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The effects of some selected herbicides and plant hormones on the growth of Prototheca wickerhamii

Henning, Penelope Anne

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After setting for half an hour, the agar was cut into small blocks which were then handled as small cubes of tissue in the following preparative processes.

1% osmium tetroxide in 0,025M phosphate buffer (pH 7,3) was used at 4°C overnight as the double fixative (appendix iii) (Palade, 1952; Sabatini et al, 1963; Juniper et al, 1970). The material was then twice washed in cold deionised water for 15 mins each. The agar cubes were dehydrated in an ice bath at 4°C, in a graded series of ethanol. Pease (1964) suggests dehydration periods of 15 mins. The blocks were placed in cold absolute ethanol and brought to room temperature with two, one hour changes.

ii) Embedding

In 1969, Spurr recommended that a low-viscosity epoxy resin be used for the embedding medium (appendix iii). The agar blocks were placed in resin for one hour to allow the resin to exchange for the ethanol. The blocks were transferred to fresh resin, and allowed to rotate, in sealed glass containers overnight. After impregnation, small 1mm cubes were transferred to Beem capsules and the capsules were filled with embedding medium. The capsules were placed in an oven at 40°C and the material was allowed to polymerise for 8 hours. Once cured, the capsules were cut off and the castings were trimmed with a razor blade (Juniper et al, 1970).

iii) Sectioning and staining

Blocks were given a smooth cutting face on a Reichert ultramicrotome using 45°glass knives set at 2°. Silver sections cut on a 2° diamond knife, were mounted on uncoated, coarse mesh, copper grids. The sections were double stained with lead citrate (Reynolds, 1963) and uranyl acetate (Watson, 1958) as suggested by Juniper et al, (1970) (appendix iii). Drops of stains were placed on a wax surface, to maintain surface tension, and were kept

covered. The grids were floated upside down on the lead citrate drops for 2 to 10 minutes, washed with 0,025M sodium hydroxide and thereafter twice washed in distilled water. The grids were then immersed in uranyl acetate drops for 10 mins, washed in distilled water and dried on fluffless filter paper.

iv) Viewing and printing

All viewing was done on a Siemens Elmskop I electron microscope. Cell size, mitochondria length and nucleus diameter were measured on rapidoprints. The mean measurements of these components in normal and herbicide treated Prototheca cells were calculated. The number of dividing, degenerating and total number of cells present on a grid were counted and the percentage of dividing and degenerating cells were calculated. Permanent prints were developed, fixed and then glazed.

v) Analysis of results

The difference between two means was used to find if herbicide treatments had any significant effects on the division and degeneration of the cells and on the size of the cellular components. Bishop (1967) calculates the variance of the difference of two means from

$$\sigma d^2 = \frac{\sigma^2 1}{n_1} + \frac{\sigma^2 2}{n_2}$$

with t being found from

$$t = \frac{x_1 - x_2}{\sigma d}$$

e) Inhibition mechanism studies

i) Herbicide replacement

Prototheca cells were grown for 3 days in 5mls of

double concentration nutrient solution. At time x, 5mls of sterile IAA was added to the experimental tubes to give 1000ug/ml herbicide. Sterile deionised water was added to the control tubes. Samples were taken at time x. Experimental and control tubes were grown for 10 or 15 or 20 hours and then spun down in sterile centrifuge tubes at 2700 r.p.m. for 10 mins. The supernatant was decanted off and the cells were washed once in 10mls fresh sterile nutrient solution of normal concentration. The cells were recentrifuged and the pellets were suspended in test tubes containing fresh sterile nutrient solution. All work was carried out in a sterile inoculation room and care was taken to keep conditions aseptic. Samples were taken at the time of replacement of herbicides with nutrient solution and at twenty four hour intervals thereafter.

ii) Substrate concentration variation

The mechanism of inhibition was examined further using a modified "enzyme-kinetic" type experiment. The substrate concentration was varied and initial concentrations chosen were 1,0; 0,75; 0,5 and 0,25. Substrate concentrations of 1,0; 0,1; 0,01 and 0,001 were also studied. 5mls inhibitor was supplied to give a concentration of 1000ug/ml IAA. Experiments with and without inhibitor for different time intervals were performed. Velocity was taken as number of cells per 0,1cc produced per time interval. The time intervals chosen were 10,15 and 20 hours. The data obtained was subjected to a simple linear regression analysis (Freese, 1967; Bishop, 1969). This analysis provides information on the slope of a graph, the 'y' axis intercept and the significance of the correlation coefficient. The results were plotted in the form of a Lineweaver-Burke graph using reciprocals of substrate concentration and velocity (number of cells per time) (Fruton and Simmonds, 1959; Patton, 1965).

RESULTS

a) Culturing Techniques

The first experiments performed determined the most suitable environmental conditions for growth of the cells with nutrient broth as the nutrient medium. Light intensity and temperature were varied. Counts of cells on a hemacytometer were taken as an indication of growth rate. The results are shown in figure 1 (see Table 1, appendix iv). Hereafter, Prototheca cells were grown in an incubator with a 2000 Lux 12 hour light/dark supply with a temperature maintained at 32°C.

The best nutrient solution, method of growth estimation, and the statistically smallest sample size were then determined. The growth rates of cells cultured in nutrient broth, Casselton and Stacey solution with glucose and sucrose, are shown in figure 2 (Table 2, appendix iv). Growth rates for these three media were also determined using a spectrophotometer. The results are depicted in figure 3 (Table 3, appendix iv). The statistically determined minimum sample size required for each nutrient solution, time interval and method of determination of growth rate were calculated (see Tables 4 and 5, appendix iv). Subsequent experiments were in nutrient broth, for 96 hours, with five samples being counted.

b) Herbicides

pH of the herbicides before and after sterilisation and on addition to nutrient broth on a 50/50 v/v basis are in Table 6 (appendix v). Prototheca wickerhamii cells were treated with different herbicides during the exponential phase of growth from 48 hours to 96 hours. Results of the experiments using herbicides in the concentration range of 100 to 1000µg/ml are in figure 4 (see Table 7, appendix v). Controls were taken at 48 hours

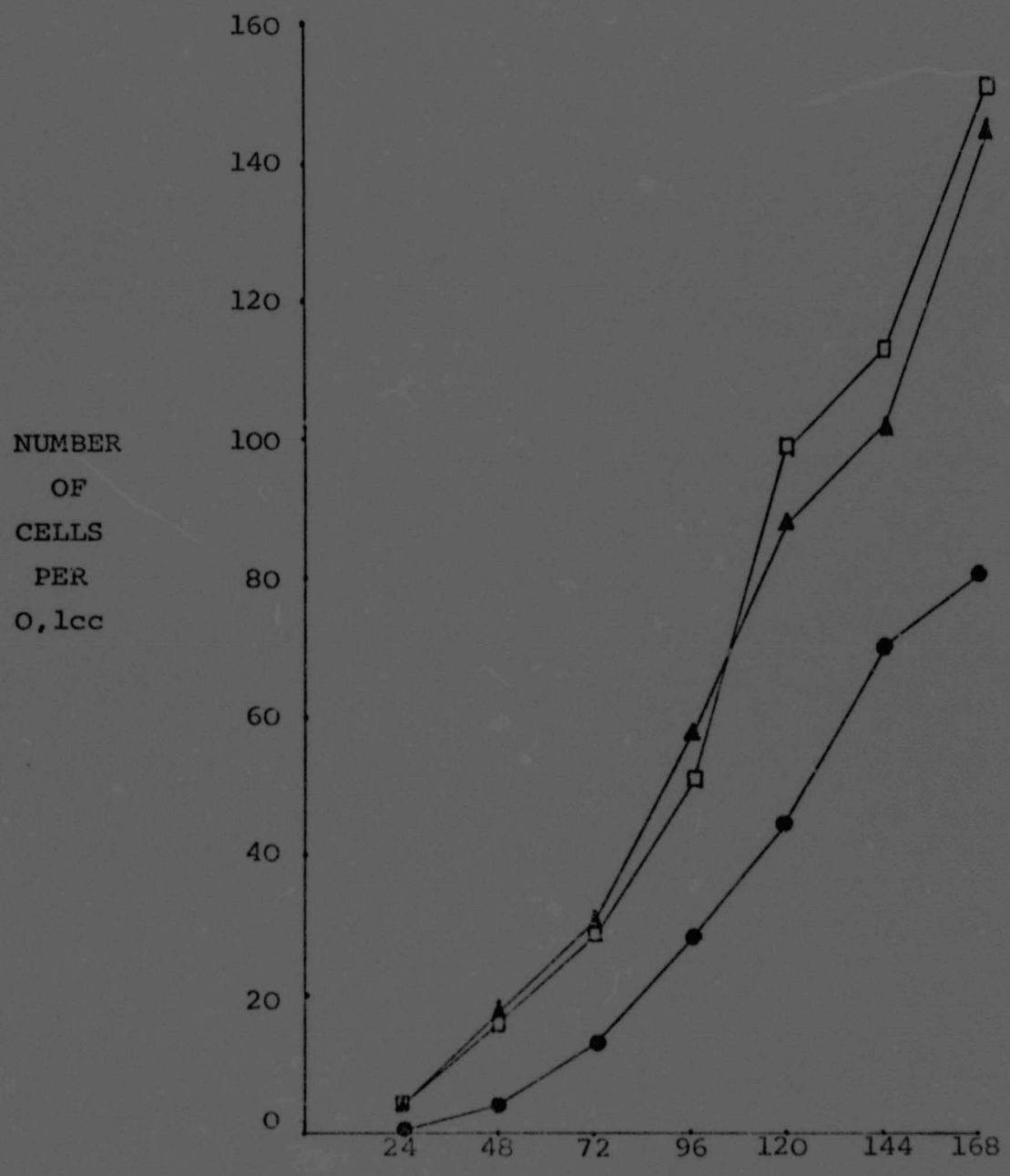


FIGURE 1 GROWTH CURVES FOR PROTOTHECA WICKERHAMII UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

LEGEND

□	= 32°C	12 hours -- 2000 Lux
▲	= 32°C	0 Lux
●	= 20°C	12 hours -- 500 Lux

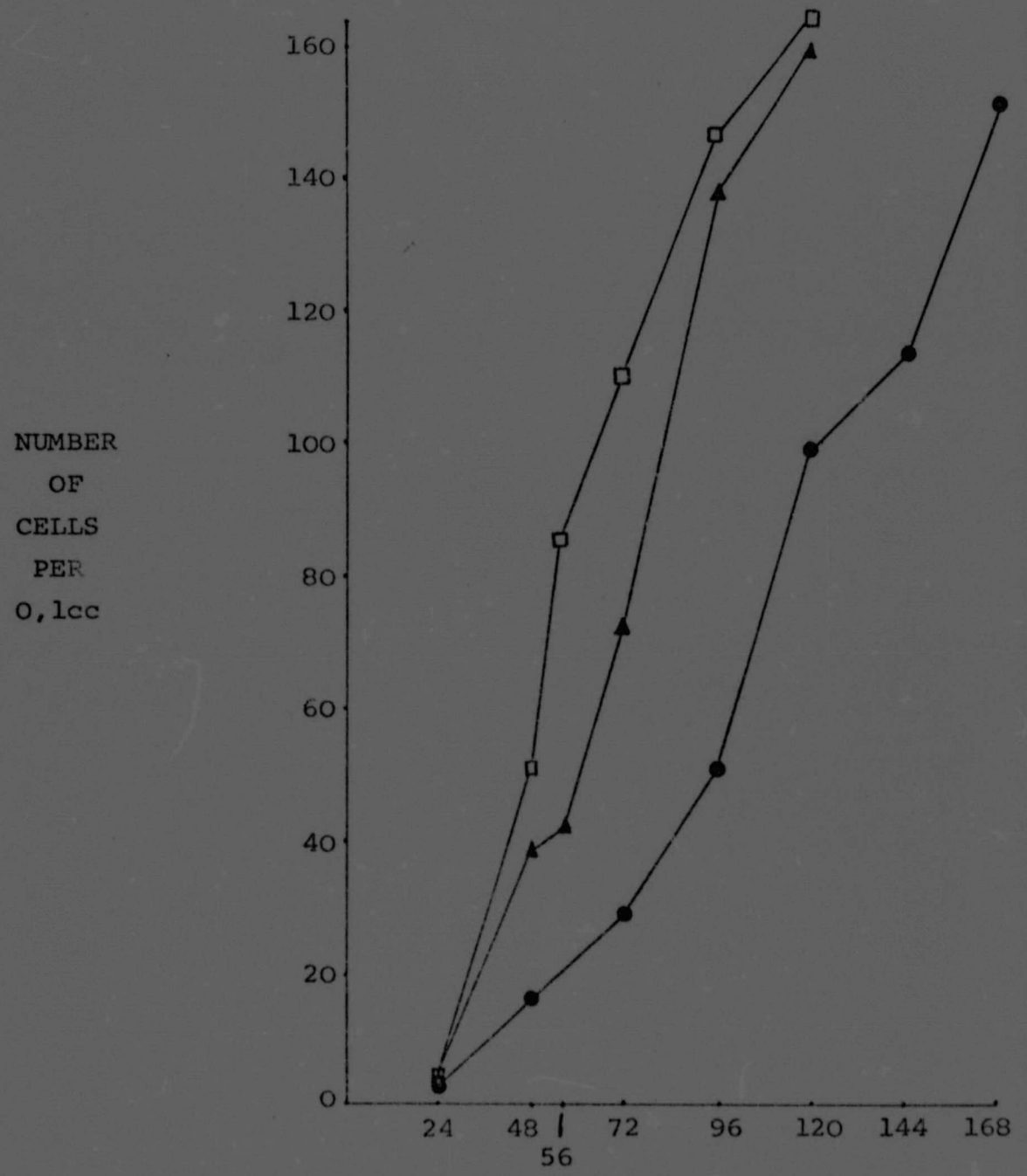


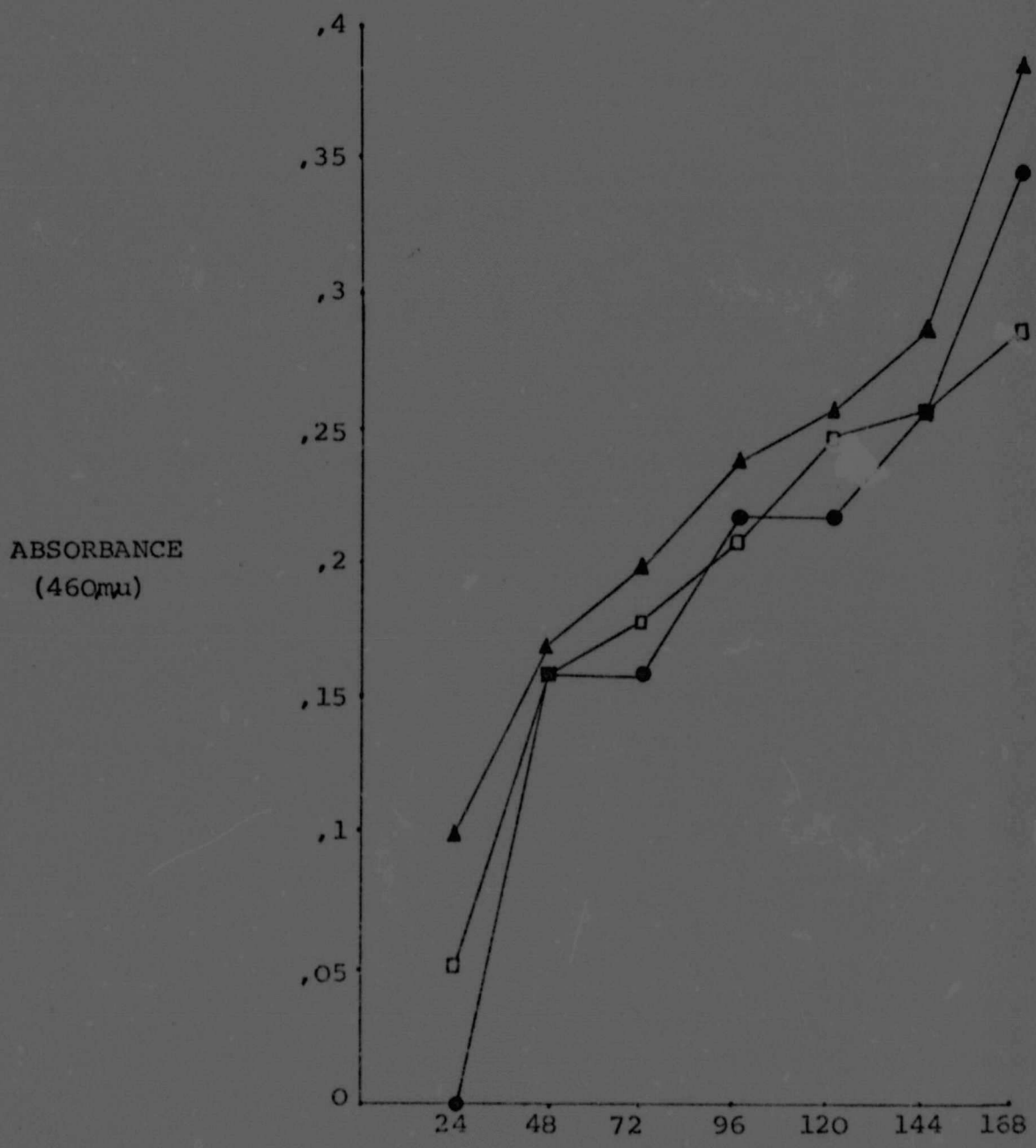
FIGURE 2 GROWTH CURVES FOR PROTOTHECA WICKERHAMII UNDER DIFFERENT NUTRIENT CONDITIONS

LEGEND

□ = sucrose in Casselton & Stacey solution

▲ = glucose in Casselton & Stacey solution

● = nutrient broth



FIGUR E 3 GROWTH CURVES FOR PROTOTHECA WICKERHAMII UNDER DIFFERENT NUTRIENT CONDITIONS

LEGEND

□ = sucrose in Casselton & Stacey solution

▲ = glucose in Casselton & Stacey solution

● = nutrient broth

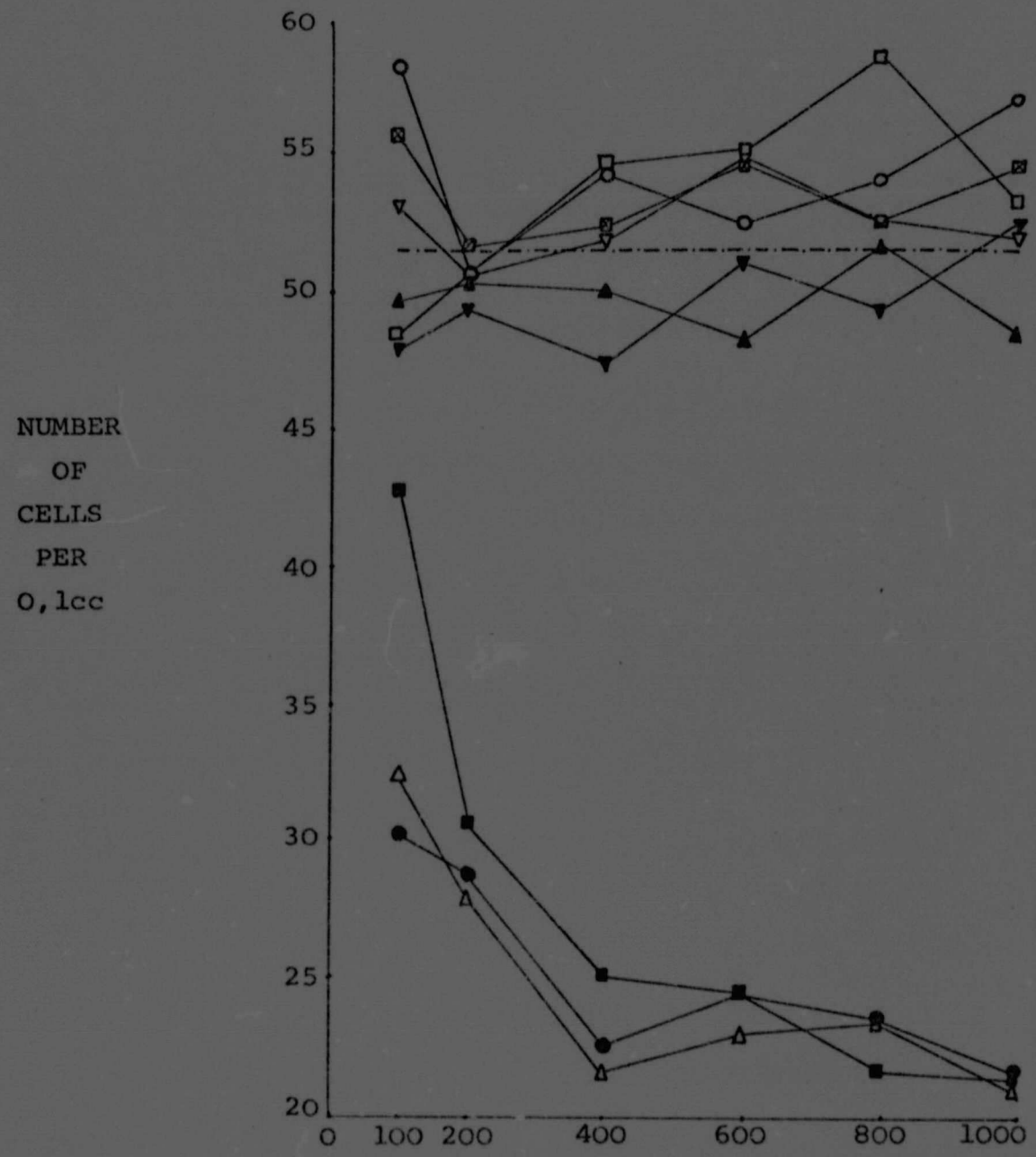


FIGURE 4 TREATMENT OF PROTOTHECA WICKERHAMII WITH DIFFERENT HERBICIDES IN THE RANGE 100 TO 1000 µg/ml

LEGEND

■ = IAA Δ = IBA ● = IPA
□ = NAA ▲ = GA_3 ○ = 2,4,5-T
▽ = Na gold salt ▼ = simazine ⁄ = control
⊠ = MH

and 96 hours for each herbicide (see Table 8, appendix v). Figure 5 shows the results of experiments using herbicides in the range of 10 to 100µg/ml and coconut milk from 10 to 50% v/v. Controls and Table 9 are in appendix v. The mean number of cells per 0,1cc for IAA, IBA and IPA at 100µg/ml was plotted in figure 6 which shows the overall effect of IAA, IBA and IPA on Prototheca wickerhamii. Higher concentrations at 2000µg/ml and 4000µg/ml of the herbicides MH, 2,4,5-T, NAA, gibberellic acid and simazine were used (Table 11, appendix v).

Time course curves with IAA, IBA and IPA using 1000µg/ml were undertaken to determine the time for inhibition by these herbicides. Figure 7 gives the results where the 12 hour light/dark period in the incubator was from 7am. to 7pm. with the herbicides being added at 10am. (Table 12, appendix v). Figure 8 shows the results when the 12 hour light/dark period was changed in the incubator so that the light was supplied from 1am. to 1pm. The herbicides were again added at 10am. (Table 13, appendix v). From figures 7 and 8, the time for absorption and effectivity of the herbicides was determined and this time was used in the formula derived to calculate the percentage inhibition of the cells by the herbicides. The 48 hour control plus delay was read off figure 1 to give 21,0 cells per 0,1cc. The 96 hour controls, which are obtained from Tables 8 and 10, were corrected for the delay in absorption and effectivity of the herbicides. Percentage inhibition by herbicides in the concentration range of 100 to 1000µg/ml are shown in figure 9 (see Table 14, appendix v). Figure 10 shows the percentage inhibition obtained from herbicides in the range of 10 to 100µg/ml and from coconut milk in the range of 10 to 50% v/v. (Table 15, appendix v). Percentage inhibition means for IAA, IBA and IPA at 100µg/ml were used in figure 11 to show the overall trend of inhibition with these three herbicides. Percentage inhibition at the higher concentrations of the selected herbicides was also calculated (see Table 16, appendix v).

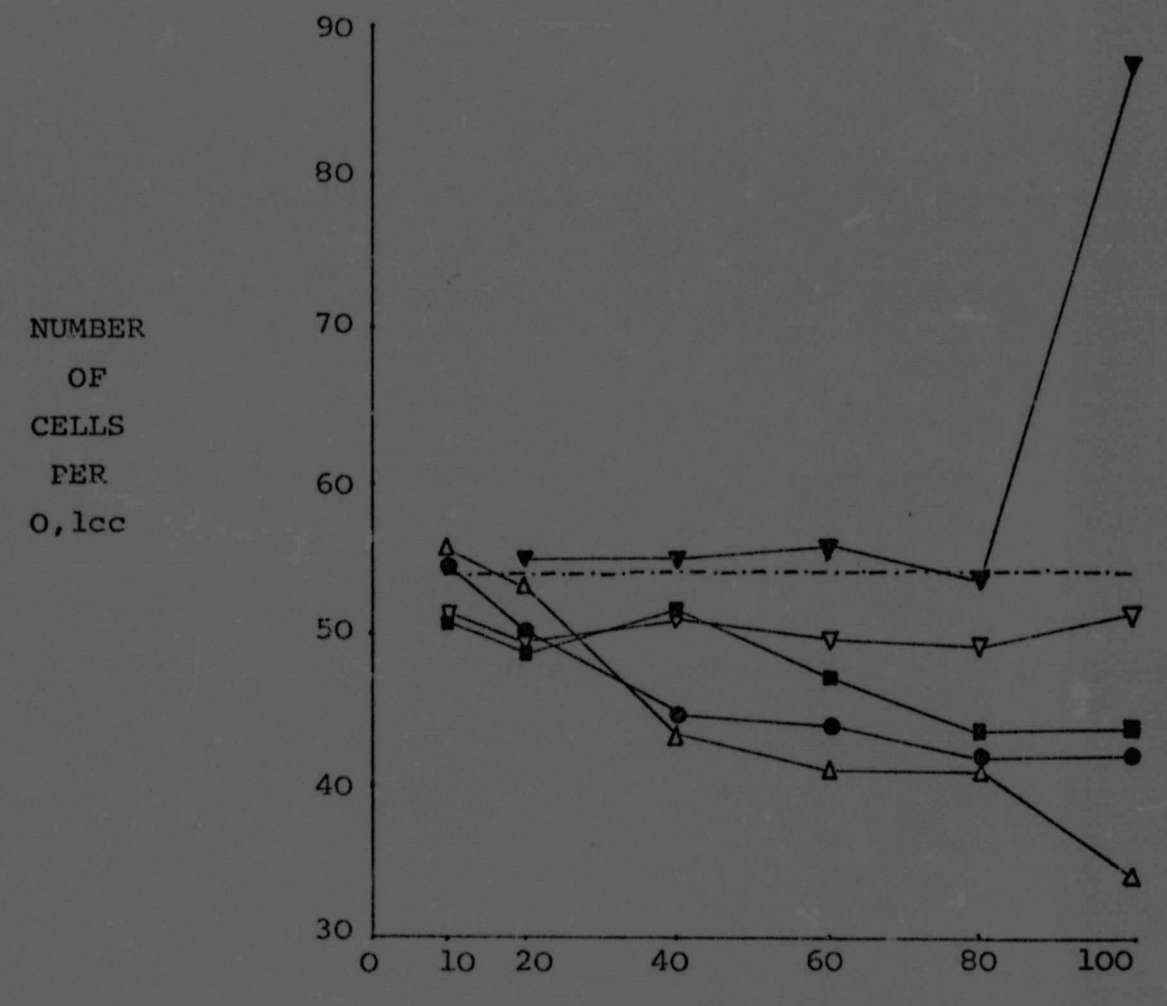


FIGURE 5 TREATMENT OF PROTOTHECA WICKERHAMII WITH DIFFERENT HERBICIDES IN THE RANGE 10 TO 100 µg/ml

LEGEND

■ = IAA Δ = IBA ● = IPA

▽ = kinetin ▼ = coconut milk ⁄ = control

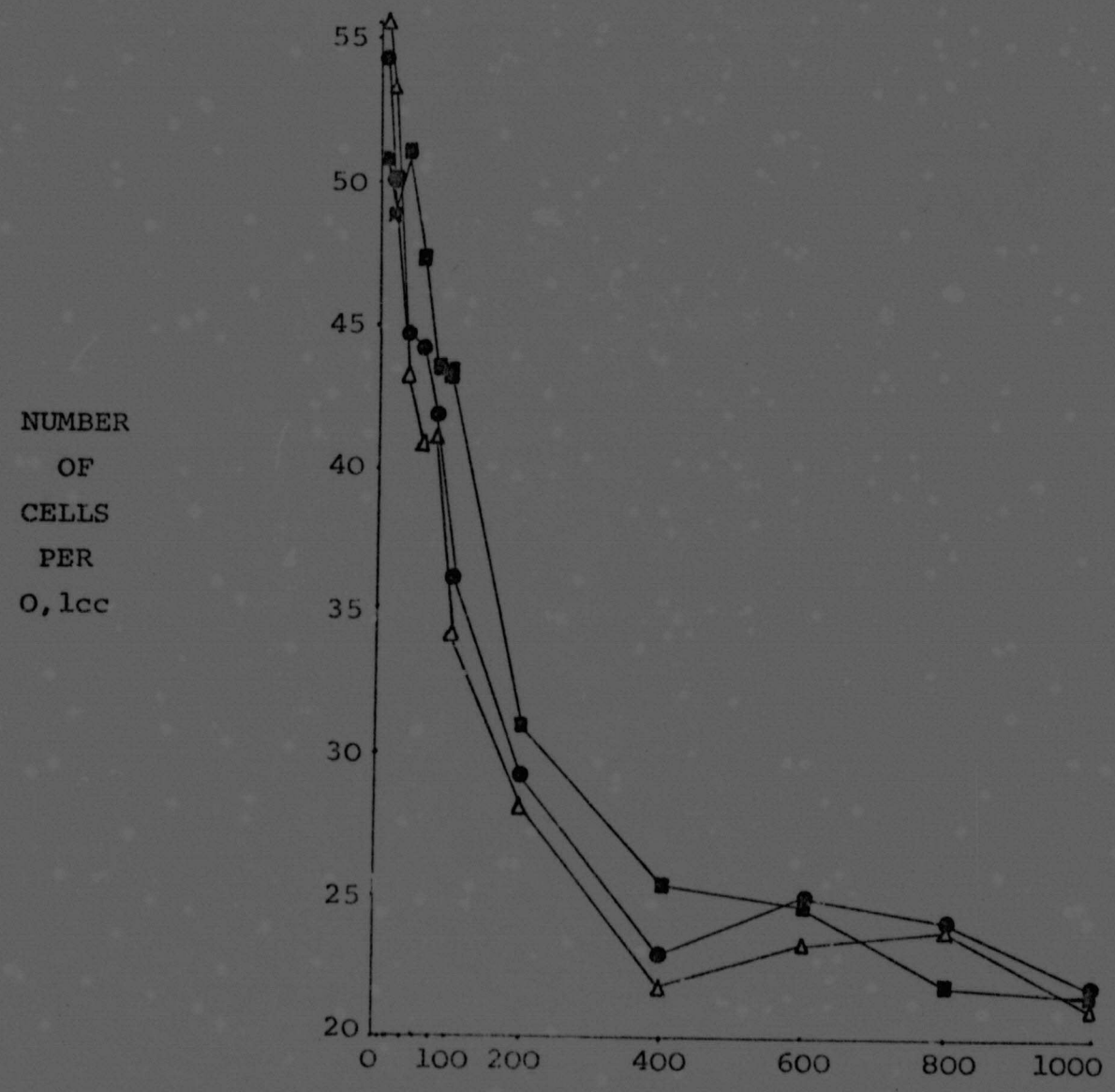


FIGURE 6 TREATMENT OF PROTOTHECA WICKERHAMII WITH THE INDOLE-ACID HERBICIDES

LEGEND

■ = IAA Δ = IBA ● = IPA

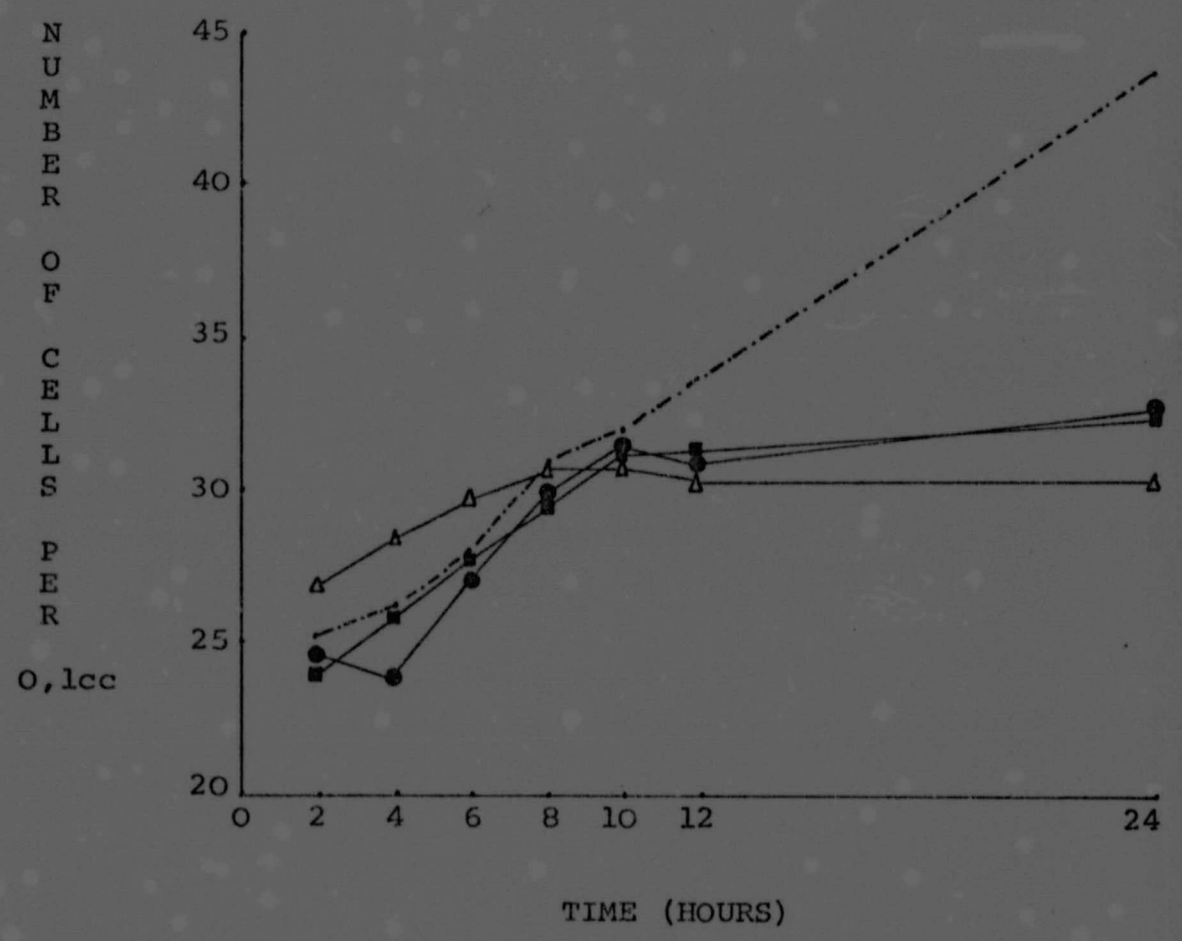


FIGURE 7 DETERMINATION OF TIME TAKEN FOR INHIBITION OF PROTOTHECA WICKERHAMII.
LIGHT PERIOD FROM 7 am TO 7 pm

LEGEND

■ = IAA Δ = IBA

● = IPA ⁄ = control

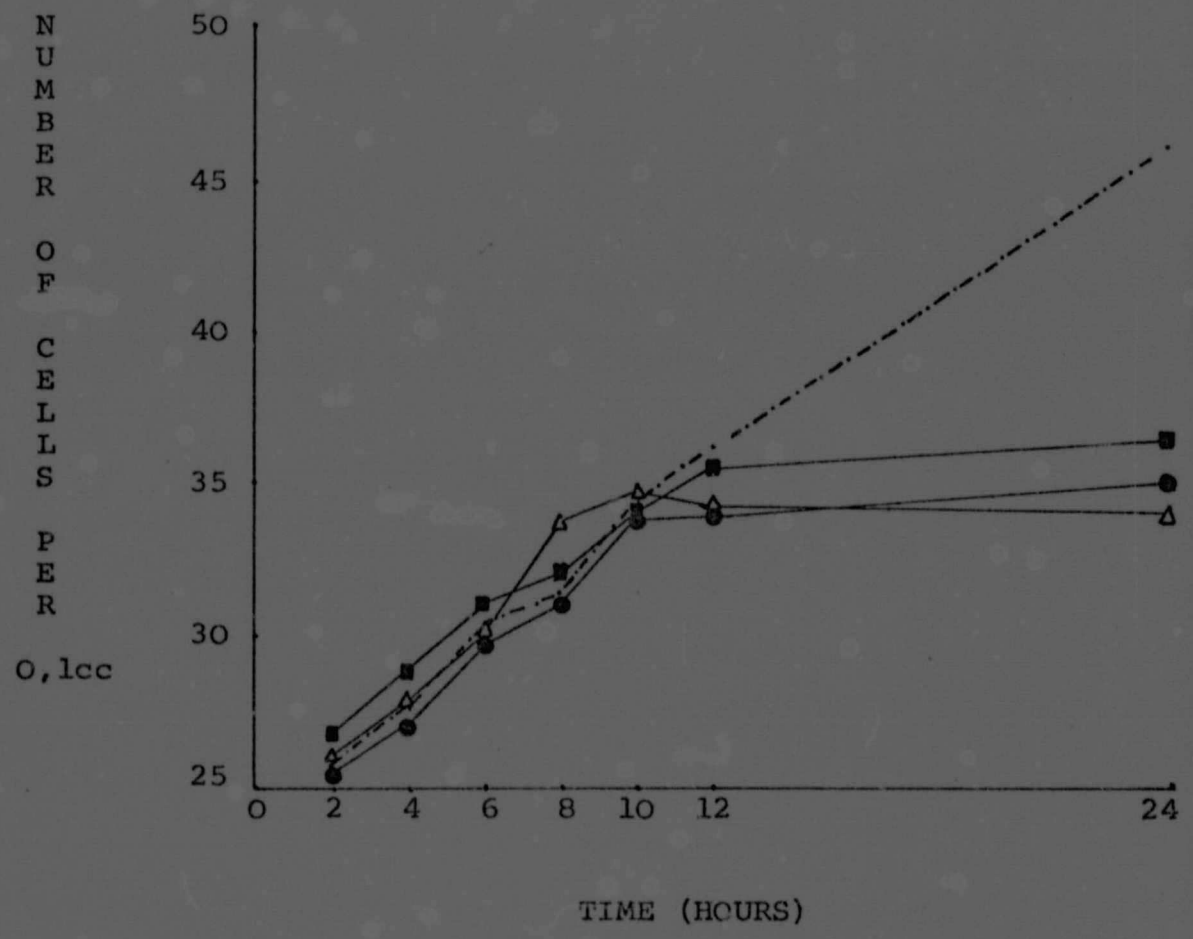


FIGURE 8 DETERMINATION OF TIME TAKEN FOR INHIBITION OF PROTOTHECA WICKERHAMII.
LIGHT PERIOD FROM 1 am TO 1 pm

LEGEND

■ = IAA Δ = IBA

● = IPA ⋰ = control

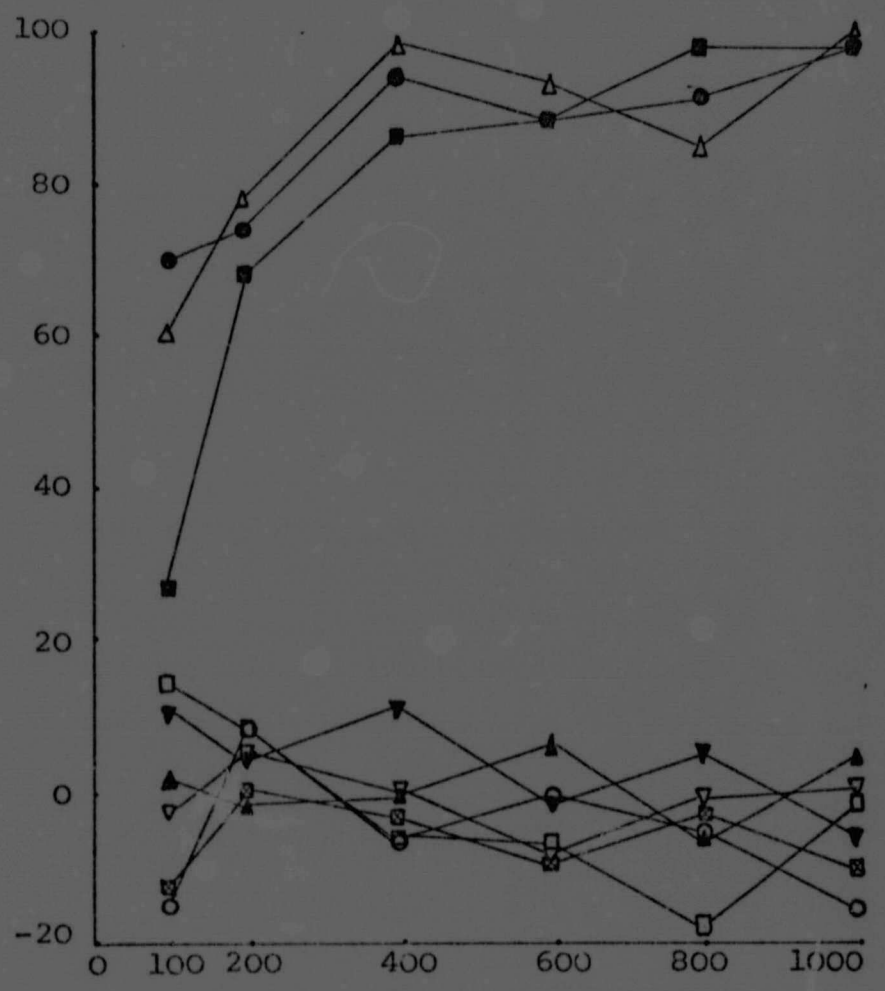


FIGURE 9 PERCENTAGE INHIBITION OF FROTOTHECA WICKERHAMII WITH DIFFERENT HERBICIDES IN THE RANGE 100 TO 1000 µg/ml

LEGEND

■ = IAA ▲ = IBA ● = IPA
□ = NAA ▲ = GA_3 ○ = 2,4,5-T
▽ = Na gold salt ▼ = simazine ⊠ = MH

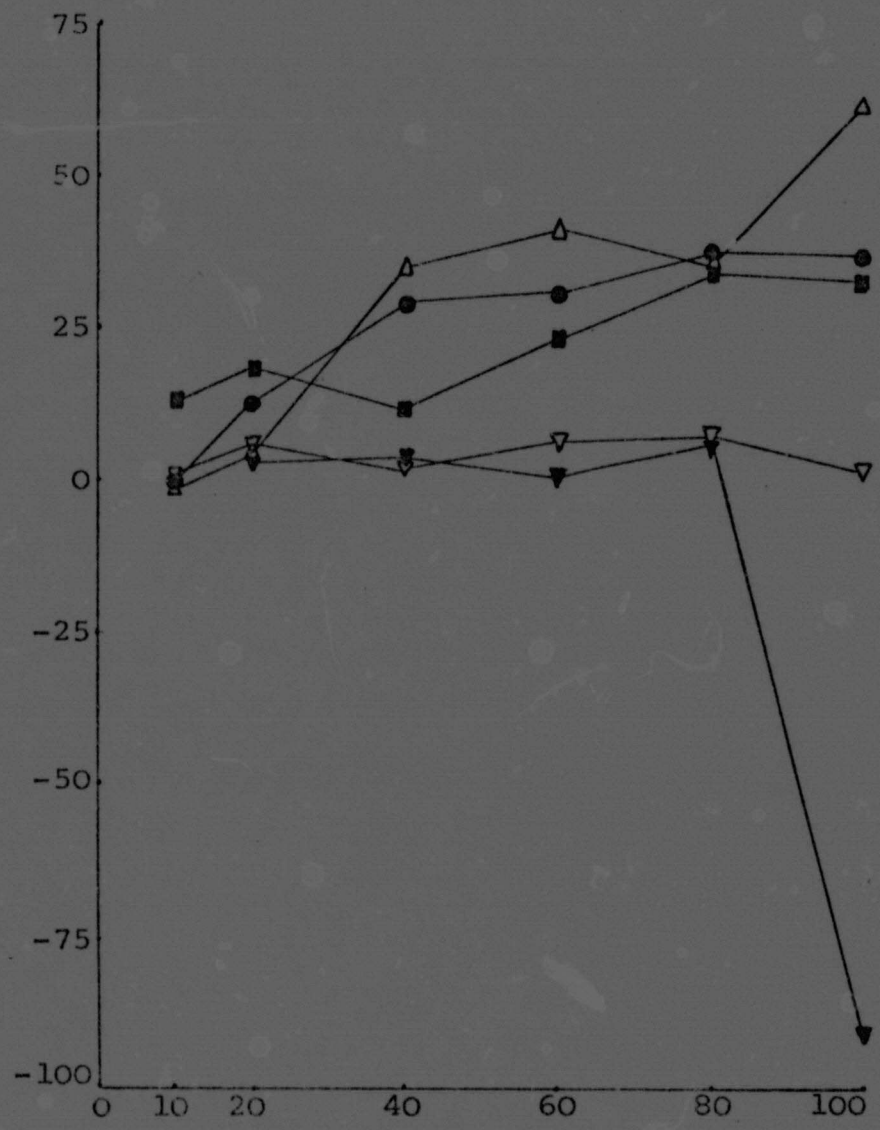
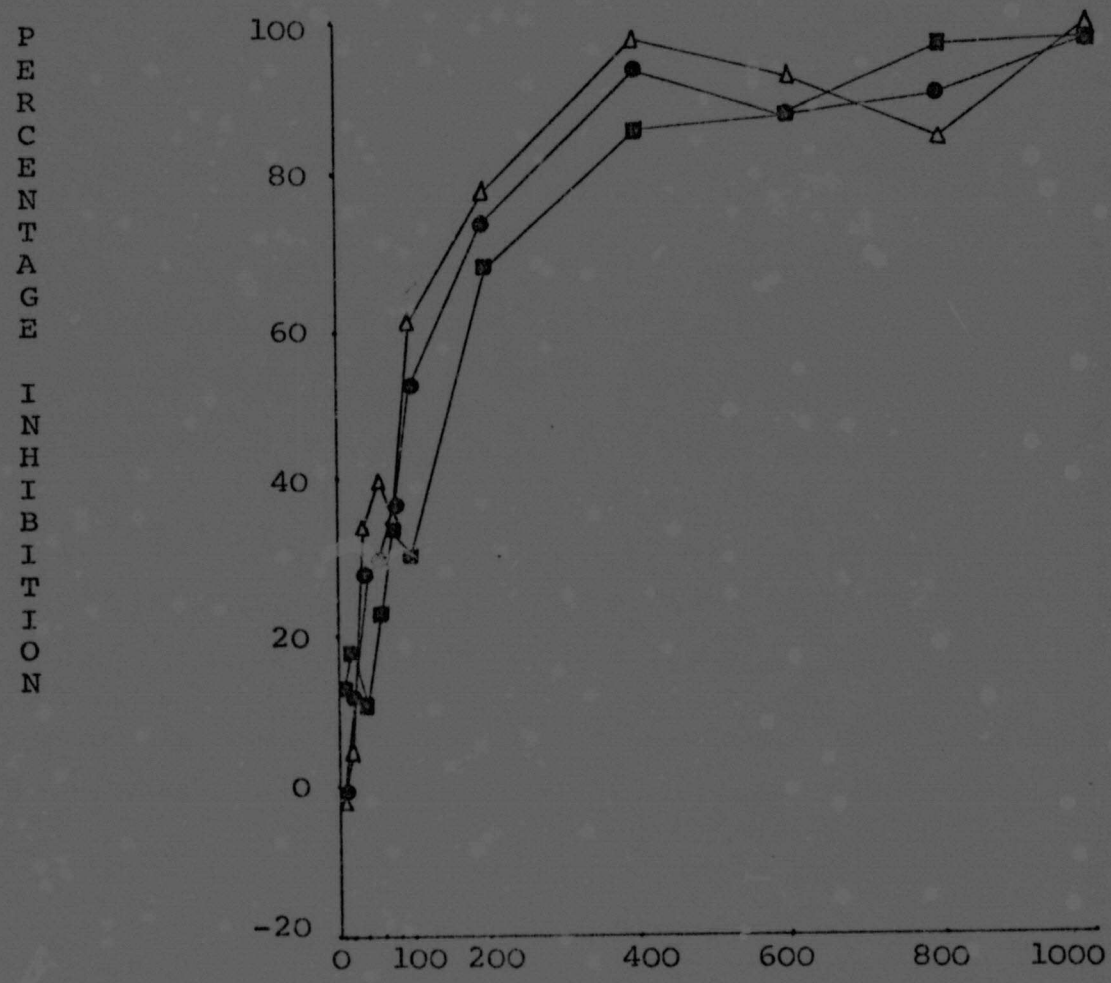


FIGURE 10 PERCENTAGE INHIBITION OF PROTOTHECA WICKERHAMII WITH DIFFERENT HERBICIDES IN THE RANGE 10 TO 100 µg/ml

LEGEND

■ = IAA Δ = IBA ● = IPA

▽ = kinetin ▼ = coconut milk



CONCENTRATION OF HERBICIDE (µg/ml)

FIGURE 11 PERCENTAGE INHIBITION OF PROTOTHECA WICKERHAMII WITH THE INDOLE-ACID HERBICIDES

LEGEND

■ = IAA Δ = IBA ● = IPA

c) Radioactive Herbicide Studies

Results of the time course curve with 400ug/ml IAA to determine the time for uptake and effectivity of the herbicide are shown in figure 12 (see Table 17, appendix vi). The experiment where the first control (a) was used gave incorrect results as the glutaraldehyde combined with the proteins in the semipermeable membrane. This changed the nature and properties of the membrane and allowed free diffusion of the isotope into and out of the cells. The results of the second experiment using 2,4-DNP as control (b) are presented in Table 18 below. There is no quenching by the cells so that correction of the results was unnecessary. The disintegration rate of the sample was evaluated by multiplying the observed count rate with background subtracted, by one hundred over the percentage efficiency of the sample. The counting efficiency of the machine for the two isotopes (IAA-C^{14} and NAA-C^{14}) was calculated to be 85%.

TABLE 18

UPTAKE OF ISOTOPE BY PROTOTHECA WICKERHAMII

	DPM IN THOUSANDS					
Indolyl-3-acetic acid-1-C^{14}	130	110	88	76	84	75
Control	56	64	31	24	29	28
Naphthyl-acetic acid-1-C^{14}	89	88	88	78	109	69
Control	49	55	45	24	49	26

As these results were obtained from a number of experiments performed at different times, they were treated as samples being drawn from a population. The difference between two means was used, at five degrees of freedom, to establish if they are different (see Table 19 below).

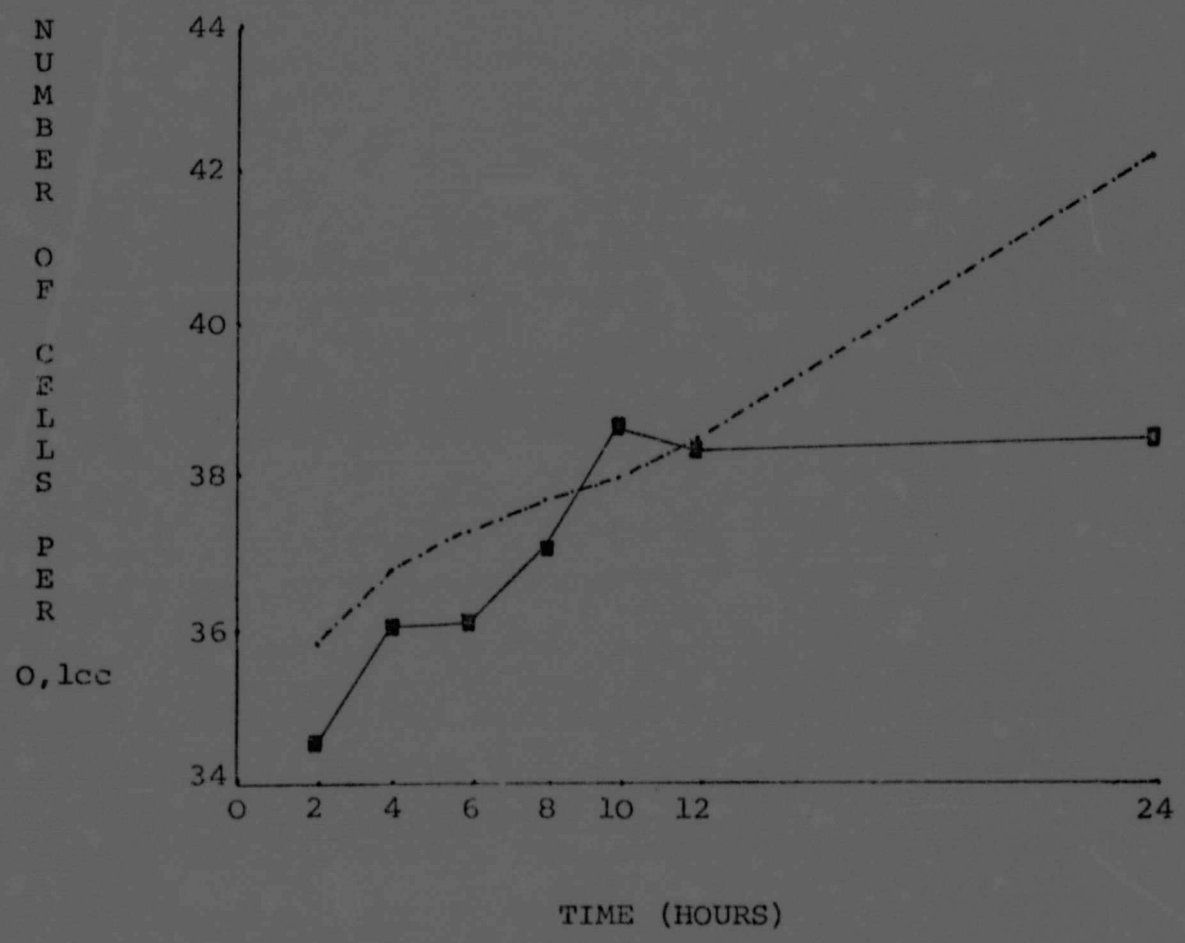


FIGURE 12 DETERMINATION OF TIME TAKEN FOR INHIBITION OF PROTOTHECA WICKERHAMII WITH 400 µg/ml IAA

LEGEND

■ = IAA ⁄ = control

TABLE 19

DIFFERENCE BETWEEN MEANS TEST

Test	t	p	Significance
IAA-C^{14} & NAA-C^{14}	0,25	<,1	N.S.
IAA-C^{14} & control	5,53	>,01	**
NAA-C^{14} & control	6,02	>,01	**
control & control	0,22	<,1	N.S.

The results of the experiment where cold treatment ($4^{o}C$) as control (c) was used are presented in Table 20 below.

TABLE 20

UPTAKE OF ISOTOPE BY PROTOTHECA WICKERHAMII

	DPM IN THOUSANDS					
Indolyl-3-acetic acid-1-C^{14}	125	123	130	109	74	67
control	49	35	49	33	30	26
Naphthyl-acetic acid-1-C^{14}	91	88	106	86	94	86
control	39	58	38	51	50	50

The results of the difference between two means test, at five degrees of freedom, of the samples are presented in Table 21 below.

TABLE 21

DIFFERENCE BETWEEN MEANS TEST

Test	t	p	Significance
IAA-C^{14} & NAA-C^{14}	1,12	<,1	N.S.
IAA-C^{14} & control	,53	>,01	**
NAA-C^{14} & control	9,96	>,001	***
control & control	1,86	<,1	N.S.

d) Electron Microscopy

Prototheca wickerhamii cells grown in 1000µg/ml IAA, IBA and IPA for 48 hours were viewed under the electron

microscope. The cells were examined for gross abnormality changes and the herbicide treated cells were compared with untreated cells grown in nutrient broth (Plates 1,2 and 3). Particular attention was paid to the mitochondria and the nucleus of these cells. Cell size, nucleus diameter and mitochondria lengths in the different treatments were measured and the mean results are presented in Table 22 (appendix vii). The cellular components of the herbicide treatments were grouped and the difference between two means was used to compare the treated and normal cells. This was to determine if the herbicides had any effect on the size of the cellular components (see Table 23, appendix vii).

Grids examined under the microscope appeared to have an increased number of dead cells in the herbicide treated cultures (Plate 4) The percentage of dividing and degenerating cells on a number of grids was calculated (Table 24, appendix vii). The effects of IAA, IBA and IPA on the division and death of the cells was analysed using the difference between two means test at eight degrees of freedom (see Table 25 below).

TABLE 25
DIFFERENCE BETWEEN MEANS TEST FOR NORMAL AND HERBICIDE TREATED CELLS

Difference Test	t	p	Significance
Normal & IAA % Dividing	1,06	<,1	N.S.
Normal & IBA % Dividing	1,72	<,1	N.S.
Normal & IPA % Dividing	0,12	<,1	N.S.
Normal & IAA % Degenerating	7,32	>,001	***
Normal & IBA % Degenerating	1[illegible],32	>,001	***
Normal & IPA % Degenerating	31,34	>,001	***

e) Inhibition mechanism studies

Results of the experiments where the herbicide IAA was added to the cultures for 10, 15 or 20 hours, and

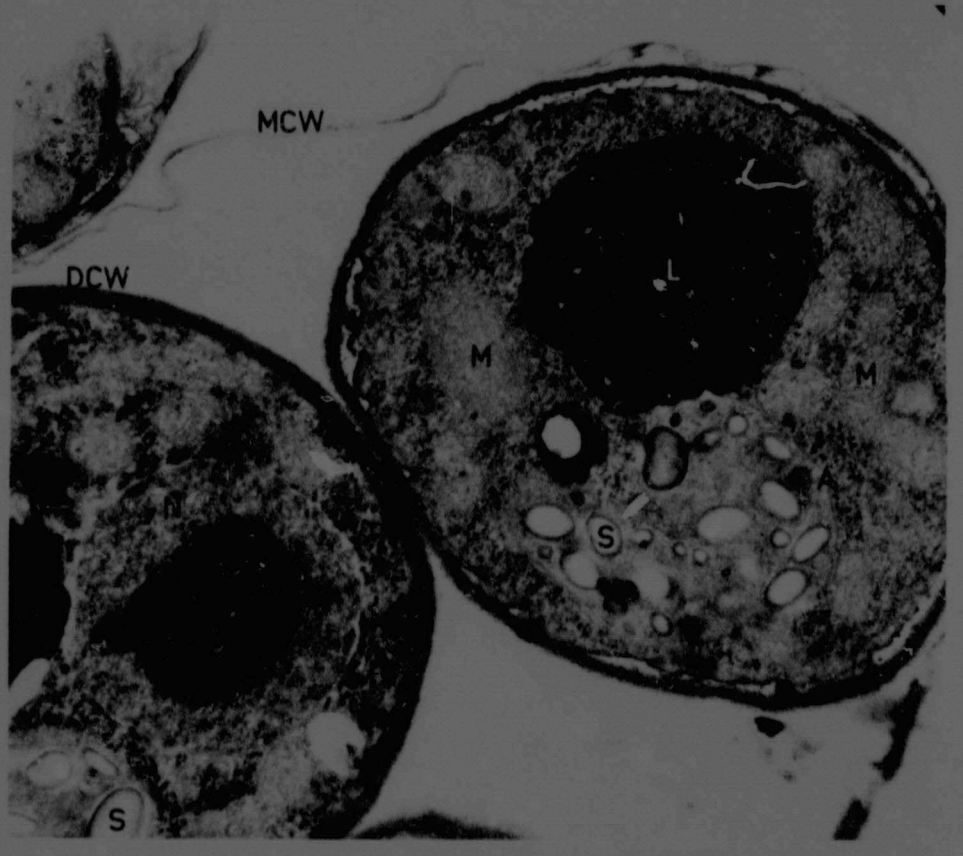


PLATE I PROTOTHECA WICKERHAMII DAUGHTER CELLS WITHIN THE MOTHER CELL WALL. MAG. X 31 000

LEGEND

A = Amyloplast membrane
DCW = Daughter cell wall
L = Lipid
M = Mitochondrion
MCW = Mother cell wall
N = Nucleus
R = Ribosomes
S = Starch

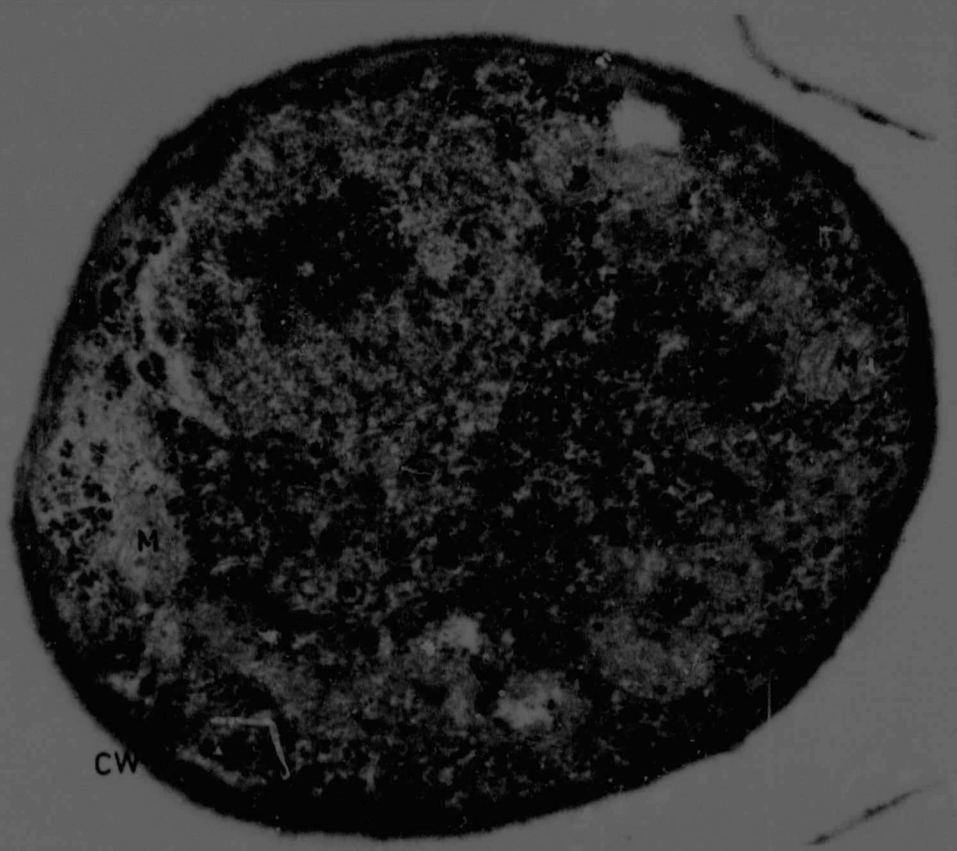


PLATE 2 PROTOTHECA WICKERHAMII CELL TREATED WITH IAA.
MAG. X 40 000

LEGEND

CW = Cell wall
M = Mitochondrion
N = Nucleus
R = Ribosomes

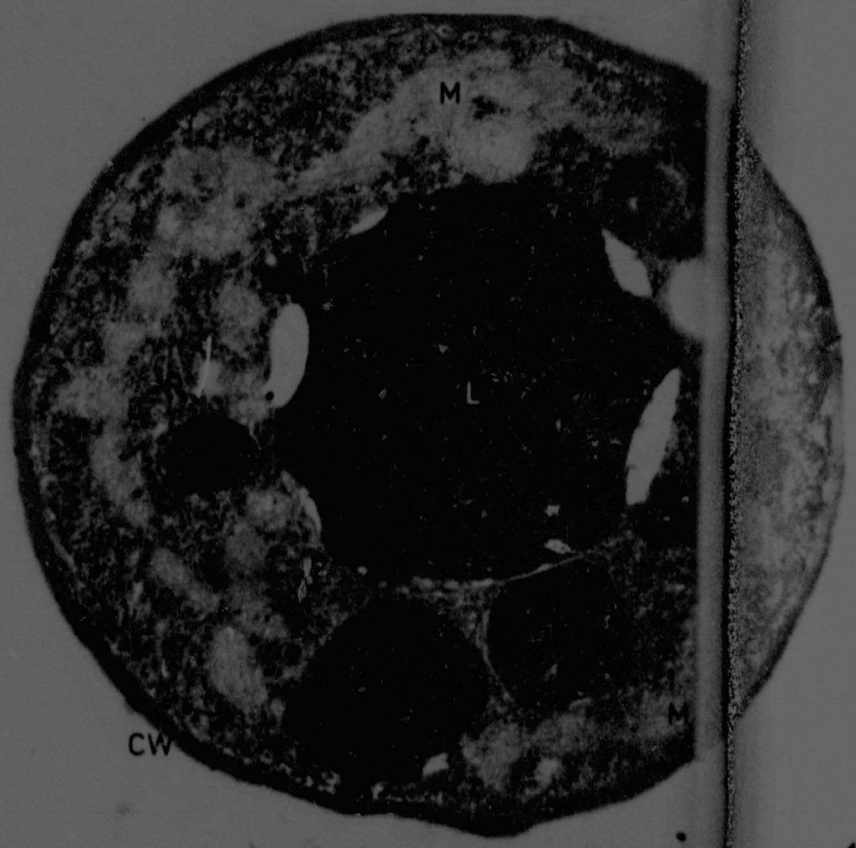


PLATE 3 PROTOTHECA WICKERHAMII CELL TREATED WITH IBA.
MAG. X 32 000

LEGEND

CW = Cell wall
L = Lipid
M = Mitochondrion
R = Ribosomes

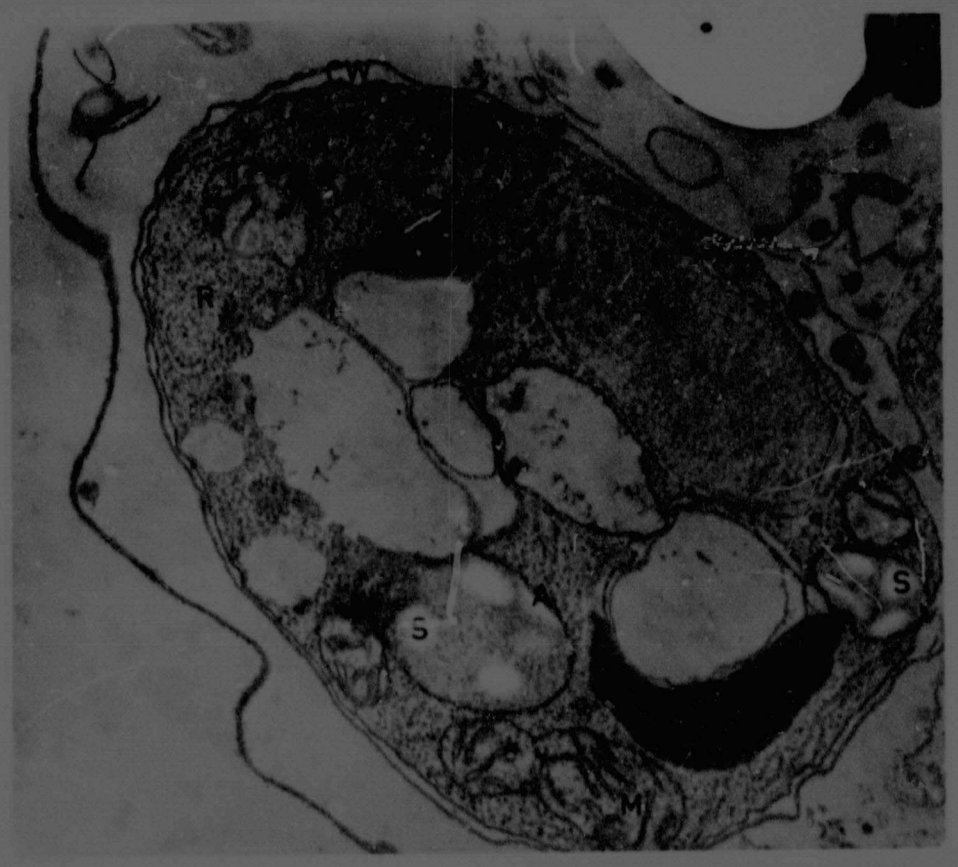


PLATE 4 DEGENERATING CELL PRODUCED ON TREATMENT OF PROTOTHECA WICKERHAMII WITH IPA.
MAG. X 40 000

LEGEND

A = Amyloplast membrane
CW = Cell wall
M = Mitochondrion
N = Nucleus
R = Ribosomes
S = Starch

was then replaced, are presented in Table 26 (appendix viii). Figure 13 shows the number of cells obtained at 24 hour sampling after herbicide replacement with nutrient broth.

From the results in the above experiment, it was decided to examine further the mechanism of inhibition involved by utilising a modified enzyme kinetic type of experiment. The results from the varied substrate concentrations of 1,0; 0,75; 0,5 and 0,25 are in Table 27 (appendix viii). The reciprocals of substrate and velocity (number of cells per time) were taken for the different time intervals. The results obtained with varied substrate concentrations of 1,0; 0,1; 0,01 and 0,001 are presented in Table 28 (appendix viii). Information on the slope, the 'y' axis intercept and the significance of the correlation coefficient was obtained from a simple linear regression analysis (Table 29) (appendix viii). The following Lineweaver-Burke type figure was constructed (Figure 14).

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Name of thesis The Effects Of Some Selected Herbicides And Plant Hormones On The Growth Of Prototheca Wickerhamii. 1974

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