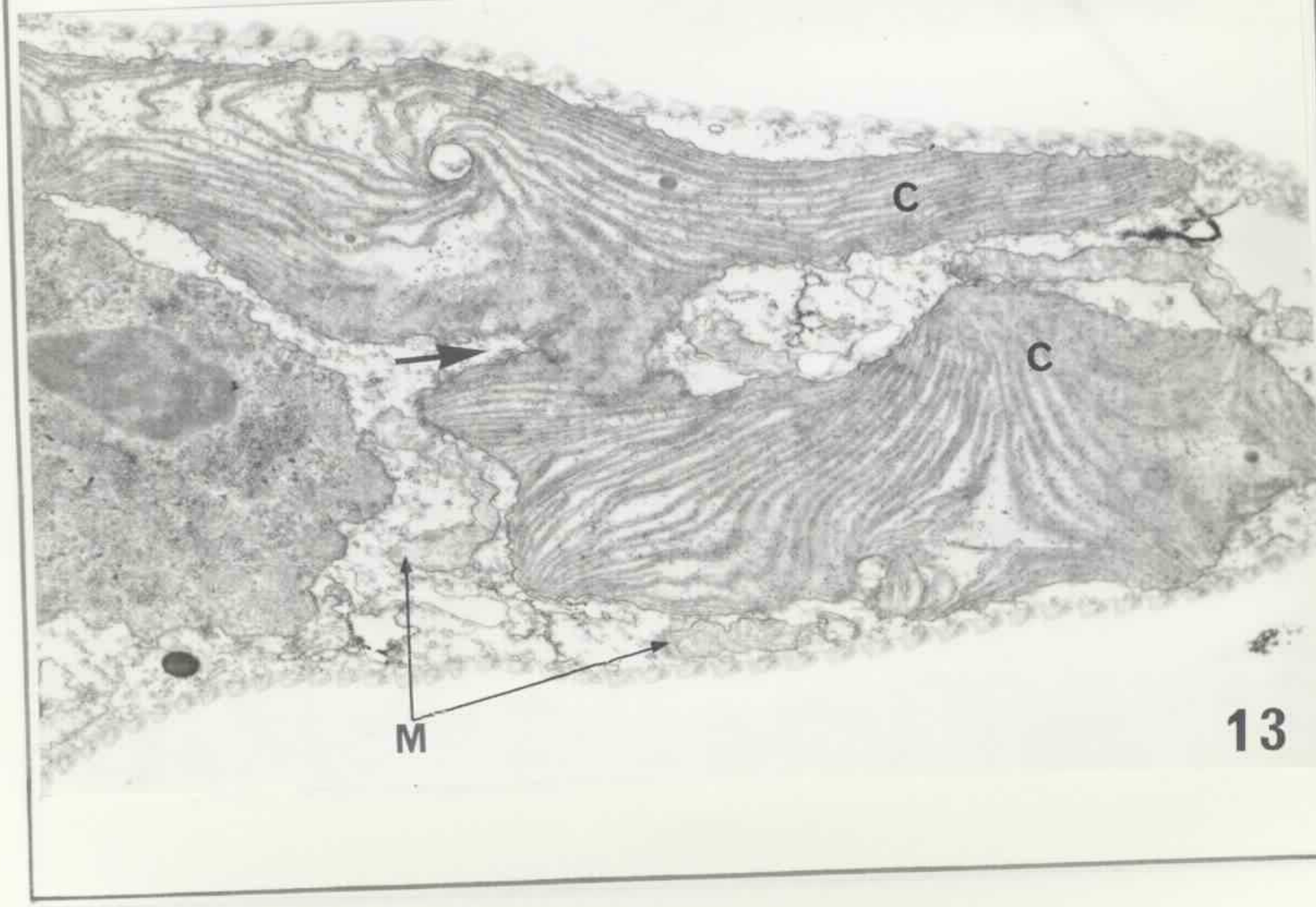
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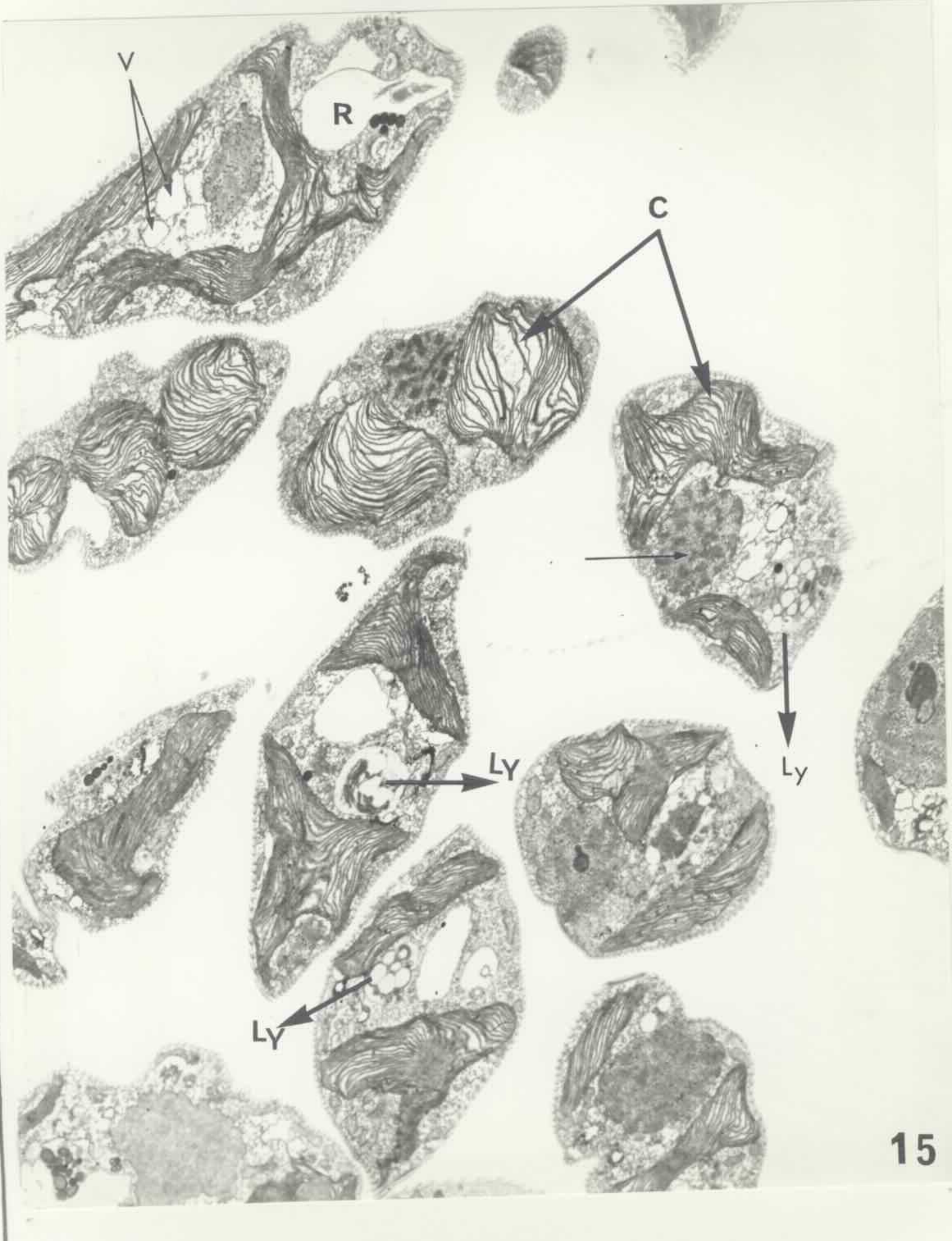


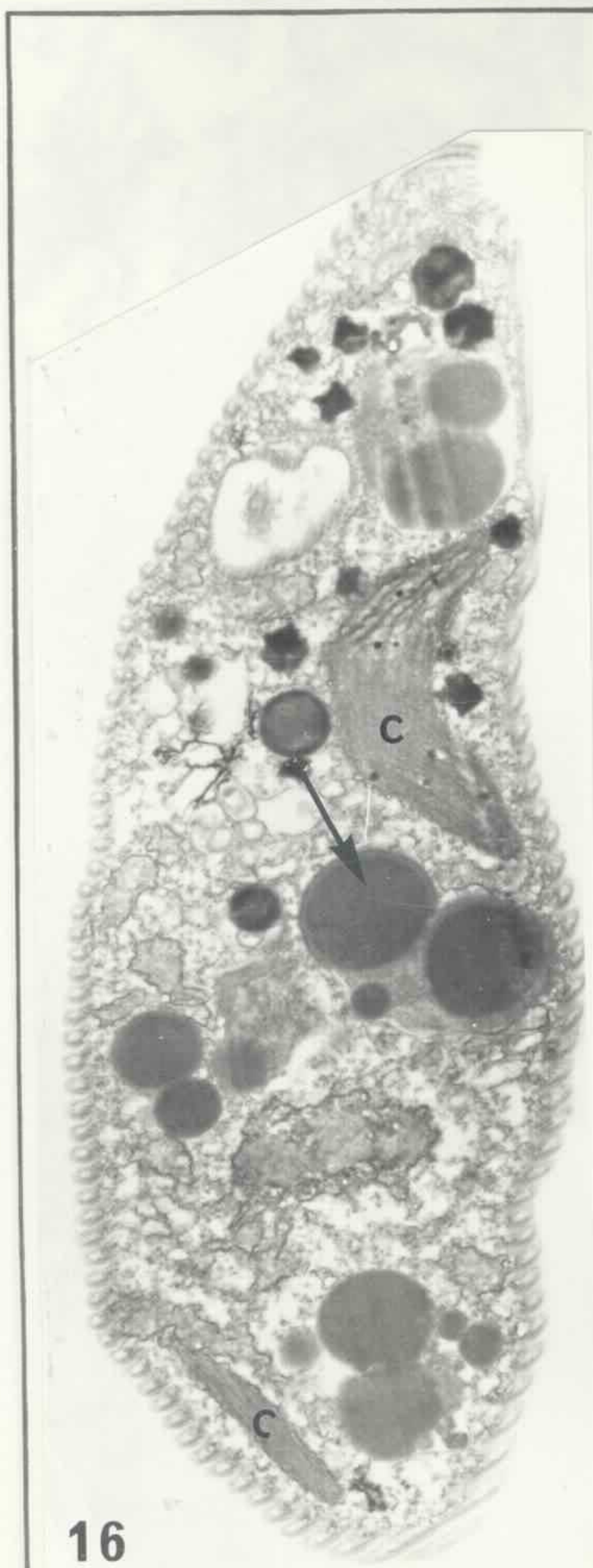
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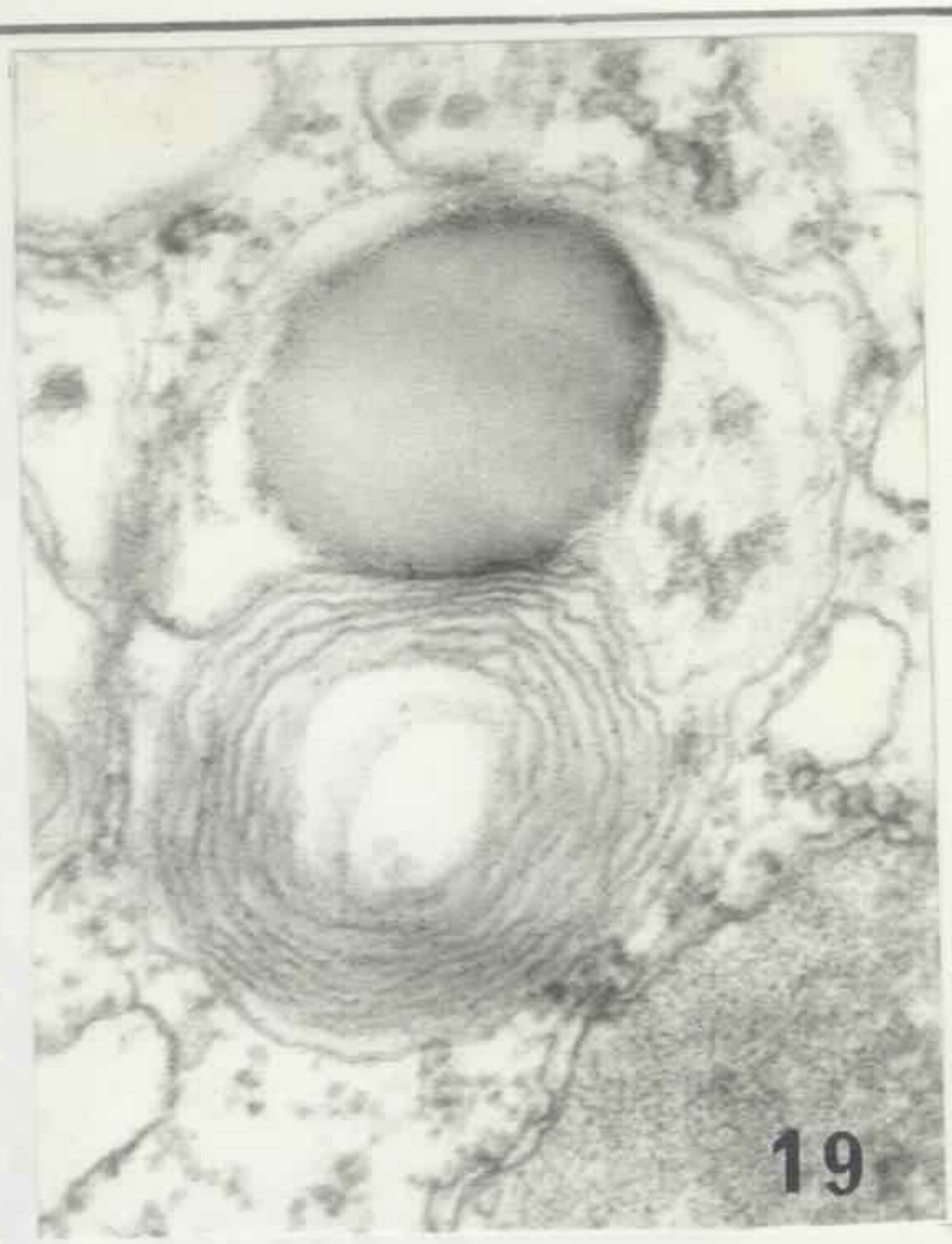


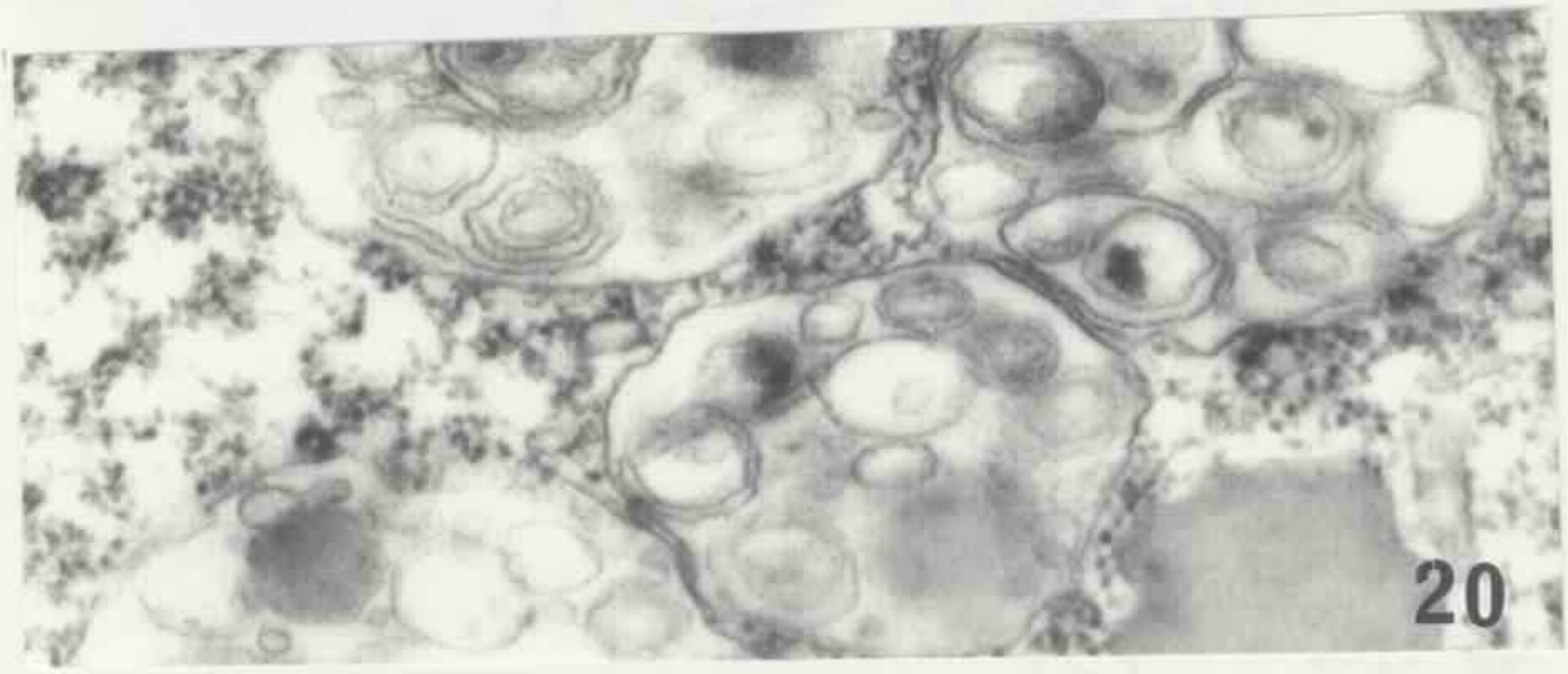
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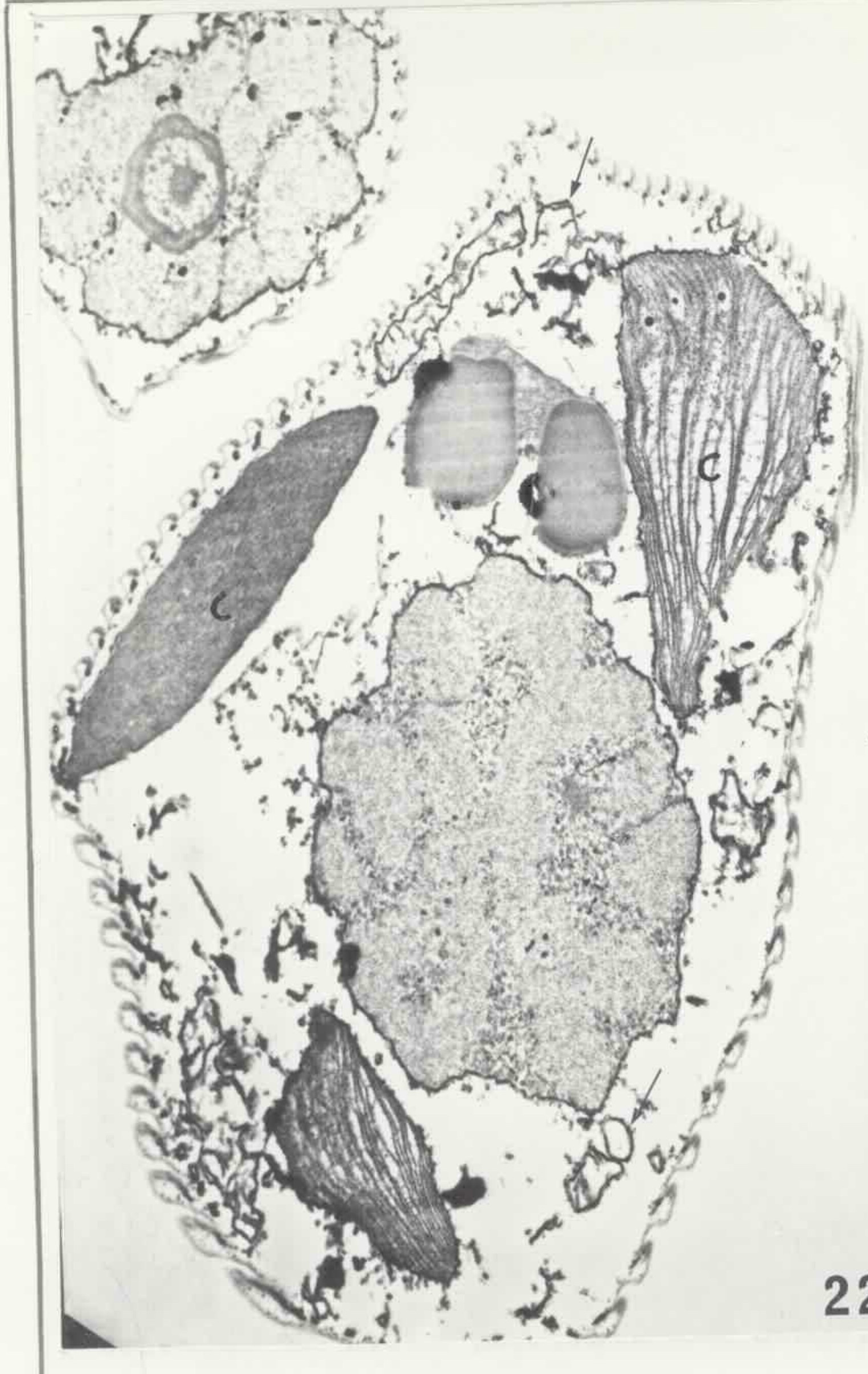
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CHAPTER 7

EFFECTS OF AMITROLE ON CHLORELLA NMI

7.1 Introduction

Amitrole (3-amino-1,2,4-triazole) is highly toxic towards many species of plants and has received considerable attention because of its effectiveness as a herbicide. Several workers, using different test organisms and different physiological and biochemical approaches, have speculated about its primary mode of action. Little positive information, however, has been offered.

Hich concentrations of amitrole strongly inhibit cell multiplication of a number of microorganisms (Weyter and Broquist, 1960; Hilton, 1960; Aaronson, 1960; Aaronson and Scher, 1960; Bond and Akers, 1961; Wolf, 1962; Castelfranco and Bisalbutra, 1965). Inhibition of cell division is the same in the light and in the dark, but concentrations which cause complete chlorosis of Ochromonas sp. do not completely block cellular division (Aaronson and Scher, 1960). The toxicity of amitrole on growth of yeast and bacteria can only be partly overcome by addition of histidine (Hilton, 1960; Weyter and Broquist, 1960; Bond and Akers, 1961). A combination of histidine and adenine is required to reverse completely amitrole inhibition

(Weyter and Broquist, 1960; Kloptowski and Bagdasarian, 1966). From these findings it has been suggested that amitrole interferes with purine synthesis. Since then most of the current research on toxic action of amitrole has been focused on purine metabolism.

For the photosynthetic organisms, Chlorella pyrenoidosa and Scenedesmus quadricauda, however, the inhibitory effect of amitrole is not reversible by the addition of histidine (Wolf, 1962; Castelfranco and Bisalputra, 1965). Furthermore, amitrole does not interfere with histidine synthesis or utilization in higher plants (McWhorter and Hilton, 1967; Brown and Carter, 1968).

McWhorter and Porter (1960) reported that amitrole interferes with carbohydrate metabolism. Frederick and Gentile (1960a) suggested a possible interference with glycolysis. From their studies with bean plants, Carter and Naylor (1961) concluded that the phytotoxicity of amitrole may be in part due to an interference in the normal metabolism of glycine and serine. An effect on nucleic acids content, as a result of interference with purine synthesis has also been reported by some workers, (Bartels and Wolf, 1965; Guerin-Dumartrait,1966).

The appearance of chlorotic leaves in new shoot growth of plants treated with amitrole has led a number of workers to investigate the mechanism of this effect. (Minton et al., 1958; Pyfrom et al., 1957; Wolf, 1960; Guerin-Dumartrait, 1960; Nguyen Thi, 1961; Guillot-Salomon and Signol, 1967).

Pyfrom et al. (1957) reported that amitrole does not affect chlorophyll content of mature leaves, but reduces chlorophyll formation in new growth, through interference with developing chloroplasts. From these results, and the findings of the other investigators, it has been suggested that chlorosis of the leaves is the result of inhibition of chlorophyll synthesis rather than the destruction of the pigments.

Wolf, (1960) and Nguyen-Thi (1961) have also noted that a reduction in chlorophyll synthesis is accompanied by a reduction in carotenoid pigment synthesis. They concluded that the effects of amitrole on chloroplast pigments is secondary to an effect upon the chloroplast itself.

Recently Guillot-Salomon et al. (1967) reported that in maize leaves amitrole inhibited the formation of the inner lamellar system in chloroplasts that were not differentiated at the time the leaves were

treated. In these studies, however, two types of chloroplasts were encountered and conclusions were reached on the effect of amitrole on chloroplasts with a predominantly Photosystem II activity.

Bartels et al. (1967) have shown that chloroplasts from plants grown in light and treated with amitrole lack fraction 1 protein and 70 S ribosomes. They also report that amitrole does not affect cytoplasmic 80 S ribosomes in either light or darkness.

In view of the various ways by which amitrole may cause inhibition of growth and death of cells and plants, and more particularly its action on photosynthetic cells, it is thought interesting to study the action of the herbicide on a unicellular alga.

Most of the previous investigations have been carried out with fairly high concentrations of amitrole ranging from 10 to 1000 ppm. As these concentrations will be rarely encountered in practical herbicide applications, low concentrations, comparable to field levels (1 to 5 ppm) have been used in the present investigation. Such concentrations would also reduce the likelihood of detecting secondary effects of the herbicide. In this study, the effects of amitrole

on ultrastructure and pigment synthesis of Chlorella NMI have been examined.

7.2 Materials and Methods

Chlorella NMI cells were harvested in logarithmic phase of growth from a synchronous culture, washed once in distilled water and resuspended in fresh medium. Inoculated medium was dispensed in Erlenmeyer flasks and treated with amitrole at the following concentrations: 0, 1 and 5 ppm. The flasks were illuminated with two 150-watts incandescent lamps, and a stream of air enriched to 5% CO_2 was flushed through the cultures during the experimental period. The temperature of incubation was 26 $\pm$ 1^{o}C. Samples were removed at intervals, (a) for pigment estimations, (b) cell count and (c) electron microscopy, as in Section 2.

7.3 Results

Cell division

Data taken after 45 hours showed that amitrole (1 ppm) did not affect the algal cells. After 100 hours, however, a slight reduction in cell number was noted. A concentration of 5 ppm of amitrole markedly reduced rate of cell division, and although inhibition

was apparently relieved, fewer cells with regard to the untreated culture were found in the treated culture after 100 hours. (Table 8).

Photosynthetic pigments. The action of the herbicide on synthesis of chlorophyll and carotenoids of the alga was also studied by determination of pigment concentrations after 45 and 100 hours. Table summarises the results. The amount of chlorophyll produced in 45 hours by the treated culture was reduced by nearly 29% at 1 ppm and 43% at 5 ppm with regard to the control. Thus after 45 hours, there was an excess of 0.82 mg of total chlorophyll in 1 ml of the control culture and only an excess of 0.58 mg/ml and 0.47 mg/ml in cultures at 1 ppm and 5 ppm respectively, with regard to the inoculum. The rate of carotenoid synthesis was decreased by 42% at 1 ppm and 45% at 5 ppm of amitrole (Table 9). After 100 hours, the inhibitory effects of amitrole on chlorophyll production were completely lifted. It was noted that no shifts in Chl a: Chl b ratio occurred with 1 ppm amitrole. However, after 45 hours, 5 ppm amitrole caused an increase in the chlorophyll a/chlorophyll b ratio, indicating a preferential reduction of chlorophyll b.

Table 8. Effects of amitrole on cell division and photosynthetic pigments in Chlorella NMI

Amitrole conc. (ppm)	Time (hours)	No.of cells/ ml.of culture ($x10^6$)	Total Chl./ ml.of culture ($x10^{-4}$g)	Ratio Chl.a / Chl.b
0	0	1.52	3.5	2.68
	45	10.80	11.7	1.95
	100	26.10	11.3	2.18
1	0	1.52	3.5	2.68
	45	10.10	9.3	1.94
	100	20.70	11.5	2.16
5	0	1.52	3.5	2.68
	45	6.43	8.2	2.15
	100	16.50	11.1	2.19

Table 9 Effects of amitrole on chlorophyll and carotenoid pigments after 45 hour incubation

Amitrole conc. (ppm)	Percent reduction of chlorophyll synthesis	Percent reduction of carotenoid synthesis
0	0	0
1	29	42
5	43	45

Light Microscopy

Light microscope examination of the 5 ppm amitrole treated cultures after 45 hours showed the presence of a few enlarged cells, mostly in the division stage. It was observed that in these dividing cells, the daughter cells increased in volume within the mother cells. They probably represent a stage where the mother cell had produced daughter cells which, however, were not liberated but remained grouped together.

Electron microscopy

Plate 7.1 represents a section of a cell from the untreated culture after 45 hours. The basic structures were similar to those of a normal cell as described in Section 4.1. In general, cells treated with 1 ppm amitrole were apparently unaffected. The internal lamellar system of the chloroplast contained well oriented bands of thylakoids. The pyrenoid was surrounded by a sheath of starch. However, some cells had a number of small lipid bodies in the chloroplast, vacuoles, membranes, and membrane bound bodies with a homogeneously fine granular matrix, in the cytoplasm (Plates 7.2 and 7.3). These structures were rarely found in normal Chlorella NMI cells. Cell division appeared to be insensitive to this amitrole concentration. (Plate 7.4).

In contrast, at 5 ppm amitrole, the total number of thylakoids per chloroplast was markedly reduced, and the organization of the chloroplast lamellae was severely disrupted. Vacuoles were present in the cytoplasm. The mitochondria were located around the periphery of the cells, an in normal untreated young cells (Plate 4.1.2). Although the integrity of the mitochondria appeared unaffected, there was a reduction in the number of cristae. (Plates 7.5,6 and 7). These micrographs also showed a marked reduction in chloroplastic ribosomes, while cytoplasmic ribosomes could still be found in large numbers.

DNA fibrils were clearly distinguished within the chloroplast. Plate 7.7 showed the presence of small vesicles and multi-vesicular bodies, which might represent small cytolosomes, as described in Euglena cells after treatment with diuron (Section 6). To supplement these observations, cells from the culture were pre-fixed with glutaraldehyde followed by potassium permanganate. Plate 7.10 confirmed the marked changes in organization of chloroplast lamellae, and the reduction in number of mitochondrial cristae observed in the glutaraldehyde - osmium fixed cells. Plates 7. 8, 9 and 10 also showed the marked reduction in thylakoids, hypertrophy of the Golgi body, vacuole in the cytoplasm, and the presence of starch around the pyrenoid. It was further observed that the process of cell division seemed to be affected by treatment with 5 ppm amitrole (Plate 7.11,). The reduction in cell number (Table 8) could be partly due to a reduced number of autospores.

After 100 hours, the fine structure of the 1 ppm amitrole treated cell had assumed a normal appearance. This is in agreement with the cell number and pigment estimation, which indicated complete recovery of the cells from the herbicide toxicity (Table 8). Although most of the cells in the 5 ppm amitrole - treated cultures appeared unaffected, a few cells were seen showing severe disorganization of internal structures. (Plate 7.12).

7.4 Discussion

The results obtained by cell number and pigment estimations of Chlorella NMI treated with low concentrations of amitrole, and examination of the ultrastuctural changes induced by the herbicide at these concentrations suggest certain conclusions.

At a concentration of 1 ppm amitrole, cell division (Table 8) mitochondria and photosynthetic apparatus of Chlorella NMI do not appear to be affected after 45 hours. Pigment estimation, however, shows a decrease in chlorophyll and carotenoid, indicating that among the cellular components, chloroplast pigments appear to be the first affected by amitrole. Severe disorganization and reduction in the number of thylakoids of the lamellar system only occur in the presence of a higher amitrole concentration (5 ppm), suggesting that the effect of amitrole on chloroplast structure is probably secondary to the effect on the pigments.

From studies on the effects of amitrole in plants and algae in recent years, different conclusions have been reached on the identification of the primary target of amitrole toxicity. Wolf (1960) found that amitrole had a similar influence on the levels of both carotenoids and chlorophylls in wheat leaves and concluded that the effects of amitrole on chloroplast pigments were secondary to an effect upon the chloroplast. A similar conclusion was reached by Nguyen-Thi (1961) with rice seedlings.

Guerin-Dumartrait, using high concentrations of amitrole (25 to 1000 ppm), concluded that in Chlorella NMI inhibition of RNA was the primary effect of amitrole. Burns et al (1971) proposed that in wheat the primary effect of amitrole was the inhibition of carotenoid synthesis.

In the present study, where low concentrations of amitrole have been used to minimise the likelihood of secondary effects, the data show that at 1 ppm, amitrole acts rapidly on the synthesis of carotenoids (42% reduction) and slowly on chlorophyll synthesis (29% reduction); but at 5 ppm, the rate of inhibition of chlorophyll and carotenoid production is nearly the same. These results indicate that carotenoid synthesis appears to be the primary target of amitrole toxicity. This is in agreement with the conclusion reached by Burns et al (1971).

The reduction of carotenoids at low concentrations of amitrole renders the chlorophyll pigments susceptible to photooxidation, as it is known that carotenoids protect chlorophyl from photodestruction (Krinsky, 1967). The secondary product of chlorophyll destruction by light may act on the chloroplast DNA and destroy or alter its genetic properties. This assumption is supported by the work of Leff and Krinsky (1967), who reported that the photooxidised chlorophyll molecule may function as a photosensitiser which reacts with chloroplast DNA, resulting in a genetic alteration. The close proximity of

chlorophyll molecules, which are confined in the lamellae, and the DNA fibrils which are attached to the lamellar membranes (Section 4.1) may account for the rapid effect of photooxidised chlorophyll on the DNA molecules.

Any genetic alteration in chloroplast DNA would affect production of ribosomal RNA, as biochemical evidence strongly suggests that the chloroplast DNA may code for the formation of ribosomal RNA (Brawerman, 1966; Kirk, 1966; Aoki and Hase, 1967; Scott and Smillie, 1968). Inhibition of formation of chloroplast ribosomes would block the synthesis of chloroplast enzymes, and possibly lead to inhibition of chloroplast proteins, involved in membrane structure, resulting in disruption of the lamellar system. The above suggestion is strongly supported by the ultrastructural evidence which shows that, although the chloroplast lamellar system is severely disorganised and reduced in size, DNA fibrils can still be observed, and while the concentration of chloroplastic ribosomes is considerably reduced, cytoplasmic ribosomes can still be found in large numbers (Plates 7.5, 6 and 7).

This is in accord with the finding that amitrole inhibits the synthesis of chloroplast ribosomal RNA but does not affect cytoplasmic 80 s ribosomes in plants. (Bartels et al, 1967).

The presence of starch around the pyrenoid in cells treated with 5 ppm amitrole (Plate 7.8) may indicate an inhibition of catabolic enzymes, possibly due to interference with formation of chloroplast ribosomes by amitrole.

This suggestion gains support from the work of McWhorter and Porter (1960) who found that amitrole interferes with carbohydrate metabolism.

The reduction in number of cristae in the mitochondria may indicate an inhibition by amitrole of proteins essential for the formation of functional cristae, resulting in an inhibition of mitochondrial adenosine triphosphate (ATP) production. This would partly be responsible for the decrease in growth rate of Chlorella cells observed in the presence of 5 ppm amitrole (Table 8).

7.5 Summary

1. A concentration of 1 ppm amitrole has no affect on cell division, but causes 42% reduction of carotenoid and only 29% reduction of chlorophyll after 45 hours, indicating that synthesis is the primary target of amitrole toxicity.

2. 5 ppm amitrole reduces number of thylakoids in the chloroplast, and causes severe disruption of lamellae organisation after 45 hours.

3. The results suggest a secondary effect on chloroplast DNA by photo-oxidised chlorophyll, with subsequent inhibition of chloroplast proteins.

PLATE 7

Key to labeling

G - Golgi body

N - nucleus

L - chloroplast lamellae

r - chloroplast ribosomes

R - cytoplasmic ribosomes

S - starch

lg - lipid globules

P - pyrenoid

F - division furrow

C - chloroplast

CW - cell wall

CM - cytoplasmic membrane

Plate 7.1 — 45 - hour light control cell. This micrograph illustrates the normal organisation of chloroplast thylakoids in bands of 2 - 6. Concentration of chloroplast (r) and cytoplasmic ribosomes (R) is high. Note small mitochondria (M) at the periphery of the cell.
Glut - OsO_4. x 48,000

Plate 7.2 — Chlorella NMI after treatment with 1 ppm amitrole for 45 hours. Chloroplast contains 'grana - like' stacks of thylakoids as in control cell. (Plate 7.1) Note bodies (Y) in cytoplasm and small lipid globules (lg) in the chloroplast.
Glut - OsO_4. x 60,000

PLATE 7

Plate 7.3 — Chlorella NMI after treatment with 1 ppm amitrole for 45 hours. Vacuole (V) in the cytoplasm. Arrows indicate membranes. Glut - OsO_4. x 60,000

Plate 7.4 — 45 hr-light, amitrole (1 ppm) - treated chlorella cell. Photomicrograph represents a cell after the first complete cytoplasmic division. Note Golgi bodies (G) have completed second division. Glut - OsO_4. x 60,000

Plates 7.5, 7.6 and 7.7 — Chlorella NMI after treatment with 5 ppm Amitrole for 45 hours. Few thylakoids are present. Concentration of ribosomes in the chloroplast is considerably lower than in the chloroplast of the control cell (Plate 7.1) Note presence of DNA fibrils (ℱ). Arrows indicate small vesicles and multivesicular bodies in the cytoplasm. Note reduction of number of cristae in mitochondria. Glut - OsO_4.

Plate 7.5 - x 48,000
Plate 7.6 - x 30,000
Plate 7.7 - x 30,000

Plate 7.8 — Micrograph of a field of chlorella NMI cells treated with 5 ppm amitrole. Note disorganisation of chloroplast lamellar system, presence of vacuole (V) in the cytoplasm, and starch sheath around pyrenoid. Glut - $KMnO_4$. x 19,980

PLATE 7

Plates 7.9 and 7.10

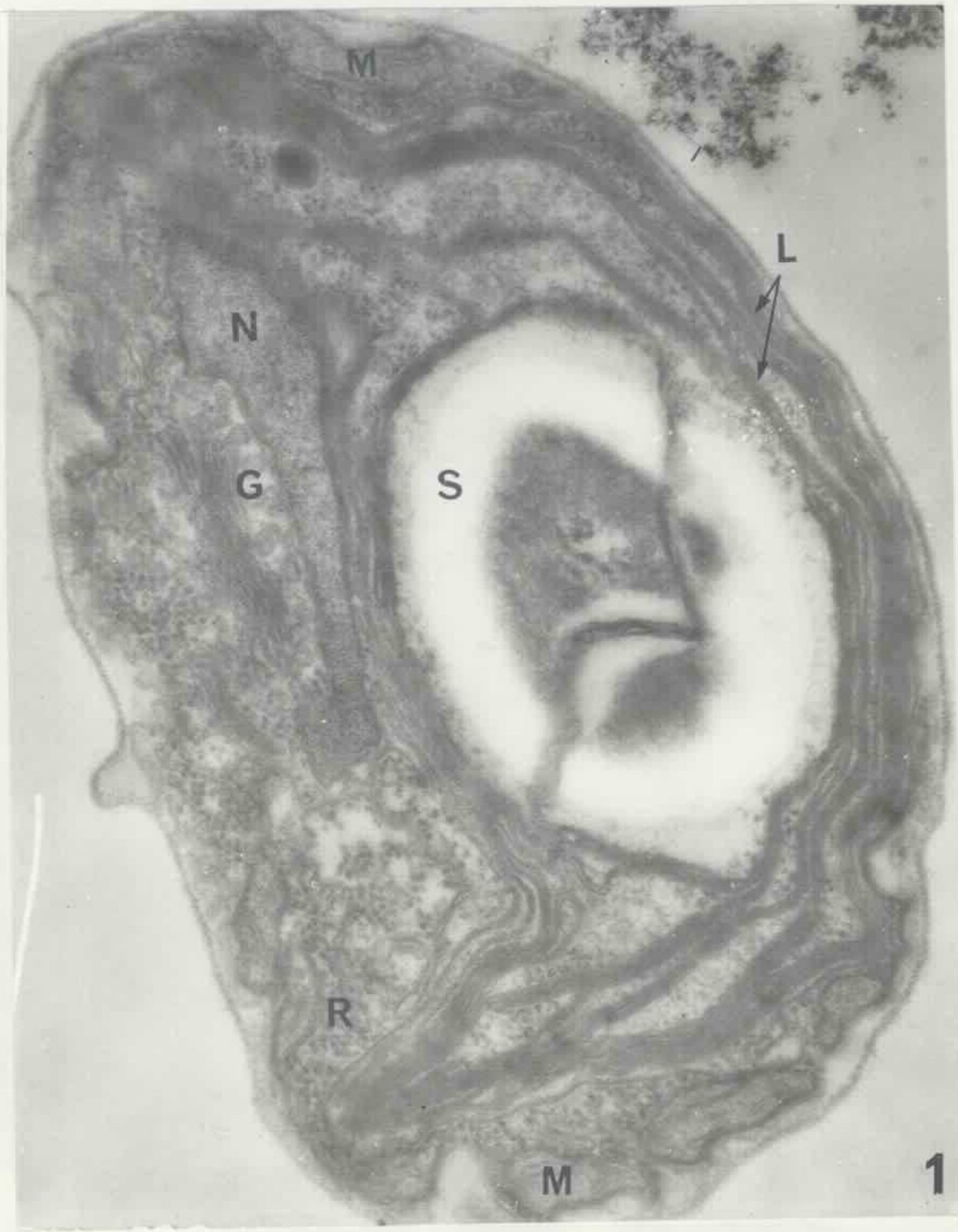
Sections show effects of 5 ppm amitrole on chloroplast lamellae after 45 hours. There is a high reduction in number of thylakoids, and severe disruption of lamellae orientation.
Note hypertrophied Golgi body (G) and mitochondria without cristae around the periphery of cell.
Glut - $KMnO_4$. x 37,050

Plates 7.11 and 7.12

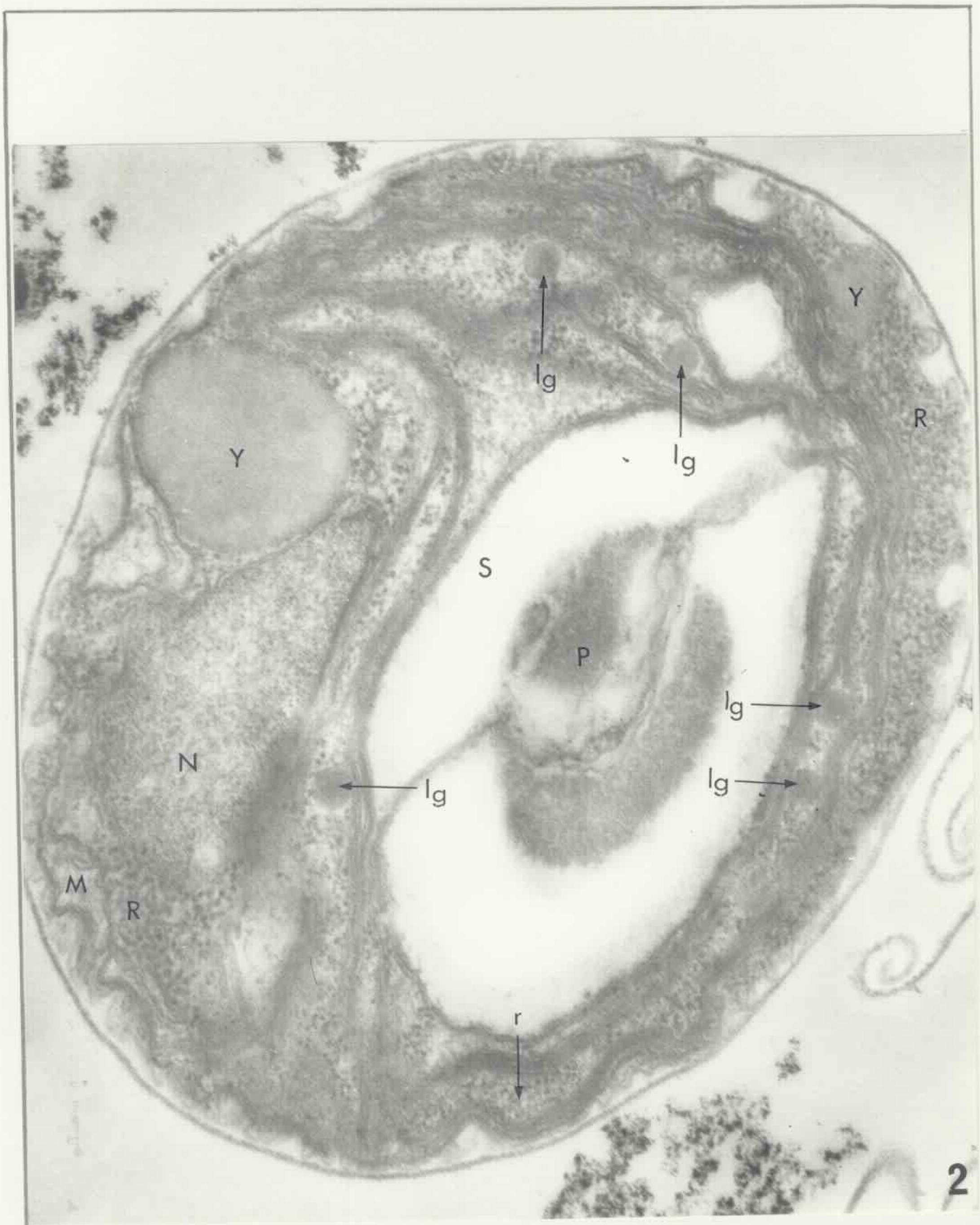
Micrographs of 5 ppm amitrole - treated cells after 45 hours. Division furrow appears abnormal and Golgi body is hypertrophied. Note large accumulation of starch and lipid bodies.
Daughter cells severely affected.
Note presence of large vacuole in cytoplasm.
Glut - OsO_4. x 30,000

Plate 7.13

Chlorella NMI, 100 hours after treatment with 5 ppm amitrole. Only a few vesicles present in chloroplast. Large vacuole in cytoplasm. Nuclear membrane disrupted at some points (arrows).
Matrix of mitochondria is almost clear.
Glut - $KMnO_4$. x 60,000



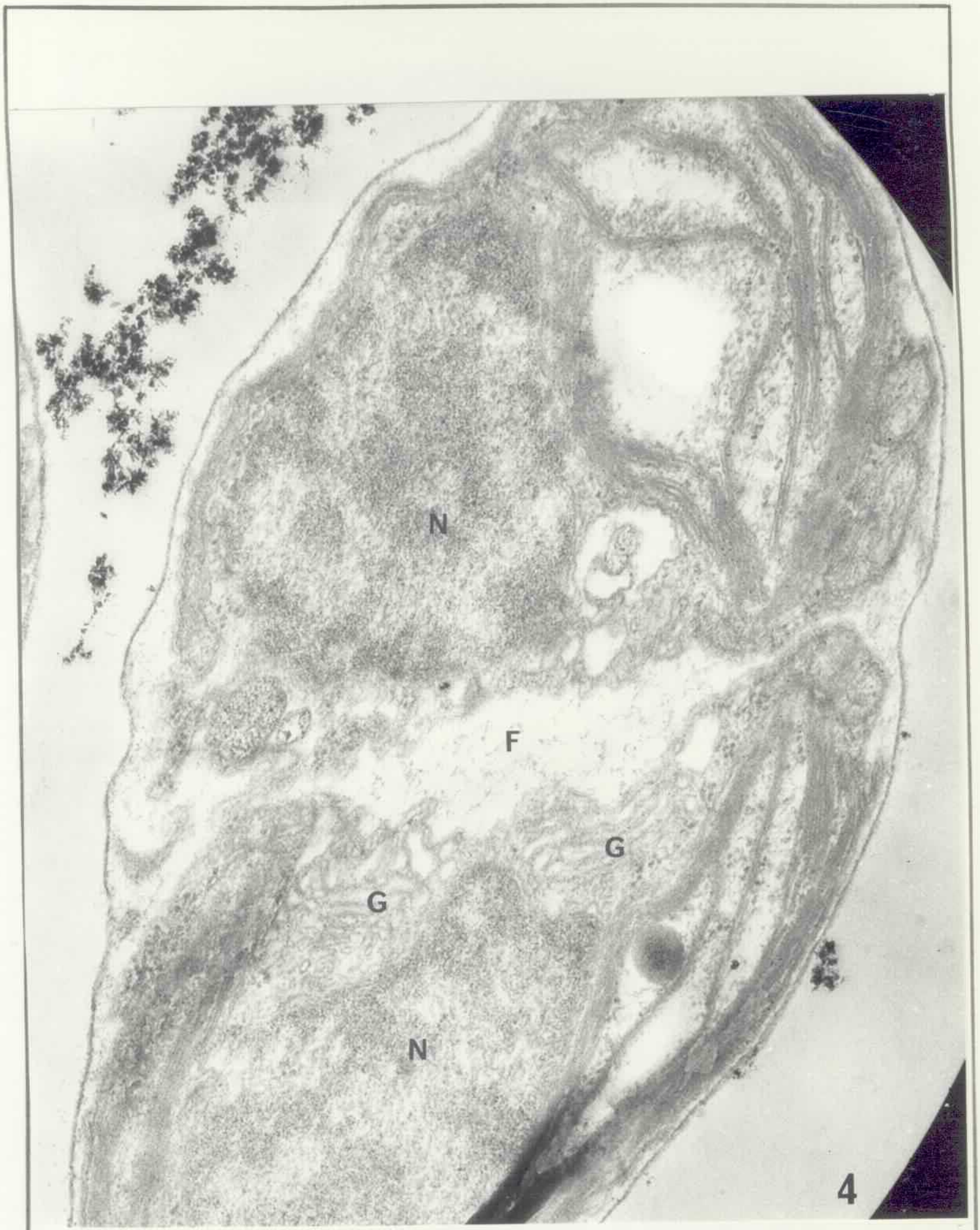
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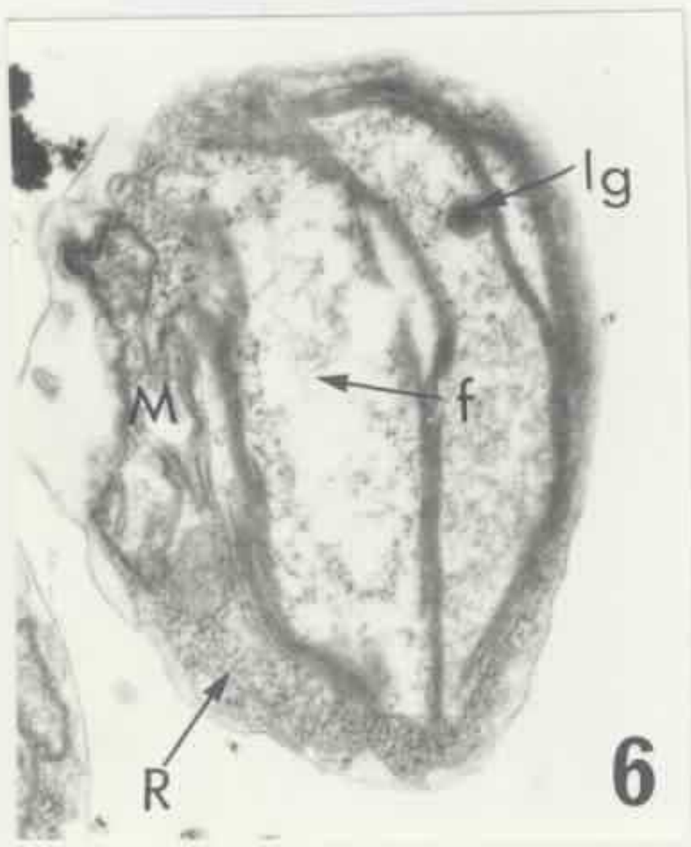


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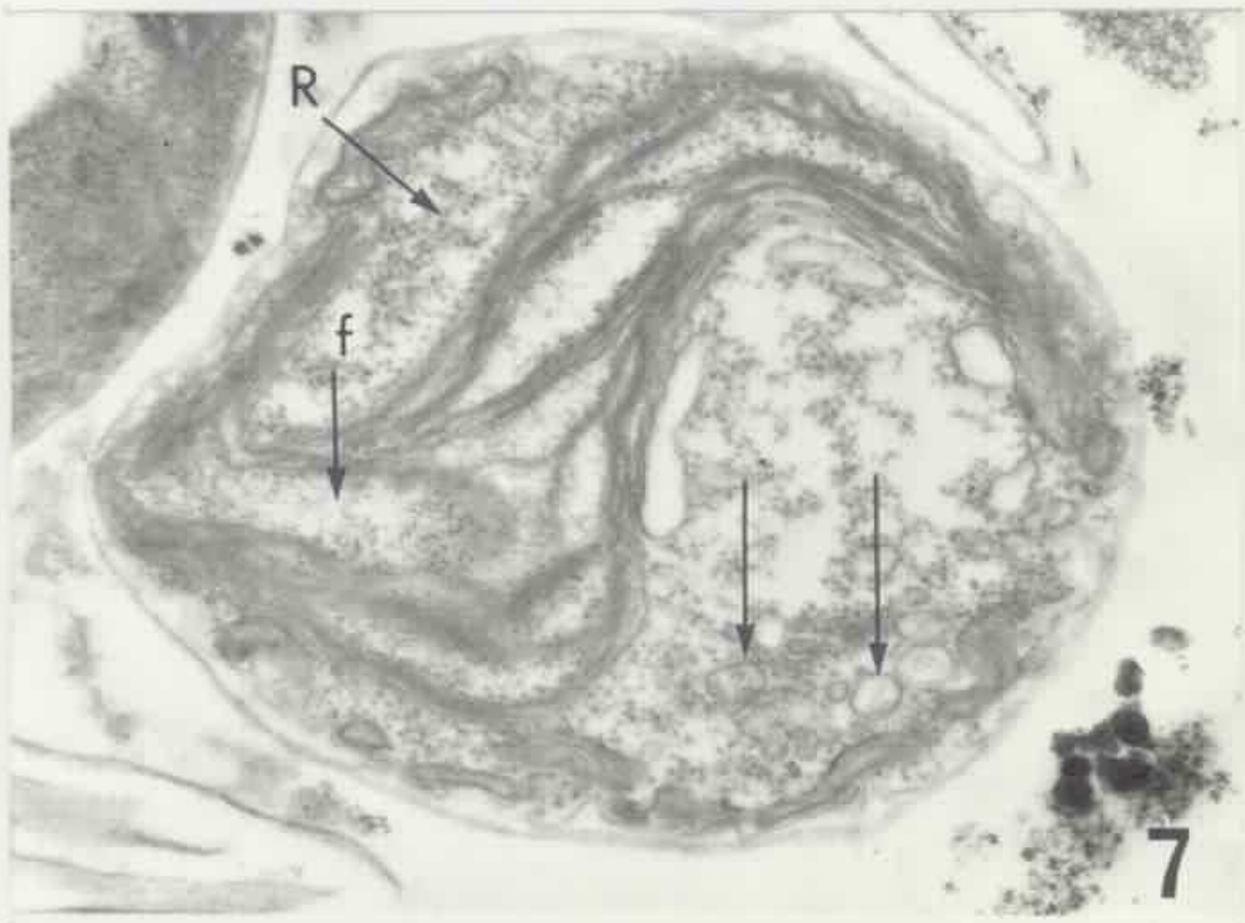

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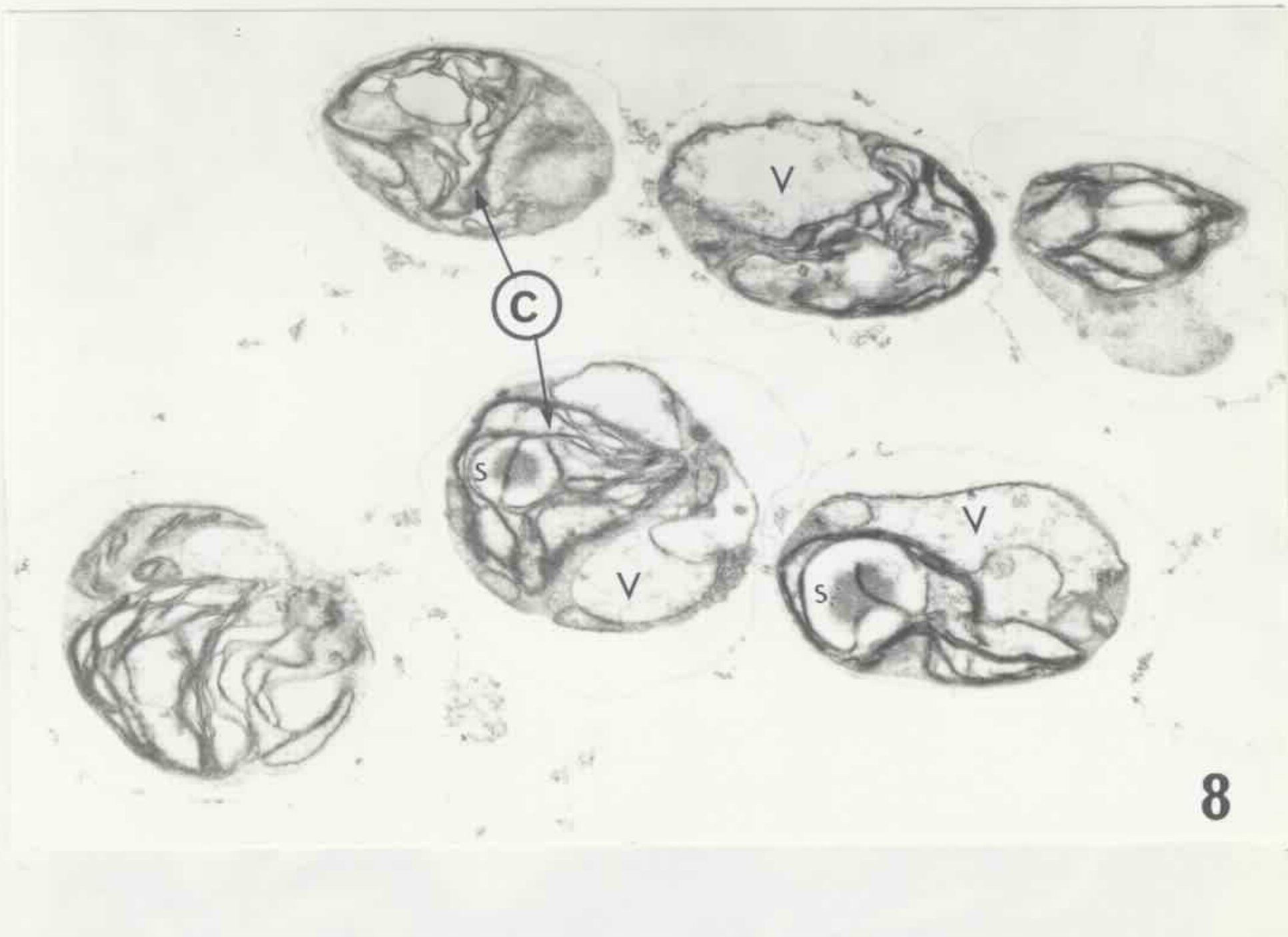
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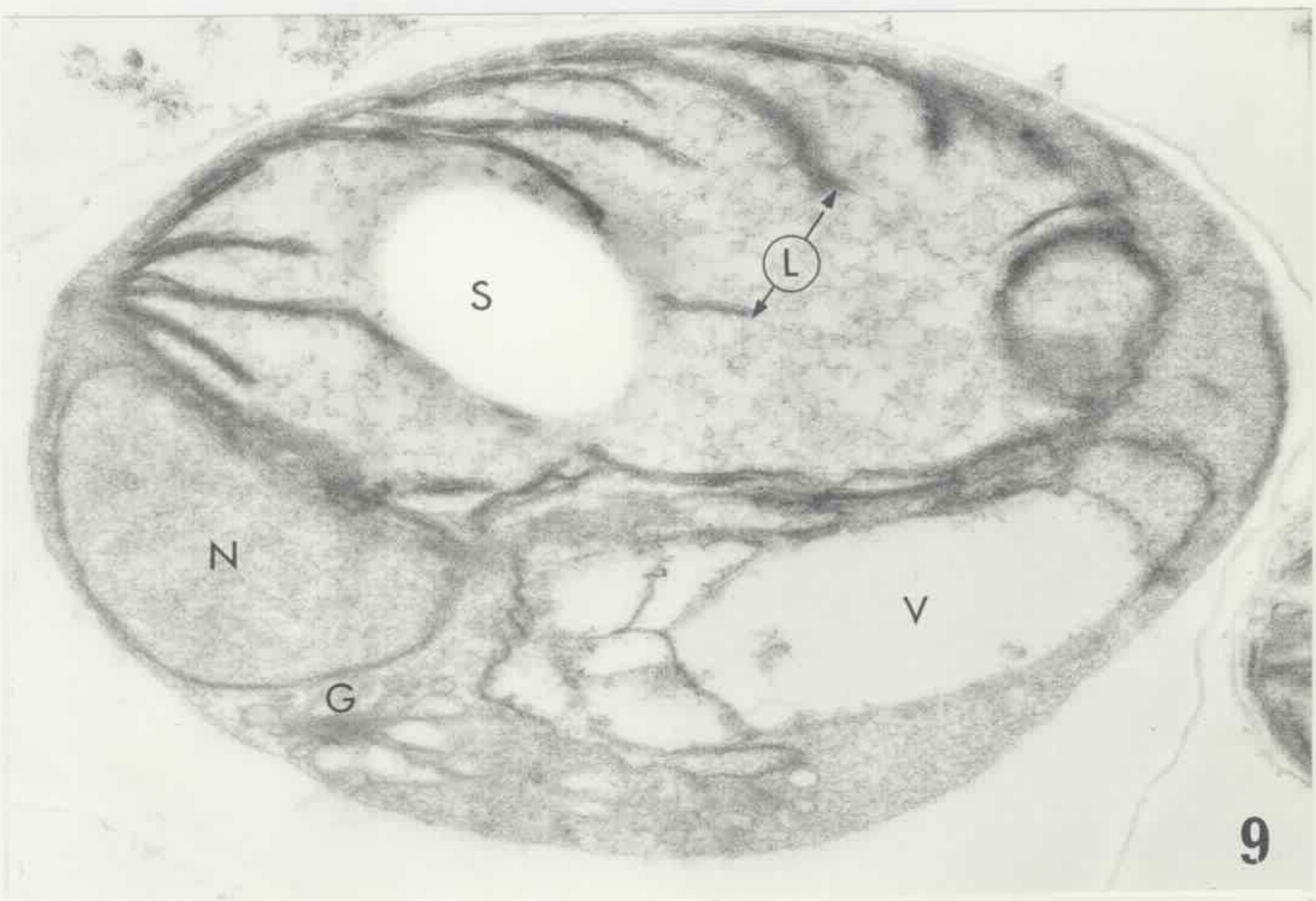
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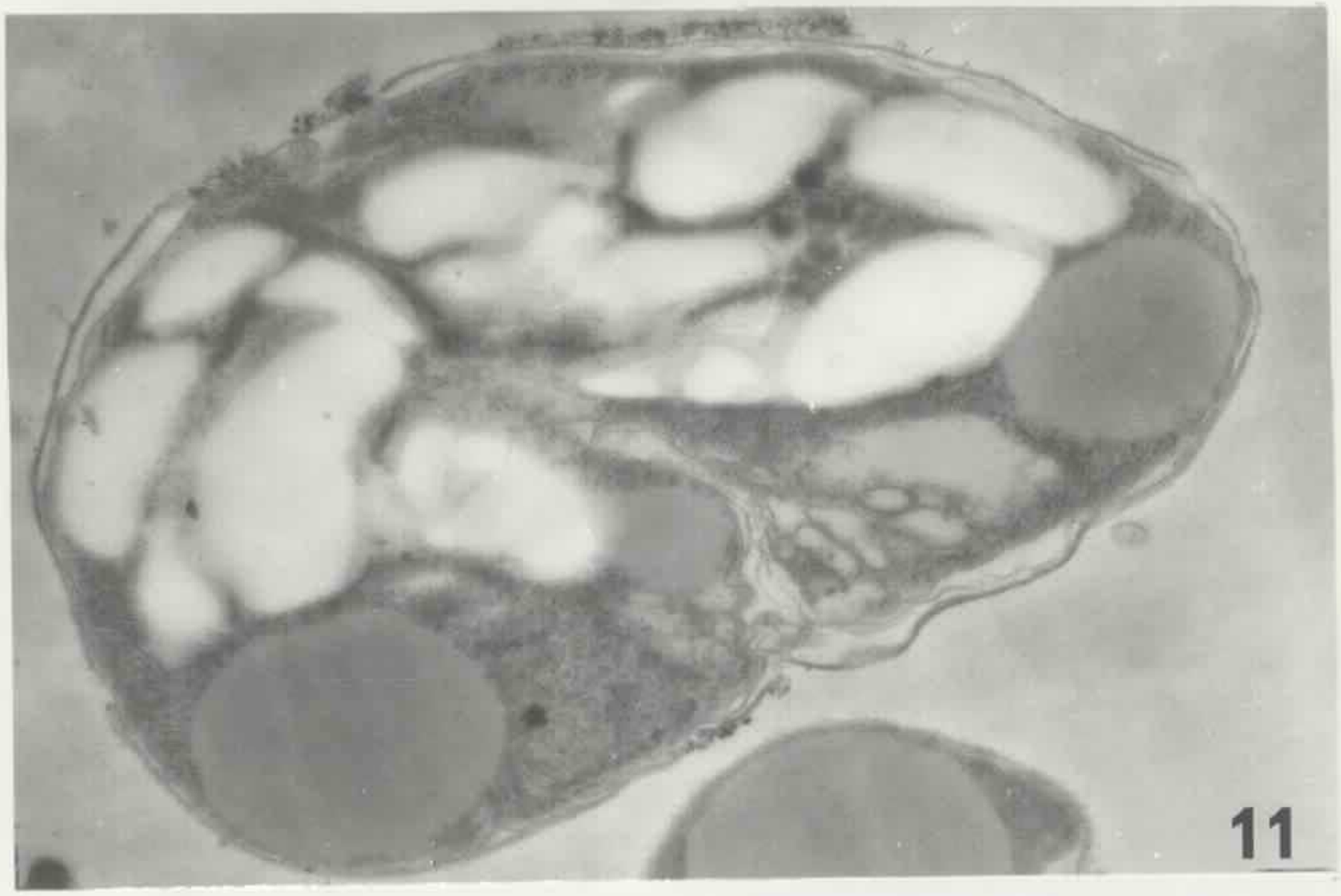


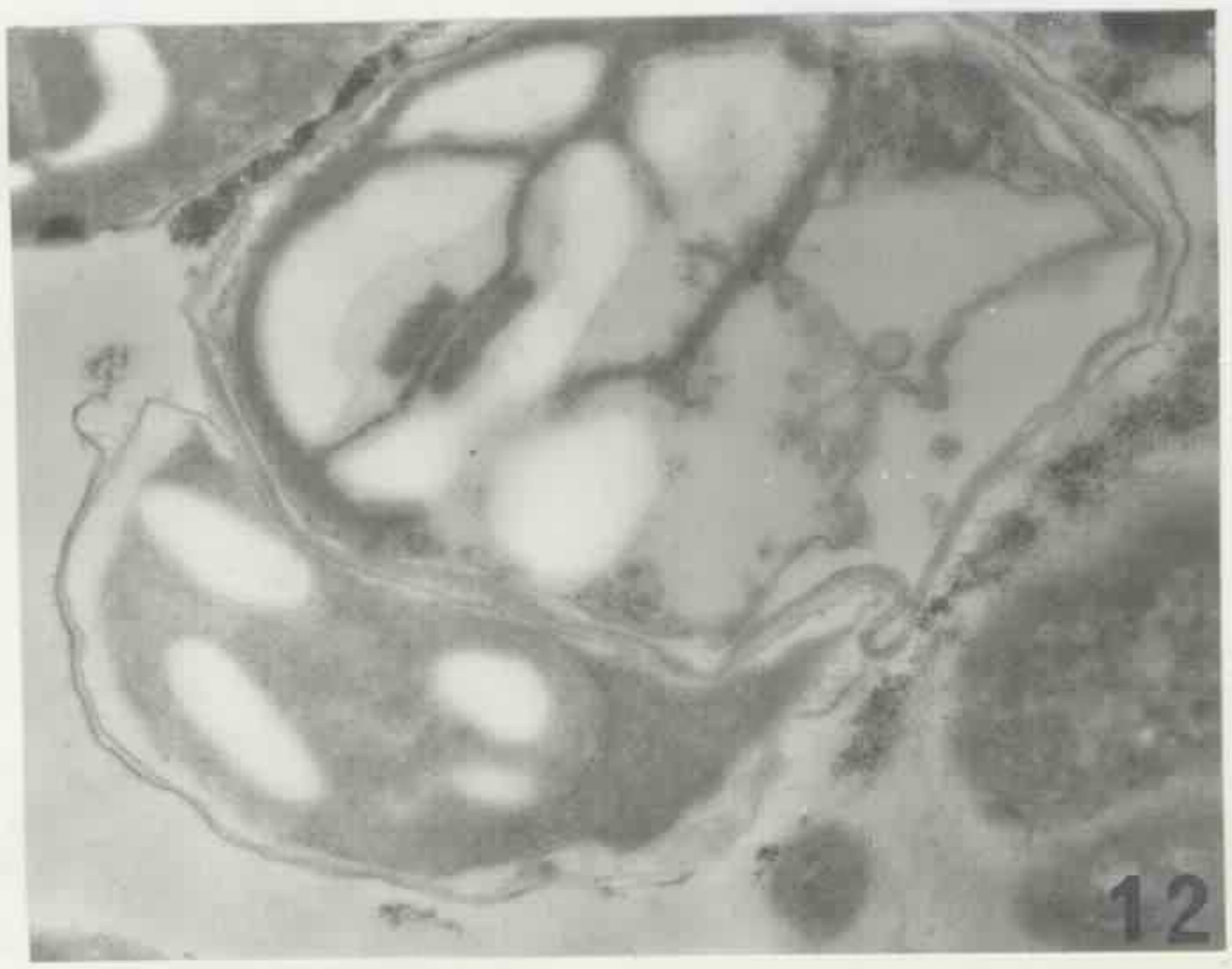
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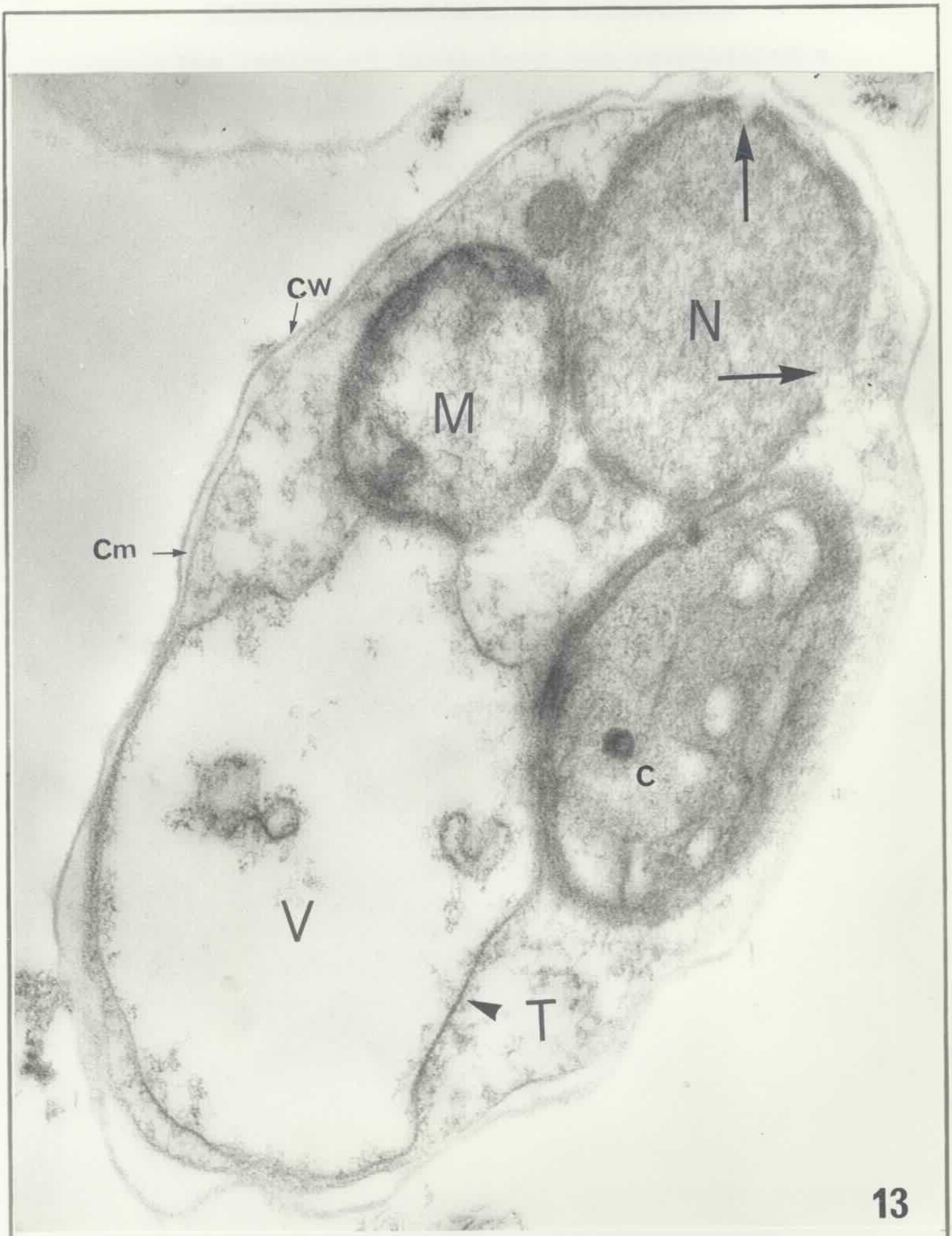


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General Discussion and Conclusions

The review of literature has established a perspective regarding the broad usefulness of various investigations, and summarised the knowledge on methods of assay, effects on soil microorganisms, behaviour, persistence, and absorption of phenylurea in soils. However, there are limitations to the bioassay techniques as indicated in Sections 1.2 (4 and 5), and (1) a lack of information about the fate of these herbicides in Australian soils, (2) a suitable method to predict their performance in the field, (3) a clear apparaisal of their mode of action. They have led to the use of unicellular algae in the study of phytoxocity of the phenylurea herbicides.

Algal Assay of Herbicides

The use of unicellular green algae as test organisms for herbicide bioassay presents certain advantages over that of higher plants. They can be grown in small vessels under closely controlled conditions. The number of organisms present in a light inoculum is large enough to eliminate the effects of individual variations. It is easy to obtain large amounts of algal material which can be used for several experiments, when comparison of results are required. The results of algal techniques are

obtained within 2 to 3 days, compared to the weeks involved in higher plant bioassays.

Chlamydomonas, Chlorella sp. Chlorella NMI are suitable as test organisms, but Chlorella NMI was preferred because of its high sensitivity to the herbicides.

Chlorella NMI in liquid algal technique gave significant responses to low concentrations of monuron, diuron and neburon (0.005-1.0 ppm) after 3 day incubation. This indicated that the alga could be an excellent test material for the detection of very small active amounts of these herbicides in soil.

Very low concentrations of these herbicides caused stimulation of algal growth as measured by optical density of chlorophyll extracts. The practical importance of the stimulatory effect of low concentrations of the herbicides on algae was shown in the assays for herbicide residues in the soil after the field trials. A stimulation in algal growth in the culture flasks containing soil samples from treated plots indicated the presence of small traces of active herbicide. Furthermore, as discussed later, the stimulatory effects gave a certain insight into the mode of action of these herbicides.

In the investigation of the culture conditions, it was found that period of incubation and inoculum size could reduce the sensitivity of the algal assay technique. 20 ml. sample of mineral solution inoculated with algae to give a final concentration of 10^5 cells/ml provided a highly sensitive assay medium when measurements were taken after three day incubation. The growth rate of Chlorella NMI and rate of algal inhibition by the herbicide were not markedly altered when the pH of the culture ranged between 5.8 and 7.5.

The algal technique has been extended to other herbicides which are known to cause chlorosis in plants, and was found to be quick and efficient for assessing the phytotoxicities of such herbicides.

The estimations of inherent toxicities of different herbicides by the algal technique compared favourably to those of higher plants. The order of toxicity obtained by the algae was: diuron> neburon> monuron> bromacil> atrazine> simazine> atratone> amitrole.

This study also demonstrated that in the phenylurea herbicides, phytotoxicity was affected by substitution on the aromatic group, but not necessarily by substitution on the N-alkyl group.

Soil adsorption

There are marked differences among the phenylurea herbicides with regard to their degree of adsorption in a given soil. This was well illustrated in the comparison of empirical Freundlich adsorption isotherms of diuron and neburon. One gram of a University garden soil adsorbed 18 times more neburon than diuron. In this soil adsorption study, the results indicated that the herbicide with the longer N-alkyl chain was more strongly adsorbed by soil.

The experiment with soils collected at different depths from the same site indicated that soil organic matter may be involved in the lowering of the herbicidal activity of diuron in soil. The positive role of organic matter in this regard was definitely established by paper disc tests and adsorption studies. In addition, the results show that herbicides which are inhibited in their actions by organic matter, are more phytotoxic in subsurface zones than in surface layers of soil.

The development of the 'Paper disc' technique provided a quick method for assay of phytotoxic amounts of herbicides in soil. It is not as sensitive (0.2 ppm for diuron and neburon) as the chemical and other bioassay methods, but it has the advantage of speed, simplicity, and low cost.

The disc technique clearly showed that the phytotoxicities of diuron and neburon were lowered as the amount of organic matter was increased in a sand-organic matter mixture. The increasing additions of clay to a sand-clay mixture had little influence on the phytotoxicity of the herbicides. It was concluded that the varying clay contents in soils had little effect on the herbicidal performance of diuron and neburon. Furthermore, the experiment showed the differential adsorption of diuron and neburon by soil constituents. One gram of organic matter (peat) adsorbed 19 times more neburon than diuron, and 1 gram of clay adsorbed 6 times more neburon than diuron.

Prediction of rates of herbicide application.

In the interpretation of the simple correlations between ED_{50} and soil property, it must be recognised that soil properties may vary together, as indicated by the highly significant correlations among soil properties (Table 12). Thus contributions of the various soil factors to toxicity reduction cannot always be determined by simple correlations. However, more information can be obtained by regression techniques. Individual contributions and combined contributions by soil properties can be evaluated by determination of

partial regression and multiple regression coefficients. From the correlation and regression studies, it was evident that organic matter contributed more to toxicity reduction than the other soil properties examined (Tables 12 and 13).

It appeared that the level of organic matter in the soils studied might be used as a practical indication of the amount of diuron which would be required on a given soil to produce a high degree of selective weed control. However, the study also showed that adsorption values gave a higher correlation with ED_{50} values and could be more accurate in prediction of herbicide performance on higher plants (Table 13).

Field Trials

Weeds that emerge with the crops often persist through a greater portion of the growing period. They compete strongly with the crops for water, light and nutrients during the early growth stages. During this period, the weeds may also be harmful as hosts of pests and diseases which are transmitted to the sensitive young seedlings. Weeds emerging after the crop is established cause only slight damage to the crop. The sooner weed competition is removed the greater will be the possibility of an improvement in yield.

From the results of the field trials it is apparent that a 100% control of weed during the entire crop cycle is not necessary to obtain an increase in yield. Moreover, the amount of herbicide required to achieve that control usually leaves harmful residues. The recommended rates of application of incorporated, pre-planting herbicides prevented the establishment of weeds before planting, and achieved an adequate weed control (70-80%) over the first 3-4 months, without injury to the crop. This early control enabled the crop to take full advantage of the absence of weed competition, and to produce a very dense canopy of leaves which could exercise an excellent biological control over secondary weed germination. Furthermore, by causing a retardation of weed growth, seed formation of the weed plants was delayed. The absence of weed seeds at harvest time would greatly reduce grading costs and a higher return would be obtained for a high quality product.

It is practical to remove residual weeds after harvest of crop plant by mechanical cultivation. Thus a combined chemical-biological-mechanical weed control can be realised.

The field trials have also shown that tirallate efficiently controlled ryegrass, but lacked activity on broad leaved weeds. Elimination of ryegrass in a wheat crop by triallate may sometimes result in an increase of broad leaved weeds and so off-set the advantage gained by the herbicide. The potentiality of diuron and simazine for weed control in wheat needs developing for it shows considerable promise and can well prove to be an economic and reliable answer to the problem of certain broad-leaf weeds. Furthermore, it shows the possibility of using diuron and simazine as part of a herbicidal mixture where its weed spectrum and crop selectivity can be complemented by another herbicide, such as triallate.

Ultrastructural studies of Chlorella NMI and Euglena.

Different fixation procedures for electron microscopy were applied to the algal cells, and the procedure involving the use of glutaraldehyde as a pre-fixative followed by osmium tetroxide or potassium permanganate gave excellent preservation of find structure.

As the herbicides to be studied are known to cause chlorosis in green leaves, possibly by inhibiting pigment synthesis or chloroplast development, it is expected that they would affect protein synthesis in

the chloroplast. Cytological studies (light and electron microscopy) were therefore used to establish the presence of a protein synthetic system in Chloroplasts of Chlorella NMI and Euglena. These studies demonstrated DNA, ribosomes and polyribosomes in the chloroplasts of the algae.

More interesting, however, was the attachment of DNA fibrils and polyribosomes to the lamellar membranes. This strongly suggests that protein synthesis and DNA replication take place at sites associated or closely associated with the lamellar membranes. Possibly, chloroplast ribosomes are formed at the site of attachment of DNA to the lamellar membrane, and there, are assembled as polyribosomes. The observations also imply that protein synthesized by these polyribosomes takes place at the same site. Direct connection between DNA molecules and the membrane system is well known in bacteria (Morgan et al, 1967), in chloroplasts of brown algae (Bisalputra and Burton, 1969) in chloroplasts of green plants (Woodcock and Fernandez, 1968; Werz and Kellner, 1968). It was suggested that association facilitated replication of the DNA molecules. In the present study, replication of DNA in Chlorella NMI chloroplast was indicated by the presence of single looped and double looped structures.

Evidence was presented to show marked differences in the mode of division of Chlorella NMI from species of Chlorella examined by other workers. Division occurred by successive bipartition. Another interesting point revealed by the electron microscopic studies was the direct involvement of the nucleus in providing material for the development of the Golgi body.

Effects of herbicides on algal cells

Very low concentrations of diuron stimulated cell division and chlorophyll production. It is proposed that the stimulation is due to a cytokinin effect of diuron molecules in the molecules, resulting in increased protein synthesis, hence increase in chlorophyll production. From a study of chlorophyll a to b ratios, it would appear that the primary effect of diuron is localised on chlorophyll. Further, the effects on chloroplastic pigments are not secondary to an effect upon the chloroplast itself. The herbicide had a preferential inhibitory effect on chlorophyll b, causing an increase in the chlorophyll a to b ratio. It is concluded that this change in chlorophyll interferes with the normal activity of Photosystem II, and also causes a disruption of the lamella stacking in the chloroplast, and the regular arrangement of the lamellae

within the chloroplast. A similar effect was observed in Euglena, but the cell also responded by forming cyrolysomes, and using the breakdown products of non-essential components of the cytoplasm to carry out the more basic vital processes for survival. This response may explain the capability of the Euglena to resist high dosages of diuron, although the chloroplasts are disrupted and broken down at these concentrations.

In the studies with amitrole, the synchronised Chlorella NMI cells were subjected to treatment at low levels of the herbicide. It was assumed that at the lowest effective concentration, only one or two metabolic reactions would be affected, and at high concentrations several reactions would be inhibited at the same time. Concentrations (1 and 5 ppm) of amitrole which did not have any or only a slight effect on cell growth were used, The results clearly indicated that carotenoid synthesis was the primary target of amitrole toxicity. Carotenoid pigments degraded faster in the amitrole-treated chloroplasts than chlorophyll. It is concluded that, as the protective action of carotenoid decreases with loss of the pigment, chlorophyll is photo-oxidised. It is also suggested that the breakdown products of chlorophyll may then affect the

chloroplast DNA, resulting in an interference with chloroplast protein synthesis.

This suggestion is supported by the work of Naylor (1964) who found that the effect of amitrole on the fronds of Lemna minor was very like that of genetical variegation.

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Appendix A

Transformation applied to data - Oats pot trial with diuron

Variable	Form of transformation
x (diuron conc. in ppm)	No transformation
y (% growth of control)	Log_{10}

Data (untransformed)

x (ppm)	y (%)
0	100
1.0	91.2
2.0	72.8
3.0	70.1
4.0	47.9
6.0	25.7

Correlation matrix

- 0.970

Regression Analysis

Source	D.F.	S.S.	M.S.	V.R.
Regression	1	.22395	.22395	65.029
Deviations	4	.01378	.00344	
Total	5	.23773		

Regression equation (x, y as transformed)

$$y = 2.0543 \quad 0.098\ x$$

Observations	Observed y	Expected y
1	2.000	2.054
2	1.960	1.956
3	1.862	1.858
4	1.846	1.760
5	1.680	1.662
6	1.410	1.466

Appendix B

1. <u>One way Analysis of variance</u> (ED_{50} for algae in 14 soils)

S.O.V.	D.F.	S.S.	M.S.	F.
Between soils	13	69.019	5.309	78.355
Error	28	1.897	0.068	
Total	41	70.916		
S.D.	0.260			
C.V.	5.264			
S.E.	0.150			
L.S.D. (0.05)	0.435			
L.S.D. (0.01)	0.587			

2. <u>One way Analysis of variance</u> (ED_{50} for algae in 20 soils)

S.O.V.	D.F.	S.S.	M.S.	F.
Between soils	19	93.245	4.908	63.058
Error	40	3.113	0.078	
Total	59	96.358		
S.D.	0.279			
C.V.	5.680			
S.E.	0.161			
L.S.D. (0.05)	0.460			
L.S.D. (0.01)	0.616			

Appendix C

1. Oats - ED_{50} values Against Organic Carbon Content (14 soils)

Regression Analysis

Source	D.F.	S.S.	M.S.	V.R.
Regression	1	32.7086	32.7086	37.513***
Deviations	12	10.4630	0.8179	
Total	13	43.1716		

Regression Coefficients	Standard errors
-0.1450	0.5475
1.3847	0.2260

Regression equation = $y = -0.1450 + 1.3847\ x$

2. Algae - ED_{50} values against Organic carbon content (20 soils)

Regression Analysis

Source	D.F.	S.S.	M.S.	V.R.
Regression	1	21.7338	21.7338	41.766***
Deviations	18	9.3667	.5204	
Total	19	31.1004		

Regression Coefficient	Standard errors
3.0477	0.3304
0.9145	0.1415

Regression equation

$$y = 3.0477 + 0.9145\ x$$

3. <u>One way Analysis of Variance</u> (% adsorption of diuron for 20 soils)

S.O.V.	D.F.	S.S.	M.S.	F.
Between soils	19	15406	810.842	57.979
Error	40	559.406	13.985	
Total	59	15965.40		

S.D.	3.740
C.V.	11.800
S.E.	2.159
L.S.D. (0.05)	6.171
L.S.D. (0.01)	8.258

APPENDIX D

Castle Hill (1970)

Prediction of herbicide rates of application - Liquid algal technique - Diuron

Soil

0	→	0.0947	→	100%
.1	→	0.085	→	90%
.2	→	0.054	→	57%
.5	→	0.0253	→	28.7%
	→		→	
1.0	→	0.0140	→	14.8%

Sand

0	→	0.0885	→	100%
0.05	→	0.0775	→	87.5%
0.10	→	0.0485	→	54.7%
0.20	→	0.0395	→	43.8%
0.50	→	0.0305	→	17.4%
0.80	→	-		-

At 50% inhibition - 1 gm. of soil adsorbs - 1.40 µg of diuron

At 100% inhibition - 1 gm. of soil adsorbs - 3.64 µg of diuron

Disc	Sand	Soil
50% inhibition -	0.8 ppm	6 ppm
100% "	1.2 ppm	18 ppm

Sand = 1.6 ml

Soil = 3.6 ml

at 50% inhibition - 1 gm of soil adsorbs - 3.74 µg of Diuron

at 100% " 1 " " " " 11.89 " "

Ratio - $\frac{100\% \text{ inhibition}}{50\% \text{ inhibition}} - \frac{11.89}{3.74}$ 3.2

Adjustment of recommended does relative to depth of incorporation (5-7.6) - $\frac{1.19 \times 6.5}{5} = 1.547$ = 1.40 lb/ac.

Narrabri (1970)

Prediction of herbicide rates of application
Algal (liquid) technique

Diuron

Soil			Sand		
0	- 0.043	- 100%	0	- 3.053	- 100%
.1	- 0.042	- 100%	.1	- 0.045	- 85%
.2	- 0.030	- 70%	.20	- 0.0275	- 52%
.5	- 0.013	- 30%	.50	- 0.0130	- 24.5%
.8	- 0.0045	- 10.5%	.80	- 0.005	- 0.0%
0.31		50%		0.22	50%

At 50% inhibition 1 gm of soil adsorbs - 0.9 µg of diuron.
At 100% " 1 " " " " 2.43 " " "

Disc		Sand		Soil
50%	-	0.5 ppm	-	5 ppm
100%	-	1.4 ppm	-	16 ppm

Sand - 1.6 ml.
Soil - 3.6 ml.

50% - 1 gm. of soil adsorbs - 3.24 µg Diuron
100% - 1 " " " " - 10.51 " "

$$\text{Rate} - \frac{100\% \text{ inhibition}}{50\% \text{ inhibition}} = \frac{10.51}{3.24} = 3.24$$

$$\text{Adjustment of recommended dose relative to depth of incorporation (5-7.6 cm)} - \frac{10.51 \times 6.5}{5} = 13.65 \text{ Kg/ba} = 1.2 \text{ lb/ac}$$

APPENDIX E

1. Untreated Chlorella NMI cell count using a Petroff-Hauser counting chamber.

ONE WAY ANALYSIS OF VARIANCE

Number of Treatments 2

Number of Replicates 12

1.2 1.4 1.2 1.2 1.3 4.6 1.4 1.7 1.6 2.2 2.4 1.8

Mean 1.8333

2.0 2.3 2.1 1.4 1.8 2.0 1.2 1.8 1.8 2.1 3.6 1.6

Mean 1.9750

Grand Mean 1.9042

ANALYSIS OF VARIANCE TABLE

S.O.V.	D.F.	S.S.	M.S.	F.
B.Trts	1	0.1205	0.1205	$0.190^{n.s.}$
Error	22	13.9893	0.6359	
Total	23	14.1097		

STANDARD DEVIATION	0.7974
COEFFICIENT OF VARIATION	41.8775
STANDARD ERROR OF THE MEAN	0.2302
L.S.D. (0.05)	0.6751
L.S.D. (0.01)	0.9176

2. Diuron-treated Chlorella NMI cell count.

ONE WAY ANALYSIS OF VARIANCE

Number of Treatments 2

Number of Replicates 12

2.5 2.6 2.8 3.8 4.0 2.4 2.5 2.8 3.5 4.7 4.0 3.1

Mean 3.2250

2.9 3.0 4.0 3.0 4.0 2.8 5.2 4.5 3.5 2.7 5.4 6.9

Mean 3.9917

Grand Mean 3.6083

ANALYSIS OF VARIANCE TABLE

S.O.V.	D.F.	S.S.	M.S.	F.
B. Trts.	1	3.5267	3.5267	$3.112^{n.s.}$
Error	22	24.9319	1.1333	
Total	23	28.4585		

STANDARD DEVIATION 1.0646

COEFFICIENT OF VARIATION 29.5026

STANDARD ERROR OF THE MEAN 0.3073

L.S.D. (0.05) 0.9013

L.S.D. (0.01) 1.2250