THE EFFECTS OF SOME SELECTED HERBICIDE & PLANT HORMONES ON GROWTH OF PROTOTHECA WICKERHAMII

BY

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I, Penelope Anne Henning, declare that the work submitted for this dissertation is my own, unaided and has not been submitted in whole or part for any degree in any university.

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SUMMARY

Prototheca wickerhamii, a derivative of *Chlorella*, is a pathogenic alga. It causes the disease protothecosis in mammals, for which there is no known cure. The effects of eleven selected herbicides and plant hormones on the growth of *Prototheca wickerhamii* were studied. Most of the chemicals were ineffective. Coconut milk, however, stimulated growth. The hormones IAA, IBA and IPA caused inhibition of growth at 400µg/ml. The site of action appears to involve the mitochondrion with competative inhibition affecting the Kreb's cycle. Further studies on the effects and quantities of these chemicals in patients suffering from protothecosis are necessary before a cure can be recommended.

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INTRODUCTION

Prototheca is a genus of colourless, heterotrophic organisms. It occurs in a number of habitats, for example, the slime fluxes of trees, faeces and fingernails of man, potato skin, acid stream water, diseased tissues associated with a case of bovine mastitis and sludges in waste stabilisation ponds (Cooke, 1968a). It is easily cultured in the laboratory and has been obtained as a laboratory mutant of Chlorella by cultural manipulations and the use of radiation (Butler, 1954).

The taxonomy of Prototheca is uncertain as its circular and creamy-white growth form resembles a fungal-like colony. Usually it is referred to as a "colourless derivative of the green algae, such as Chlorella" (Davies et al, 1964; El-Ani, 1967; Migaki et al, 1969; Poyton, 1970). Prototheca has been referred to as a chlorotic alga belonging to the Protothecaceae, a family related to the Chlorellaceae (Cooke, 1968a; Fritsch, 1968). Scheifer and Gedek, (1968) and Povey et al, (1969) however, consider Prototheca to be a connecting link between the fungi and the algae, but more closely related to the fungi. Nadakavukaren and McCracken (1973) are of the opinion that the ultrastructure of Prototheca is that of a non-photosynthetic alga and not that of a fungus. Cooke (1968a) considers Prototheca to be an alga occupying the same ecological niche as many yeasts or yeast-like fungi, but retaining an algal relationship by its method of reproduction. El-Ani (1967) and Arnold and Ahearn (1972) report that the method of asexual reproduction is similar to Chlorella.

Since Prototheca has lost the ability to produce chloroplasts and pyrenoids, it has adopted a heterotrophic mode of behaviour (Cifferi, 1956; Emmons et al, 1970). Because of its structure and hence its growth habit, Prototheca is associated with the infectious disease, protothecosis. In dogs, protothecosis occurs as focal

abscesses in the viscera with some spread to local lymph nodes (Povey et al, 1969). The disease in cows produces a type of mastitis associated with the udder. Autopsies performed on slaughtered cows showed lesions in the body, especially at lymph nodes (Frank et al, 1969; Migaki et al, 1969). In man, protothecosis causes lesions in the dermal layers of the skin and can spread to the lymph nodes (Davies et al, 1964; Davies and Wilkinson, 1967; Klintworth et al, 1968).

The use of herbicides and plant growth regulators in the control of undesirable plants has increased many fold in recent years. Substituted methylureas caused varied effects on Chlorella vulgaris var. viridis (Geoghegan, 1957). Chlorella pyrenoidosa was shown to be influenced by 2,4-D (Wedding and Black, 1961; Swets and Wedding, 1964). Vance and Smith (1969) inhibited the growth of Chlorella pyrenoidosa with Amitrol-T. Subject to the nitrogen source in a nutrient medium amitrole has also been shown to inhibit the growth of Prototheca zopfii (Casselton, 1964; 1966; 1967).

This study was made to observe the effects of different herbicides and plant hormones on the growth of Prototheca wickerhamii. The concentration of herbicide necessary to cause death in algae is very low. It may be possible to apply some selective chemicals to Prototheca cells in high enough concentrations to inhibit the cells. These concentrations should probably be below that necessary to harm mammalian host cells.

The organism studied was Prototheca wickerhamii (hereafter referred to as Prototheca) obtained as an isolate from lesions on the forehead of an African patient by the Medical Research Council, University of the Witwatersrand, Johannesburg.

LITERATURE REVIEW

Light microscopic histological studies of Prototheca have shown many structural features such as storage products and cellular contents, size and method of reproduction (Migaki et al, 1968; Povey et al, 1969). Various authors report that Prototheca cells are spherical, ovoid or ellipsoid in shape and measure from 2µm to 11µm (Klintworth et al, 1968; Emmons et al, 1970; Poyton, 1970). Prototheca wickerhamii cells are medium to large in size (Cooke, 1968). El-Ani (1967) measured wild type Prototheca wickerhamii autospores of 3,3µm to 4µm, while the mother cells varied between 7,7µm to 13µm. The diameter of Prototheca wickerhamii cells may change with varying nutrient conditions but usually approximates to the published size (Arnold and Ahearn, 1972). Mature Prototheca wickerhamii cells obtained in culture, from dermal lesions of a patient, have a diameter measuring 6,6µm to 12,1µm (Mars et al, 1971).

Electron microscopic studies show that lamellar photosynthetic structures comparable to those of autotrophic algae are not present (Klintworth et al, 1968). Starch granules bounded by double-layered membranes, proplastid-like structures and amyloplast-type storage plastids can be seen at the fine structural level (Webster et al, 1968; Nadakavukaren and McCracken, 1973). Protothecal cells have been reported to contain numerous lipid droplets and lipoidal electron dense granules (Davies et al, 1964; El-Ani, 1967; Webster et al, 1968).

Mitochondria are usually located near the outer margin of the cell (Webster et al, 1968; Casselton and Stacey, 1969; Nadakavukaren and McCracken, 1973). They show typical discoidal cristae of random orientation (Webster et al, 1968). Isolated mitochondria from Prototheca zopfii measure 1,3µm by 0,6µm (Lloyd, 1964). The classical cytochromes and ubiquinones of the electron transport chain are present (Arnold and Ahearn, 1970).

El-Ani (1967) reports that the life cycle of Prototheca wickerhamii is the same as that of Chlorella. Uninucleate autospores are formed by nuclear division, accompanied by mother cell enlargement and followed by cytoplasmic cleavage (El-Ani, 1967). Multinucleate cytoplasmic segments may initially be formed if nuclear division proceeds for some while before cytoplasmic cleavage begins (El-Ani, 1967). Autospores are liberated, after cleavage, by bursting of the mother cell wall (Davies et al, 1964; Cooke, 1968b; Arnold and Ahearn, 1972). The number of autospores produced varies from one to twenty which on release assume the shape of the parent cells, increase in size and undergo an assimilative phase before cleavage again takes place (Cooke, 1968b; Arnold and Ahearn, 1972).

Although Prototheca resembles the achloric mutants of Chlorella in morphology and mode of reproduction it differs, however, in that it requires thiamine (Vitamin B_1) for its growth (Anderson, 1945a; Cifferi, 1956; El-Ani, 1967; Cooke, 1968a; Klintworth et al, 1968; Casselton and Stacey, 1968; Arnold and Ahearn, 1972). In 1935, Barker found that some constituents of yeast autolysate are essential for the processes of cell production in Prototheca. Anderson (1945a) studied the nature of this requirement further and showed that growth in the presence of thiamine alone, approximated closely the development with yeast extract, observed by Barker. A linear relationship exists between cell yield and thiamine concentration up to $1x10^{-7}$M. At this concentration, growth is practically as heavy as the maximum obtained with $3x10^{-5}$M vitamin (Anderson, 1945a). Pore (1972) related Prototheca to Chlorella protothecoides as both have a thiamine requirement.

Excellent growth at 25°C to 37°C has been noted for Prototheca cultures, with no growth occurring at temperatures below 10°C and above 38°C (Cooke, 1968a; Frank et al, 1969; Arnold and Ahearn, 1972). The

temperature range 29°C to 32°C is accepted as optimum (Barker, 1935; Davies et al, 1964; Cooke, 1968a; Klintworth et al, 1968; Emmons et al, 1970). Prototheca cultures are generally incubated at 25°C to 30°C (Anderson, 1945a; 1945b; Cifferi, 1956; Casselton, 1964; Callely and Lloyd, 1964).

In 1964, Shihira - Ishikawa and Hase found that the growth of "bleached", yellow Chlorella protothecoides cells in darkness was not significantly different from that in 2,000 Lux light. Epel and Krauss (1965; 1966) found that white light, from cool white fluorescent bulbs, at 1,200 ft.c. inhibited cell division in Prototheca zopfii. The action spectrum for this inhibition was localised in the blue end and the near UV region of the spectrum. Further work by Epel and Butler (1969; 1970) showed that respiratory inhibition in this organism was directly correlated with a photodestruction of cytochromes a_3, b and c and had indirect consequences on cell division, protein and nucleic acid synthesis. Casselton (1964; 1967) and Stacey (1969) grew Prototheca zopfii cultures in the dark.

Barker (1935) reported that with an increase in acidity of the nutrient medium from neutrality to pH 4 there is a marked decrease in the rate of glucose decomposition by Prototheca zopfii. Casselton and Stacey (1969) use a nutrient medium with a pH 6,1. Anderson (1945b) grew his cells at neutrality while Callely and Lloyd (1964a) raised the pH of their nutrient medium to 7,2. Some workers, however, report that growth of Prototheca is essentially inhibited at pH levels above 7 (Klintworth et al, 1968; Emmons et al, 1970).

In 1966, Stacey and Casselton showed that Prototheca zopfii was unable to utilize nitrate as the sole nitrogen source. They further found that a number of Prototheca species could not utilise either nitrate or nitrite but were able to metabolise adenine, ammonium

sulphate and ammonium chloride as nitrogen sources (Casselton and Stacey, 1969). Investigations in 1956 by Cifferi showed that four species of Prototheca failed to grow on media with only glucose, inorganic salts and amino acids. When peptone, as a nitrogen source, was added to the medium, growth improved. Prototheca zopfii has been maintained on malt and yeast extract, peptone and glucose (Cooke, 1968a). Nadakavukaren and McCracken (1973) grew Prototheca zopfii on a medium consisting of acetate, peptone and yeast extract. Prototheca wickerhamii utilises a number of agar extracts which may be supplemented with nutrients, peptone and malt (Poyton, 1970).

In 1935, Barker reported that monosaccharides are the only carbohydrates which are utilised by Prototheca with glucose giving maximum cell yield, while sucrose was not metabolised. The fatty acids acetic, propionic, n-butyric and iso-butyric are used with the iso acid being less readily oxidised than the corresponding straight acid (Barker, 1935; Cooke, 1968a; Arnold and Ahearn, 1972). The oxidative assimilation of these compounds was studied in 1936 but Barker was unable to elucidate the metabolic mechanism involved.

Anderson (1945b) suggested that the partial oxidation of such simple compounds as acetic acid by Prototheca would give rise to intermediate products from which the synthesis to carbohydrates could proceed. Investigation of the carbohydrate metabolism of Prototheca established the presence of most of the enzymes of the glycolytic pathway and of the hexose monophosphate shunt (Cifferi, 1962). As is expected the enzymes of the "Calvin Cycle" were absent in this organism. In 1964, Callely and Lloyd demonstrated the presence of all the enzymes of the Krebs cycle in Prototheca zopfii and evidence was lead that this cycle is the major pathway for the oxidation of acetate.

In 1963 and 1964, Callely and Lloyd examined

extracts from acetate and butyrate-grown Prototheca cells showing isocitrate lyase activity. This is one of the key enzymes of the glyoxylate cycle which enables acetate, or "acetate" precursors, to serve as growth substances to replenish T.C.A. intermediates which are utilised in cellular processes (Callely and Lloyd, 1963). Propionate in Prototheca zopfii is converted into the T.C.A. intermediate Acetyl CoA via the vitamin B_{12} independent malonic-semialdehyde pathway (Callely and Lloyd, 1964b). The Krebs and glyoxylate cycles are the major routes for acetate and propionate assimilation by Prototheca zopfii (Lloyd and Callely, 1965). Butyrate is degraded to two C_2 units by isocitrate lyase of the glyoxylate cycle before it enters the T.C.A. cycle (Lloyd and Callely, 1965). Cullimore (1966) showed an efficient adaptive butyrase enzyme system in Prototheca.

Prototheca species thus require thiamine and metabolise via the glycolytic pathway, hexose monophosphate shunt, tricarboxylic acid cycle, glyoxylate shunt and the malonic semi-aldehyde pathway using a vitamin B_{12} independent route (Arnold and Ahearn, 1972).

The available literature on protothecosis suggests that the infection may be secondary with Prototheca entering the host through a wound or opening (Davies et al, 1964; Davies and Wilkinson, 1967; Klintworth et al, 1968; Emmons et al, 1970). It apparently disseminates slowly, causes little destruction of parenchymatous tissue and does not evoke a febrile response by the host. All naturally occurring cases of protothecosis, thus far reported, have been chronic in nature and fatal in dogs (van Kruiningen et al, 1969; van Kruiningen, 1970; Emmons et al, 1970). Reported experimental studies indicate that species of Prototheca have slight or no virulence for laboratory animals (Davies et al, 1964; Scheifer and Gedek, 1968; Frank et al, 1969; Emmons et al, 1970).

A number of cases of protothecosis have been

reported for dogs and cattle (Povey et al, 1969; van Kruiningen et al, 1969; Frank et al, 1969; Migaki et al, 1969; van Kruiningen, 1970). In man, however, infection with algae is virtually unknown with only three cases, involving organisms belonging to the genus Prototheca, being reported. In the first report infection was present on the foot of an African rice worker in Sierre Leone (Davies et al, 1964) and in the second, it affected the lower leg of a Caucasian woman from U.S.A. who was simultaneously suffering from diabetes mellitus and carcinoma (Klintworth et al, 1968). The third case is an African manual labourer from the Transvaal with infection of the forehead and scalp (Mars et al, 1971). Prototheca segbwema was the organism isolated in the first case, while the latter two cases involved Prototheca wickerhamii. Tindall et al (1971) reported three further cases of protothecosis in man, but as yet, they are unconfirmed.

Mars et al (1971) reports a thinning of the epidermis with inflamation in the dermis. The infection disseminates slowly and has progressed from the scalp and forehead to behind the right ear and to the chin and the chest. Potassium iodide provides a temporary cure with some retrogression of the dermal lesions. Anti-bacterial antibiotics, fungicides, antiprotozoal drugs, cytotoxic agents and radiotherapy showed no apparent effect on the infection in the patients (Davies and Wilkinson, 1967; Klintworth et al, 1968).

Casselton (1964) showed that the systemic herbicide 3-amino-1,2,4-triazole (amitrole) inhibited the growth of Prototheca zopfii. Further work showed that amitrole is a competative inhibitor of enzymes involved in histidine and adenine synthesis (Casselton, 1967). The inhibition of Prototheca zopfii growth by amitrole may be reversed by histidine (Casselton, 1964). Addition of adenine, however, does not reverse the inhibition (Casselton, 1966).

Respiration and protein synthesis are some of the metabolic processes affected by herbicides and plant hormones. Chemicals which interfere with respiration and the mitochondrial electron transport system may be electron-transfer inhibitors; uncoupling agents; or energy-transfer inhibitors (Moreland, 1967). IAA, IBA, NAA and gibberellic acid, as selective hormone type weedkillers, are effective uncouplers of oxidative phosphorylation (Moreland, 1967). The herbicide 2,4,5-T is a more effective uncoupler of oxidative phosphorylation and like MH, it also inhibits oxygen and phosphate uptake (Hilton et al, 1963; Audus, 1964).

Many chemicals such as 2,4,5-T, MH and atrazine inhibit oxidation of pyruvate, α ketoglutaric acid and succinate in the T.C.A. cycle with indications that they inhibit the whole enzyme complex rather than individual enzymes (Audus, 1964; Moreland, 1967). MH competes for receptor sites of respiratory enzymes (Audus, 1964). Malic dehydrogenase, an enzyme of the Krebs cycle, is inhibited by IAA, IBA and NAA (Bonner and Bandurski, 1952). 2,4,5-T treatment produces a decrease in the activity of glycolytic enzymes with a corresponding increase in hexose mono-phosphate shunt enzymes (Audus, 1964). High concentrations of 2,4,5-T will reduce both glycolysis and the pentose phosphate pathway with the former being more actively inhibited (Hilton et al, 1963; Audus, 1964).

Moreland (1967) reports of specific influences exerted by IAA on nucleic acid metabolism with reference to DNA dependent RNA synthesis. Endogenous cytokinins and gibberellins also regulate DNase, RNase, RNA and protein synthesis. MH may suppress mitosis in actively growing plant tissues by "breaking" chromosomes with visible heterochromatin (Audus, 1964). A major site of 2,4,5-T action is the nucleus, and it affects protein synthesis by reduction of amino and amide nitrogen (Hilton et al, 1963). IAA and NAA inhibit glutamic dehydrogenase, an enzyme involved in protein synthesis (Bonner and Bandurski, 1952),

while MH reacts with sulfhydral groups of proteins (Audus, 1964).

MATERIALS AND METHODS

a) Culturing Techniques

i) Growth of Prototheca wickerhamii

Prototheca wickerhamii was relatively easy to culture in the laboratory and grew well in a number of nutrient media. In 1969, Casselton and Stacey used a nutrient solution consisting of 7,26g KH_2PO_4; 2,32g K_2HPO_4; 0,41g $MgSO_4.7H_2O$; 0,2g sodium citrate; 12mg $FeSO_4.7H_2O$; 3,37mg thiamine; 10g glucose and 1ml A_4 trace element solution in one litre distilled water to maintain their Prototheca cultures. A slight modification of Arnon's trace element solution was used here (Hewitt, 1952). Nitrogen was added in the form $(NH_4)_2SO_4$ at a concentration of 100mg N/l (Casselton and Stacey, 1969). After autoclaving at 15lbs per sq ins for 15 mins, the pH of the complete medium was measured at 6,2. Prototheca cells were also grown in a modified Casselton and Stacey (1969) solution, where the source of carbohydrate, sucrose, was provided at 10g/l.

Prototheca cells will grow on nutrient agar (Poyton, 1970) or on a medium containing yeast extract and peptone (Nadakavukaren and McCracken, 1973). The nutrient broth used in these cultures consists of 1g beef extract; 2g yeast extract; 5g peptone; 5g NaCl. 28g of this powder is made up to 1 litre which after autoclaving, has a pH of 7,2. All nutrient solutions used in these Prototheca cultures contained $1x10^{-5}$M thiamine as the final concentration of vitamin (Anderson, 1945a).

Cells were inoculated as one loopful of suspension into a test tube containing 10mls of nutrient solution. Initially, the cultures were maintained in nutrient solutions on a laboratory bench. Room temperature was approximately $20^{o}C$, while 500 Lux daylight was received. Cultures were then grown in an incubator at an

optimum temperature of 32°C. 2000 Lux of light on a 12 hour light/dark period, approximating natural conditions, was supplied (Epel and Krauss, 1966). Cells were also cultured in the dark at 32°C.

ii) Estimate of growth

Growth rate of the Prototheca cultures was determined by sampling at regular time intervals. The cells were fixed in 2,3% glutaraldehyde and were counted on a hemacytometer, where the volume over a square mm is O,lcc. Growth of the cells was also determined by absorption spectrophotometry. Maximum absorption of light by the fixed cells in the spectrophotometer was at 460mμ.

iii) Sample size

Mounsey (1967) and Freese (1967) determine sample size from this equation:

$$\sqrt{n} = \frac{t\,\sigma}{E}$$

where E is the difference allowed from the mean, n is the size of sample required and t has n-1 d.f. The standard deviation for a population from a few samples was determined from Bishop (1969).

b) Herbicides

i) Herbicide preparation

For the herbicide studies the cells were grown in 5mls of nutrient solution instead of 10mls. This was to facilitate the addition of herbicides later on in the experiment. The recommended weight of nutrient was utilised, however, even though the volume of liquid was reduced, and this had the effect of doubling the concentration of nutrients provided. The addition of 5mls of herbicides to the test tube gave the concentration of

herbicide required and the normal nutrient concentration (Vance and Smith, 1969).

The herbicides selected for study were: maleic hydrazide (MH); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); 2-chloro-4,6 bis (ethylamino)-s-triazine (simazine); sodium chloroauric acid (Na gold salt), while the plant hormones used were: indolyl-3-acetic acid (IAA); indolyl-3-butyric acid (IBA); indolyl-3-propionic acid (IPA); naphthylacetic acid (NAA); kinetin; gibberellic acid (GA_3) and coconut milk (appendix i). Some difficulty was experienced in making up these solutions as most of these chemicals are sparingly soluble in water (Stecher, 1968). It was decided to dissolve the herbicides in de-ionised water, rather than ether or ethanol, as Prototheca metabolises ethanol and the overall effect of these solvents on the cells was unknown (Barker, 1935; Emmons et al, 1970; Arnold and Ahearn, 1972). Gentle heating of these solutions, however, dissolved the powders or crystals. The herbicides were neutralised with sodium hydroxide, except for coconut milk, as the addition of alkali may have had an effect on the medium.

Initially, the herbicides were added to the cultures through a 0,65µm millipore filter. The herbicide was "injected" from a sterile syringe through a sterile millipore filter holder and needle into the test tube. Some difficulty was experienced in maintaining bacteria-free cultures. This problem was overcome by sterilising the herbicide solutions at 15lbs per sq ins for 10 mins. Heat resistant precision 5ml glass syringes and stainless steel hypodermic needles were sterilised at the same time. These chemicals have melting points above 120°C (Stecher, 1968) and the autoclaved solutions remained clear and showed no signs of denaturation. pH readings were taken before and after sterilisation and on addition of herbicide solutions to nutrient cultures.

ii) Treatment of cultures

Cells were inoculated into 5mls nutrient and grown for 48 hours. Varying volumes of the sterile stock herbicides were then added to the cultures and the volume in the tubes was made up to a total of 10mls with sterile deionised water to obtain the desired herbicide concentration. Control tubes were made up with sterile deionised water. The cultures were grown for a further 48 hours during the exponential phase of growth, the cells were then fixed and cell growth was measured.

iii) Herbicide concentrations

Stock solutions containing 2000µg/ml (ppm) herbicide were made up in deionised water (Vance and Smith, 1969). Except for kinetin which is highly insoluble in water, so that a stock solution of 200µg/ml was obtained with much difficulty. Coconut milk was supplied as a percentage of total solution. The simazine concentration was based on the active 80% wettable powder present (Vance and Smith, 1969).

Low concentrations of IAA, IBA and IPA in the range 10µg/ml to 100µg/ml were also studied. MH, 2,4,5-T, NAA, gibberellic acid and simazine were made up to higher concentrations of 2000µg/ml and 4000µg/ml. Due to insolubility and preparation problems kinetin, Na gold salt and coconut milk could not be used above 1000µg/ml.

iv) Time course curves

Time course studies with the inhibitory herbicides at 1000µg/ml were undertaken. Sampling took place at 2,4,6,8,10,12 and 24 hours after addition of herbicide to the culture. The time for absorption and effectivity of the herbicide was determined.

v) Percentage inhibition

Percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Cont } 96 - \text{Cont } 48+d) - (\text{Test} - \text{Cont } 43+d)}{(\text{Cont } 96 - \text{Cont } 48+d)} \times \frac{100}{1}$$

At the time of addition of herbicides to the cultures, 48 hours, and at the end of the run, 96 hours, the cells present in the control tubes were determined. The delay in absorption and effectivity (d) of the herbicides was obtained from the time course curves. The number of cells present in the control after 48 hours plus the delay (d) was calculated.

c) Radioactive herbicide studies

i) Time course curve

A time course study using 400µg/ml IAA was undertaken to establish if the time for absorption and effectivity of the herbicide was the same as that for 1000µg/ml IAA. Samples were taken at 2,4,6,8,10,12 and 24 hours.

ii) Isotope preparation

10µCi 3-Indolyl (acetic acid-1-C14) as a freeze-dried ammonium salt was obtained with a specific activity of 268µCi/mg. The isotope compound was dissolved in de-ionised water with an appropriate amount of IAA to give a concentration of 800µg/ml IAA. The preparation of 1-Naphthyl (acetic acid-1-C14) was slightly different as the compound was supplied as a benzene solution. The 100µCi of prepared compound, with 290µCi/mg specific activity was dissolved in deionised water. Addition of "cold" NAA produced a stock concentration of 800µg NAA per ml solution. The pH of the stock solutions was raised

to 7,2 with sodium hydroxide.

iii) Treatment of cultures

Prototheca cells were inoculated into 5mls of twice the nutrient broth concentration and grown for 7 days. 5mls of sterile radioactive herbicides were then added to the cultures. The desired herbicide concentration of 400 µg/ml was obtained and the activity of isotope in each test tube was 0,5µCi. These cultures were then grown for a further 10 hours.

iv) Liquid Scintillation preparation

The cultures were spun down at 2700 r.p.m. for 10 mins (Anderson, 1945a). The radioactive supernatant was decanted off. The cells were washed with sterile nutrient broth containing 10^{-5}M thiamine and 400µg/ml herbicide (either IAA or NAA). The cells were spun down and washed three times. The pellet was then suspended in 15mls Bray's Scintillator (appendix ii) (Bray, 1960; Wang and Willis, 1965; Smit, 1972). The low potassium vials were capped and stored in a dark cold room overnight before being read in a Packard Tri-Carb Liquid Scintillation counter.

v) Controls

Three methods of control were studied to find the most suitable one.

a) Control cultures were fixed with 0,5ml 25% glutaraldehyde while the experimental cultures were centrifuging. The cells were left to stand for half an hour before 5mls of radioactive herbicide was added to the tube.

b) 0,05mls of a stock 2,4-dinitrophenol plus calcium chloride solution was added to the control tubes

while the "tests" were spinning down. This gave a concentration of 10^{-4}M 2,4-DNP and $2x10^{-3}$M $CaCl_2$ (James, 1953; Andreae and van Ysselstein, 1960; Sutcliffe, 1962; Grunwald, 1970; White and Taniguchi, 1972). The cells were left to stand for half an hour before the radioactive herbicides were added to the cultures.

c) At the same time as the experimental cultures were inoculated with isotopic solutions, 5mls of sterile radioactive herbicides was added to the control cultures to give a total volume of 10mls. These control tubes were placed in a cold room at 4^{o}C for 10 hours.

Like the experimental tubes, these controls also contained 400µg/ml herbicide and 0,5µCi activity. Control cultures were prepared for liquid scintillation counting by the same method employed for the experimental cultures. The difference between two means was used to find if there is any significant active uptake of the herbicides by the cells (Bishop, 1969).

d) Electron microscopy

i) Fixation

Prototheca wickerhamii cells were prepared after the method described by Pease (1964) and Juniper et al (1970). Cells grown in nutrient broth and selected herbicides were centrifuged at 2700 r.p.m. for 10 mins into a loose pellet (Anderson, 1945a). The supernatant was decanted off and the cells were fixed for two hours at 4^{o}C in 10mls of 3% glutaraldehyde in 0,025M Millonig's phosphate buffer (pH 7,3) (appendix iii) (Sabatini et al, 1963; Pease, 1964; Sjöstrand, 1967; Juniper et al, 1970). Three washes with 10mls of 0,025M phosphate buffer (pH 7,3) at 4^{o}C, of 15 mins each were given. This was to remove all traces of glutaraldehyde. The cells were spun down between each wash and the supernatant was decanted off. The pellet was suspended in a little warm 2% agar.